Stem Cell Biology and Regenerative Medicine

Hossein Baharvand
Nasser Aghdami *Editors*

Regenerative Medicine and Cell Therapy

Humana Press

Stem Cell Biology and Regenerative Medicine

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To the memory of Dr. Saeid Kazemi Ashtiani, a wonderful colleague, a great stem cell biologist, and an inspirational advocate for stem cell research in Iran.
To our professors, teachers, students, and families.

Preface

There are numerous promising researches that show that science and medicine of the future will likely be based upon regenerative medicine and cellular therapies. Regenerative medicine is devoted to the replacement of diseased cells, tissues, or organs in congenital or acquired disease, or the repair of tissues in vivo by augmentation of natural or induction of latent regenerative processes. This new interdisciplinary field of research and clinical therapies that focus on stem cells and regenerative biology is just beginning at the dawn of the twenty-first century. In our previous book, *Advances in Stem Cell Research* (2012, Springer), we have addressed and discussed current advances and topics pertaining to stem cells, covering topics such as stem cell nano-engineering, pluripotent stem cells, and cellular reprogramming. In this book, *Regenerative Medicine and Cell Therapy*, we aim to explain clinical applications and experiences of stem cell therapy, taking into consideration neurological, ocular surface, skin, cardiac, musculoskeletal, liver and gastrointestinal diseases, and diabetes, in addition to germline and cord blood stem cells. The contributions to this book, all written by renowned experts in their respective disciplines, describe and explore various facets of regenerative medicine and cell therapy. This book will be an especially valuable resource for biomedical researchers and clinicians.

We want to sincerely thank all the authors who have contributed to this volume for their devoted efforts and their excellent contributions. We hope that you, as a reader, will enjoy this book. We are also grateful to Drs. Hamid Gourabi, Abdolhossein Shahverdi, and Ahmad Vosough Dizaj for having faith in and supporting us throughout this project. We also wish to acknowledge the great support provided by many at Humana Press. A special thank you goes to our dedicated colleagues at Royan Institute for Stem Cell Biology and Technology who, with their tireless commitment for stem cell research and therapy, have become crucial factors in encouraging us to edit this *Regenerative Medicine and Cell Therapy*. We are grateful to Zahra Maghari for her assistance with collecting the chapters and in follow-up.

Hossein Baharvand
Nasser Aghdami

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Chapter 1
Cell Therapy for Neurodegenerative Disorders

Ilyas Singec

Abstract The last decade has seen tremendous progress in stem cell biology, targeted genome editing, bioengineering, and systems neuroscience supporting the notion that cell therapy of various disorders of the central nervous system (CNS) may become clinical reality in the near future. In particular, the advent of induced pluripotent stem (iPS) cells and access to large quantities of patient- and disease-specific cellular material offers unique opportunities for developmental biology and regenerative medicine. It is now possible to investigate the molecular underpinnings of monogenic and complex human diseases using stem cell-derived neural phenotypes. Molecular insights from such studies will leverage the development of diagnostic tools, biomarkers, drugs, and cell replacement with the ultimate goal to halt or reverse the course of devastating maladies. In this book chapter, I shall discuss the opportunities and emerging challenges of stem cell-based therapies and highlight common neurological diseases that may benefit from such iatrogenic interventions.

1.1 Introduction

Owing to advances in modern medicine over the last decades, higher life expectancy has increased the proportion of the aged human population in industrialized countries. This profound demographic change allows the prognosis that chronic

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incurable disorders of the central nervous system (CNS) will have an unprecedented social and economic impact on society and the health care systems. In light of this prospect, regenerative medicine depends on innovative strategies and medical breakthroughs that can directly translate into novel therapeutics.

The human brain is characterized by enormous cellular and synaptic complexity and any attempt for repairing or replacing nervous tissue is among the most formidable goals in medicine. Neurons in the human brain are postmitotic and as old as the diseased patient [1]. In general, the human CNS has limited regenerative potential after injury and the pathobiology of neurodegenerative diseases is intricate and difficult to study. In most cases, human samples are derived from postmortem tissue with inherent problems such as poor tissue preservation and lack of standardization. In addition, postmortem specimens often reflect the end-stage of a given disease thereby limiting the study of prodromal changes.

The clinical manifestations of CNS diseases are determined by the underlying anatomical location of the lesion, the affected cell type(s), the age of onset, genetic background (familial or sporadic), and the environmental context (e.g. toxins, pesticides, cellular stressors). With regard to understanding disease etiologies, unraveling the interplay between complex genetic and environmental factors is particularly challenging. Available drugs for the treatment of neurological and psychiatric diseases are limited in that they only provide symptomatic relief but fail to target the underlying disease cause. Currently, the development of novel drugs in the pharmaceutical industry particularly for CNS diseases is experiencing major difficulties because of the poor success rate of drugs entering clinical trials [2]. This is in part due to the limited predictive value of small animal models for drug discovery emphasizing the fact that rodent models often do not recapitulate the critical aspects and peculiarities of the human condition [2–6]. Together, it is apparent that cell therapy, drug discovery, and mechanistic studies of human disease would greatly benefit from readily accessible live human neural cells amenable for basic research in a laboratory setting.

Human embryonic stem (ES) cells are the prototypical pluripotent cells and were first isolated by Thomson and colleagues [7]. Because of the ethical issues associated with the derivation of ES cells from human embryos, only a few laboratories were able to create such cell lines following strictly regulated guidelines. In addition, the dependence on limited embryo material did not allow the prospective isolation of human ES cell lines representing the large variety of familial and sporadic human diseases. The more recent discovery by Yamanaka and colleagues that human somatic cells can be reprogrammed into embryonic-like iPS cells by a few defined transcription factors represents a major breakthrough for biomedical research [8]. Nuclear reprogramming and the streamlined production of iPS cells hold great promise for clinical cell therapy and disease modeling. The availability of experimental platforms with functional human neurons derived from affected patients will greatly advance high-throughput and high-content screening efforts and drug discovery [9]. Ultimately, routine access to patient- and disease-specific iPS cells will pave the way to rigorous cell therapy and tissue engineering paradigms and realize the concept of “personalized medicine” in the twenty-first century.

1.2 Renewable Cell Sources

Pluripotent stem cells have unlimited self-renewal capacity and can generate all somatic cell types of the human body. In contrast to pluripotent cells, multipotent neural stem cells (NSCs) grown as neurospheres are characterized by more limited proliferative and developmental potentials [10, 11]. Research involving human ES cells over the last decade has helped to establish and define culture conditions that maintain human pluripotency ex vivo for extended periods of time under defined cell culture conditions [7, 12–14]. Significant progress has been made in characterizing the molecular circuitry of transcription factors, epigenetic regulators, and signal transduction pathways that maintain the pluripotent state in human ES cells [15–17]. For instance, the transcription factors OCT4, SOX2, and NANOG form an interconnected auto-regulatory circuitry controlling chromatin structure and gene expression signatures of pluripotency. In parallel, the field of nuclear reprogramming made continued progress by demonstrating that pluripotency can be induced in various somatic cells by cell fusion or improved methods of somatic cell nuclear transfer [18–21]. These approaches clearly established that factors present in the cytoplasm of pluripotent cells or mammalian oocytes can reprogram the nucleus of fully differentiated cell into an embryonic-like state. Yamanaka and colleagues were the first to demonstrate in seminal experiments that pluripotency can be induced in skin fibroblast by transient expression of four transcription factors: OCT4, SOX2, KLF4, and C-MYC [8, 22]. It was then reported that forced expression of a different set of transcription factors (OCT4, SOX2, NANOG, LIN28) can also gives rise to iPS cells [23]. Reprogramming by defined transcription factors and production of iPS cells is a robust and straightforward method and has been reproduced by a number of different laboratories exploiting viruses and virus-free gene delivery techniques. Understanding the iterative molecular processes governing successful nuclear reprogramming is currently a major effort in stem cell biology. From a developmental biology perspective it still remains extraordinary that the combined action of only four transcription factors can result in such a dramatic change of cellular fate and identity.

Detailed characterization of iPS cells has firmly established their pluripotent nature in various available assays for mouse and human cells. Tetraploid complementation is the most stringent assay for pluripotency. While this experiment is obsolete for human cells due to ethical reasons, mouse iPS cells passed this pluripotency test [24]. Ongoing work is currently revealing similarities and differences between ES and iPS cells and it is likely that these findings will reflect the biological variability among pluripotent cell lines [25]. Nevertheless, it is critical to establish the safety and genomic stability of iPS cells generated from a number of different cell types and different biological ages. The integration-free delivery of transcription factors during the reprogramming progress is critical in order to avoid insertional mutagenesis and oncogene reactivation. Rapid technical progress made over the last years indicates that virus-free reprogramming approaches will be widely used in the near future. Similarly, humanized cell culture conditions are

necessary in order to increase safety and propagate newly derived iPS cells under xeno-free conditions avoiding the risk of cross-species infections.

Allogeneic organ transplantations are traditionally associated with life-long immunosuppressive therapy. Since the derivation and application of patient-specific iPS cells holds great promise for autologous cell therapy, it is tempting to speculate that this strategy would obviate the immune rejection problem. However, a recent study reported immunogenicity of undifferentiated iPS cells upon grafting into syngeneic mice [26]. This cautionary observation is important but it remains unclear why immunogenicity was triggered in mice with the same genetic background. From a clinical perspective, undifferentiated iPS cells would not be direct candidates for cell therapy and it remains to be shown if grafting of xeno-free fully differentiated iPS cell-derived progeny can also lead to an immunological response.

1.3 Neural Differentiation Strategies for Pluripotent Cells

Scientists are now able to create iPS cells in unlimited numbers and these cells share many characteristics with human ES cells. However, our current knowledge regarding directed differentiation is still insufficient for fully utilizing pluripotent cells for clinical applications. It can be summarized that after more than a decade of research with human ES cells, robust and reproducible differentiation protocols have not been established.

Neural differentiation can be described as a process occurring in three main steps: (1) neural induction; (2) neural specification/patterning; (3) terminal differentiation. During gastrulation the three germ layers (ectoderm, endoderm, mesoderm) are formed in the early embryo. Neural induction describes the key event by which pluripotent cells enter the neural lineage [27]. Embryoid bodies (EBs) are free-floating cellular aggregates derived from pluripotent cells emulating the gastrulation process by generating precursors of the three germ layers in vitro. The use of EBs yields a heterogeneous mixture of cells in an uncontrolled fashion thereby providing lineage-restricted precursors at relatively low efficiencies. Plating of EBs on coated substrates and the subsequent spontaneous formation of neural rosettes have been used to select for neural precursors [28]. Alternatively, cell surface markers can be used to isolate neural precursors for further expansion [29]. Earlier work showed that co-culture of human ES cells with murine stromal cells can promote neural induction but this strategy is also inefficient, variable, and protracted [30]. Furthermore, high concentrations of recombinant Noggin and/or small molecule inhibitors of transforming growth factor-beta (TGFb) pathways have been shown to increase the efficiency of neural induction [31–34]. Although the more recent efforts to replace recombinant proteins by small molecules represent an important practical progress towards more defined neural induction protocols, the molecular mechanism of human neural induction as modeled by pluripotent cell lines is far from being understood. Specifically, the signal

transduction pathways and factors that positively and negatively control these processes remain elusive and have not been studied systematically.

Once formed, the neural tube undergoes specification/patterning along the rostro-caudal and dorso-ventral axis in the developing embryo. Neural patterning is guided by secreted morphogens that form gradients across the neural tissue thereby inducing specific transcription factors and neural phenotypes. The principles of neural patterning using human pluripotent cell lines are based on knowledge accumulated on animal models and require validation using human cells [27]. The potency of morphogenetic factors such sonic hedgehog (SHH), fibroblast growth factor 8 (FGF8), and retinoic acid (RA) has been confirmed in the context of human neural precursors. However, because of the large variability of published neural patterning protocols, it is important to strive for a more standardized approach for the use of morphogenetic factors (i.e. effective concentrations, treatment duration, appropriate developmental stage). Replacing recombinant proteins by small molecules is highly desirable with regard to standardization and large-scale applications and should be the main goal in all stages of neural differentiation including terminal synaptic differentiation. Robust and reproducible differentiation protocols will ensure the generation of pure populations of specific neuronal, astroglial, and oligodendroglial cells. It should be emphasized that the reproducible generation of astroglia and oligodendroglia from pluripotent cells is particularly challenging and the molecular pathways involved are poorly understood [35]. For instance, a recent study suggested that prolonged cultivation of up to 180 days is necessary to produce immature astrocytic cells from pluripotent cells [36].

1.4 Biomaterials and Disease Modeling

Stem cells in developing and adult organisms are thought to reside in highly specialized niches, which directly affect their survival, regulation, and physiological function [37]. This complex 3-dimensional microenvironment is defined by signals mediated by cell–cell contact as well as diffusible factors. There is increasing awareness that ordinary in vitro cell culture conditions fall short in providing the appropriate physico-chemical context for stem cell growth and differentiation. In fact, stem cell-based therapeutics including cell replacement, tissue engineering, and organogenesis may require the exploitation of versatile biomaterials. Hence, a more integrative approach that combines stem cell biology with other disciplines such as bioengineering will leverage effective cell-based therapies [38]. The realization of the importance of the stem cell niche has already spurred the design and application of biomaterials and experimental platforms in order to model specific aspects of the in vivo environment in high throughput [39]. The combined use of biodegradable matrices and cytokines presented to developing cells as spatially arranged gradients is likely to play important roles in tailoring personalized therapies. Similarly, to repair large parenchymal cavities after cystic

degeneration of the developing brain (e.g. stroke), it might be necessary to implant stem/progenitor cells that are seeded on biodegradable matrices [40].

There is great excitement about the potential of iPS cells not only for cell replacement but also for disease modeling. Although iPS cell-based disease modeling is a newly evolving field with many open questions, the rapid progress that has been made is remarkable and encouraging. For instance, a flurry of recent reports has demonstrated that cellular pathology of human diseases can be modeled by using disease- and patient-specific iPS cells [41–45]. The rationale behind in vitro disease modeling is to identify a cellular phenotype associated with disease and to correct this phenotype or defect by drugs, genome editing, or other interventions. Improved genetic techniques allow site-specific targeting of the human genome with zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs) [46, 47]. Manipulation of defined genetic loci will facilitate the concurrent design of loss- and gain-of-function experiments using human pluripotent cells. As a consequence, disease modeling can be performed under genetically defined conditions with isogenic pluripotent cell lines further increasing the confidence into the cellular assay and the observed phenotype [48]. Nevertheless, the genetic defect alone may not be sufficient to reveal the disease phenotype during the time frame of an in vitro experiment. Pluripotent cells typically give rise to young neurons and further maturation may require prolonged cultivation, which would be a limiting factor for practical applications. To enhance synaptic differentiation, neuronal differentiation protocols may benefit from more complex cell culture conditions and biomaterials. Furthermore, since the aging process is a major risk factor for many neurodegenerative diseases, application of cellular stressors might be useful to mimic the aging process in a dish [49, 50].

1.5 Cell-based Gene and Drug Therapy

Widespread cellular engraftment into the CNS has been demonstrated for immortalized neural cell lines, progenitor cells isolated from the developing fetal brain, and neural precursors derived from human ES cells [51–55]. It is remarkable that these cells remain highly migratory upon transplantation into the normal and lesioned brain. In fact, it has been reported that grafted and endogenous NSCs preferentially home to sites of brain injury [56, 57]. Molecules secreted during the inflammatory response by immune competent cells (e.g. microglia) and astroglia are the likely chemo-attractant candidates in this process. For instance, the chemokine stromal cell-derived factor 1-alpha (SDF1-α) has been shown to play an important role in attracting NSCs to pathology [58]. Animal models of various lysosomal storage diseases and myelination defects benefit from widespread engrafted NSCs [54, 55, 59]. StemCells Inc., a California-based company, initiated Phase I clinical trials for Batten disease and Pelizaeus-Merzbacher disease using their proprietary HuCNS-SC, a cell line originally derived from human fetal brains [59].

The migratory potential of NSCs together with their amenability for genetic manipulation offers unique opportunities for combining gene and cell therapy [60].

Hence, migratory NSCs expressing foreign genes after ex vivo genetic manipulation can be exploited for targeted therapies of neurodegenerative as well as neoplastic diseases. In elegant experiments, Aboody and colleagues demonstrated the utility of migratory NSCs expressing cytosine deaminase as a vehicle for chemotherapy of invasive glioma [56]. This enzyme converts a nontoxic prodrug into a powerful chemotherapeutic compound (5-flurouracil) allowing highly selective killing of cancer cells and brain tumor-forming cells ("cancer stem cells") chased by engrafted NSCs. Glioblastoma multiforme is the most malignant human cancer characterized by highly invasive growth, lack of adequate treatment, and poor survival rate. Stem cell-based therapy holds great promise to treat high-grade human gliomas and nonneural cancers that have established brain metastasis (e.g. breast cancer). Clinical trials are currently under way investigating these therapeutic opportunities [61].

1.6 Challenges of Clinical Cell Therapy

To develop innovative therapies such as cell replacement, it is essential to perform extensive preclinical testing in animal models. Robust rodent disease models, for instance, the Parkinson's disease (PD) rat model with unilateral striatal lesions after 6-hydroxy-dopamine injection into the medial forebrain bundle, has proven highly valuable and biologically informative for cell transplantation and behavioral evaluation [62]. Once the therapeutic modality under investigation has shown salutary effects in rodents, consideration of nonhuman primate models would be the next step in the translational process towards clinical application. Careful assessment of cell therapies in nonhuman primate models is particularly important considering the enormous differences regarding organ size and anatomy between human and rodent brains.

If stem cell-based therapies are going to enter the clinical stage, preclinical tests have to provide strong evidence that benefits will outweigh the potential risks. However, some potential risks will remain until results of independent multicenter clinical trials are collected over time and conclusively analyzed with regard to safety, efficiency, and reproducibility. Ongoing efforts to coordinate and better understand fetal tissue grafting in PD will be highly valuable for future stem cell-based projects (http://www.transeuro.org.uk/). In general, it is challenging to employ a clinical cell transplantation study considering patient recruitment, sample size, and standardization of procedures. For instance, there is ongoing debate if clinical cell transplantation for PD should be carried out as open-label versus double-blind placebo-controlled trials [63].

Cell therapy of neurodegenerative disorder requires rigorous safety standards according to the guidelines of the US Food & Drug Administration (FDA). If primordial cells such as highly proliferative pluripotent stem cells are the parental source of grafted cells, any residual undifferentiated cell that remains in a cell suspension harbors the risk for tumor formation and uncontrolled growth as shown in an animal model of PD [64]. Therefore, establishing differentiation strategies

that generate homogenous cell populations at high purity and defining surface marker-based cell sorting protocols are preeminent safety requirements. The company Geron Corp., in Menlo Park (California, USA) set out to establish safety standards for human ES cell-derived oligodendrocytes for spinal cord-injured patients in a clinical trial approved by the FDA. Unexpectedly, Geron terminated recently that clinical trial because of financial considerations.

Unbiased and accurate assessment of the efficacy of cell therapy poses another challenge in the translational process. The selection of the appropriate patient cohort is as important as the timing of grafting. Depending on the disease and patient history, functional and behavioral assays should be established, analyzed, and validated over longer periods of time. In general, investigating the biology of human behavior is a challenging task. Treatment efficacy should be routinely monitored by clinical criteria, functional clinical imaging (e.g. positron emission tomography with ^{18}F-fluoro DOPA in PD) and ultimately by detailed postmortem analysis of the tissue using electrophysiological and histological techniques. Unambiguous histological detection of transplanted cells expressing a reporter gene would be ideal to evaluate the extent of cell survival, integration into the host tissue, and anatomical restoration. As mentioned above, uncontrolled growth of grafted cells can be a major problem with deleterious consequences. Another adverse event of cell therapy might be that grafted cells establish improper synaptic contacts or impair existing neural circuitries. For instance, graft-induced dyskinesia in PD has been reported as a serious complication of cell therapy [65]. It has been suggested that unwanted co-transplantation of serotoninergic cells with DA neurons and the uneven distribution of grafts forming DA releasing "hotspots" might favor the development of dyskinesia [66, 67].

Depending on the underlying disease, the most adequate route of administration and gentile cell-delivery techniques need to be determined in order to maximize survival of grafted cells and minimize injury to the host tissue [68]. Neurodegenerative diseases are often accompanied by microglia activation and chronic inflammation [69]. Although the brain is an immune privileged organ, chronic inflammation and the acute trauma caused by cell transplantation can disrupt the blood–brain barrier. Therefore, it is reasonable to believe that the host environment and the inflammatory response are important determinants of cell survival and functional integration after transplantation. Besides inflammation, the transfer of potential toxic products from host cells to grafted cells has been debated as another possible mechanism by which neurotransplantation might be compromised. For instance, there is ongoing debate about the role of alpha-synuclein inclusions and Lewy bodies that appear in grafted cells in PD patients over time [70–72]. Depending on the source of the graft, another mechanism for poor graft survival might be immune rejection and has been debated extensively in the PD literature. Immunosuppression for 6–12 months is considered to be an important parameter for successful cell therapy treatment [67]. However, the advent of iPS cell technology and generation of patient-specific cells might overcome this problem in the future. Finally, experiments in Parkinsonian rats have suggested that considering the patient's hemispheric dominance and postoperative rehabilitation are important

parameters for maximizing cell therapeutic strategies and restoring complex sensorimotor functions [62].

1.7 Neurological Diseases

Increasing evidence suggests that neuronal and synaptic dysfunction in neurodegenerative diseases are chronic and protracted processes occurring over a long period of time. Impaired neurotransmission and excitotoxic insults often precede the manifestation of clinical symptoms and can culminate in widespread apoptotic cell loss. Plausible models have been suggested that describe how chronic synaptic dysfunction can progressively develop into severe stages of neurodegeneration [73–75]. Patients suffering from PD show first clinical signs when more than 70 % of the dopamine (DA) neurons in the substantia nigra are already lost. Therefore, it is crucial to search for new diagnostic methods and detect CNS diseases in their early stages. Disease-specific markers would offer opportunities to halt or slow down disease progression at a preclinical stage. In fact, therapeutic interventions at critical early time points might even be the most advantageous strategy for correcting synaptic dysfunction by grafted cells capable of delivering growth factors and neuroprotective molecules such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF1), and glial-derived neurotrophic factor (GDNF).

I will next discuss prototypical neurodegenerative diseases that may benefit from cell therapies and iPS cell-based disease modeling. It is important to note that in each of these CNS diseases different cell types, anatomical compartments, and neurotransmitter systems are affected. Hence, future cell therapy strategies need to be tailored and firmly established considering the patient's history and the pathophysiology of the targeted disease. For ongoing clinical trials using NSC-based therapies see also in Aboody et al. [61].

1.7.1 Alzheimer's Disease

In 1906, the German psychiatrist and neuropathologist Alois Alzheimer described the clinical and histopathological hallmarks of a disease that was later named after him. Alzheimer's disease (AD) is the most common neurodegenerative disease worldwide. According to the Alzheimer's Association more than 5 million patients are currently diagnosed with AD in the USA alone. As a consequence, more than $170 billion are spent annually for the health care costs of AD (http://alz.org/) [76]. There is no cure for AD and effective treatment options are not available. Clinically, AD is characterized by progressive impairment of cognitive function, memory loss, and dementia. Although the precise molecular underpinnings of AD are not conclusively understood, the disease is associated with the formation

of neurofibrillary tangles ("hyperphosphorylated tau protein") and amyloid plaques and the unequivocal diagnosis can only be confirmed by directly analyzing postmortem brain tissue. Early onset and late onset types of AD can be distinguished based on the manifestation of the disease before or after the age of 60. In general, a positive family history and age are risk factors for AD. Some genetic risk factors that support the excess formation of amyloid protein in the brain ("amyloid hypothesis") have been linked to early onset AD. For instance, in Down syndrome (Trisomy 21) patients carry an extra copy of the amyloid precursor protein (APP) which leads to histopathological changes reminiscent of AD and dementia before the age of 40 [77]. Late onset or sporadic AD is the most common type and the genetic and environmental causes are not well understood. Progressive neuronal and synaptic loss in AD is widespread in neocortical and subcortical areas (i.e. basal forebrain, hippocampus, amygdala) with resultant atrophy in the frontal, temporal, and parietal lobes. Therefore, targeting multiple neurotransmitter systems and brain regions with cell therapy must be considered for AD. In addition to replacing lost neuronal cells and synaptic networks, neurotrophic factors secreted by grafted cells might contribute to the rescue of injured host neurons or modulate the rate of adult hippocampal neurogenesis [78–82]. Mild cognitive impairment occurs at early stages of AD and detecting this clinical stage might represent an opportunity for cell therapeutic interventions. On the other hand, it is plausible that the efficacy of any cell transplantation approach could be masked when treating late-stage patients with irreversible pathologies [76]. This again underscores the importance of timing and patient selection as important factors of cell therapy.

Patient-specific iPS cells have been generated from individuals with AD [83]. As an alternative to pluripotent cells, direct generation of specific neuronal subtypes by defined transcription factors has emerged as an interesting new approach [84, 85]. A recent paper has demonstrated the utility of this strategy by converting AD patient fibroblasts with specific mutations into functional neurons and demonstration of increased amyloid production by affected cells [86]. This study exemplifies the utility of direct lineage conversion for disease modeling but scalable protocols for producing a variety of cellular phenotypes are required for cell replacement. Therefore, stem cell therapy for AD is a very challenging goal due to the widespread nature of the lesions and cell loss. It is also important to consider that ongoing inflammation and toxicity due to resident amyloid plaques might impact the survival and function of cells grafted into a "hostile" environment [69].

1.7.2 Parkinson's Disease

PD is the second most common neurodegenerative disease affecting 1–2 % of the population over the age of 60. More than 95 % of the cases are sporadic and with unknown cause, whereas 1–5 % are familial and linked to genetic mutations [87]. PD leads to severe impairments of motor function reflected by the cardinal

symptoms bradykinesia, rigidity, and resting tremor. Neuropathologically, a selective and progressive loss of DA neurons of the substantia nigra pars compacta (A9) in the ventral mesencephalon results in insufficient DA concentrations in the dorsal striatum (caudate-putamen), the axonal target region of these DA neurons [88]. Figure 1.1 illustrates the complex DA system of the mesencephalon and caudate-putamen of a nonhuman primate. Although pathological changes have been reported in other systems including the olfactory bulb and enteric nervous system [89, 90], the main motor deficits in PD are owing to a deteriorated nigrostriatal projection. Treatment with L-dopa and deep brain stimulation in the subthalamic nucleus are currently the standard therapy options for PD to alleviate motor symptoms [91]. The potent drug L-dopa, which is the precursor of DA, loses its efficacy over time and may induce undesirable side effects such as involuntary dyskinetic movements [92]. Based on remarkable results with grafted fetal midbrain tissues in the early 1990s, cell transplantation and restoration of striatal DA levels has emerged as a rationale therapy [67]. A major goal of cell therapy strategies in PD is to overcome the limited availability of midbrain-type DA neurons and to circumvent the ethical issues that are inherent to working with fetal grafts. Since pluripotent cells can be propagated indefinitely under appropriate culture conditions and differentiated into DA neurons, currently this cell source appears to be the most promising approach with immediate clinical relevance.

Generation of DA neurons from mouse and human ES cells and functional grafting in rodent models of PD has been demonstrated in earlier work [30, 64, 93]. Similar transplantation studies have been recapitulated with DA neurons derived from mouse and human iPS cells [94–96]. More recently, induced DA (iDA) neurons were generated by forced expression of the transcription factors MASH1, NURR1, and LMX1A in mouse and human fibroblasts. This technology seems to allow transition from one cell type to another without reverting cells to a progenitor cell state [97, 98]. This approach is promising but the absence of self-renewing progenitor cells may pose limitations for cell replacement requiring large numbers of homogenous cell types. In addition, virus-free methods need to be established for efficient transcription factor delivery along with documentation of long-term phenotypic and genetic stability of induced neurons. Eventually, it remains to be shown if human DA neurons, derived either by reprogramming or direct lineage conversion (i.e. iDA neurons), are functional in primate models of PD and avoid adverse events such as graft-induced dyskinesia [65–67]. Optimizing cell viability and widespread synaptic integration of grafted cells are key issues that will strongly impact the outcome of transplantation studies in large animal models.

1.7.3 Huntington's Disease

Huntington's disease (HD) was first described by George Huntington in 1872 and is a devastating genetic disorder with autosomal-dominant inheritance. It typically manifests in middle age with motor and behavioral symptoms such as abnormal

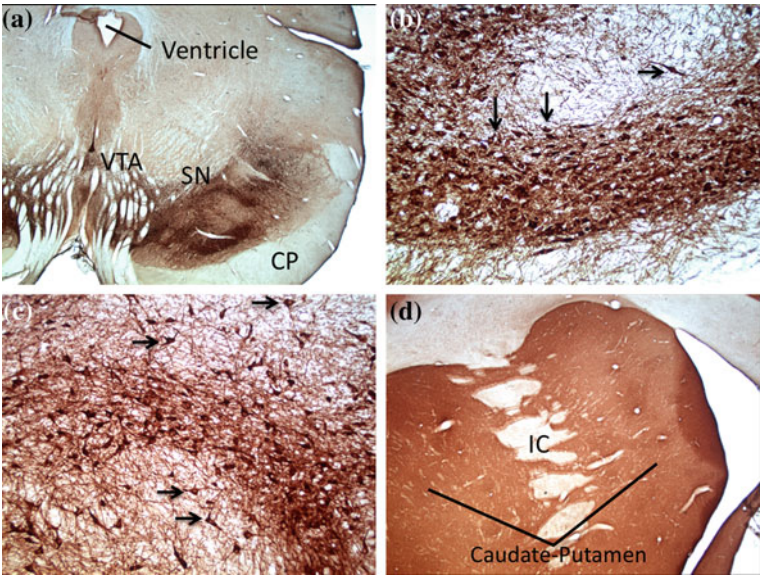

Fig. 1.1 The dopaminergic system of the midbrain (**a**–**c**) and caudate-putamen (**d**) in the monkey brain. Immunocytochemistry for tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis, illustrates the complexity of the dopaminergic system. Dopamine neurons of the substantia nigra (arrows in **b** and **c**), which are lost in Parkinson's disease, project their axons over long distances and innervate the caudate-putamen. Histological samples are from a 4-years-old vervet monkey (*Cercopithecus aetiops*) and were kindly provided by Prof. B. Volk and Prof. D. Neumann-Haefelin, University of Freiburg, Germany. Abbreviations: *VTA*, ventral tegmental area; *SN*, substantia nigra; *CP*, cerebral peduncle; *IC*, internal capsule. Magnification: **a**, **d**, 2.5; **b**–**c**, 10

involuntary jerking and writhing movements called chorea. The progressive decline of cognitive function leads to dementia and major psychiatric problems over the time course of 10–20 years after disease onset. The genetic mutation on chromosome 4 encompasses increased CAG repeats and dysfunction of the Huntingtin (HTT) gene. The three bases cytosine, adenine, and guanine (CAG) code for the amino acid glutamine and the number of CAG repeats correlate with the severity of disease symptoms, with CAG repeats in the range of 40–120 as being pathological [99]. As a consequence, some cortical neurons and selectively vulnerable medium-sized spiny neurons expressing the neurotransmitter γ-aminobutyric acid (GABA) undergo cell death in the striatum (caudate nucleus and putamen). These striatal neurons also co-express the specific marker DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein, 32 kDa) and the neuropeptides enkaphalin or substance P [5]. Impaired BDNF provision to these striatal neurons via corticostrial projections has been shown as an additional pathogenetic mechanism [100]. The availability of iPS cells from HD patients offers novel perspectives to understand cellular and molecular disease mechanisms [83].

Currently, there is no cure for HD and treatment options for HD are primarily focused on symptomatic relief with neuropsychiatric drugs (antipsychotics, antidepressants), speech therapy, and physical rehabilitation. Rodent and nonhuman primate models of HD that are based on lesioning the striatum with excitotoxins (i.e. ibotenic acid, quinolinic acid) showed encouraging cell transplantation results and led to early phase clinical trials [101]. These open-label clinical trials were performed over the last decade and indicated slowed disease progression when grafting fetal striatal neuroblasts derived from ganglionic eminences at 7–10 weeks postconception [102]. Although beneficial effects were transient and observed in only a few HD patients, this experience is a valuable foundation for future cell replacement strategies [102–104]. At present, practical problems such as finding a renewal source for donor cells of the correct phenotype, standardization of procedures, and criteria of patient selection are the main obstacles that need to be overcome before personalized cell transplantation regimens for HD can move toward the clinic.

1.7.4 Motor Neuron Diseases

Amyotrophic lateral sclerosis (ALS), also called Lou Gehrig's disease, is a devastating neurodegenerative affliction affecting large motor neurons (MNs) localized in the primary motor cortex (upper MNs) and ventral horns of the spinal cord (lower MNs). The prognosis for ALS is poor and more than 90 % of the patients die within 2–5 years after diagnosis. The vast majority of the cases are sporadic and the underlying cause is unknown. Of the familial cases ($\sim$10 %), missense mutations in the Cu/Zn superoxide dismutase gene SOD1 have provided important insights into the pathogenesis of ALS. Oxidative stress, mitochondrial dysfunction, and excitotoxic damage are likely to be important mechanisms that ultimately lead to MN death [105, 106]. Importantly, it turned out that cell autonomous deficits of MNs are not the only pathogenetic cause of ALS. In fact, astrocytes neighboring MNs critically support their health and function but can also mediate noncell autonomous toxic effects during disease and thereby contribute to MN loss [106–109]. Functional MNs have been generated from mouse and human ES cells [110, 111]. Neuronal cells sharing molecular characteristics with *bona fide* MNs (e.g. expression of transcription factor HB9, choline acetyltransferase) were generated also by exploiting iPS cells and transcription factor-based induced neurogenesis [112, 113]. Together, these cells generated ex vivo are highly valuable for disease modeling and drug discover but it is currently unclear if MN replacement per se might be a realistic clinical approach. To restore functional motor circuits, de novo formation of long-distance axonal projections would be necessary to innervate appropriate muscle fibers in the periphery. Reconstruction of motor pathways is particularly challenging in the molecular environment provided the adult mammalian CNS. Surface molecules expressed by oligodendrocytes (e.g. Nogo proteins) inhibit axonal growth after injury [114]. Nevertheless, transplantation of MNs or glial cells that exert

neuroprotective and trophic effects might be within the realm of possibility. It is promising to see that adult mammalian motor pathways in acute rodent models of MN damage can be repaired with fetal and mouse ES cells when exploiting cell types with correct identities implanted at the appropriate time point [115–117].

Spinal muscular atrophy (SMA) is an autosomal recessive disorder representing the second most common MN disease. Four different types of SMA are known with SMA type I, also called Werdnig-Hoffmann disease, being the most severe form. Mutations of the survival of motor neuron (SMN1) gene and reduced protein expression levels are the underlying cause of SMA and severity of disease correlates with the degree of muscle weakness and early infantile onset. In contrast to ALS, MNs in the ventral horns of the spinal cord (lower MNs) are selectively affected in SMA [118]. Typically, clinical symptoms such as muscle weakness, reduced muscular tone, and progressive muscular atrophy manifest in infants. The use of iPS cells is an attractive approach to better understand disease mechanisms that lead to MN death in SMA patients. Ebert et al. [42] established iPS cell lines from patients with type I SMA, differentiated them into MNs, and observed progressive loss of these cells in vitro. This cellular assay might be useful for developing novel drugs or studying off-target effects of currently used compounds. To date, there is no effective treatment for SMA and current options are supportive in that they alleviate some disease symptoms with physical therapy and rehabilitaton, ventilators due to respiratory problems, and other types of continued medical care. Similar to the inherent problems mentioned above for ALS, cell therapy for SMA has also to consider the complex nature of motor pathways and axonal projections. It remains to be shown if replacement of MNs, supportive cells (e.g. glial cells) secreting neurotrophic factors, or gene therapeutic approaches can exert beneficial effects in SMA patients.

1.7.5 Retinal Diseases

The human retina is a highly organized multilayered tissue composed of various specific cell types (e.g. photoreceptors, interneurons, Müller glia, retinal pigment epithelium [RPE]). Because of the limited self-repair capacity of the retina, stem cell therapy is a very promising approach to restore visual function or prevent blindness [119–121]. Diseases that may benefit from stem cell therapy include retinitis pigmentosa, diabetic retinopathy, and age-dependent macular degeneration (AMD). Progress has been made in generating RPE and photoreceptors from pluripotent stem cells but these stepwise protocols need further optimization and standardization in order to increase efficiency, cell purity, and safety [122–124]. Although a cell therapy approach for highly specialized phenotypes such as photoreceptors is very challenging due to the intricate anatomical organization of the retina, these cells may indirectly benefit from the paracrine and trophic effects imparted by stem cells [121]. On the other hand, direct cell replacement and functional integration of stem cell-derived RPE is a very promising strategy. AMD is the most common cause of

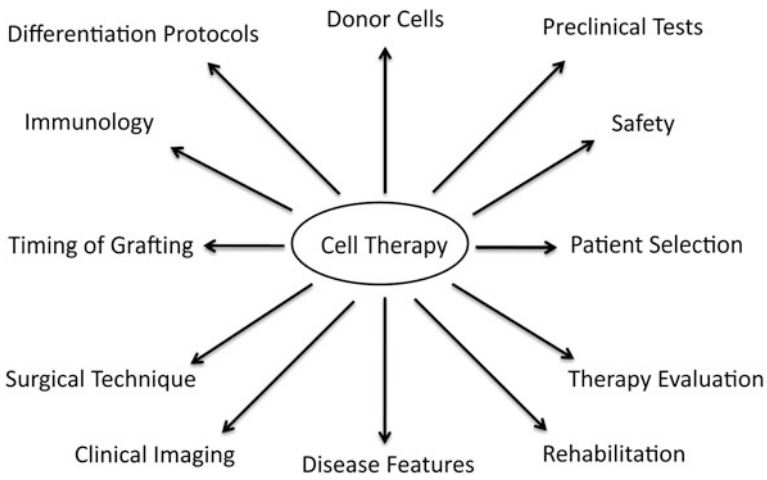

Fig. 1.2 The multiple challenges of cell therapy. Significant progress has been made over the last years regarding the various aspects of cell therapy (e.g. iPS cells as potential patient-specific donor cells). However, further multidisciplinary collaborations involving scientists and clinicians are necessary to advance cell therapies for neurodegenerative disorders

blindness in people over the age of 50. RPE cells are the specific cell type affected in this disease. Anatomically, the RPE is composed of polarized cells located between the photoreceptors and the choroid and maintain visual function by serving as a blood-retina barrier. Since RPE cells exist in a single monolayer that is surgically accessible, the efficient and controlled generation of these cells from iPS cells could pave the way to a rational autologous cell replacement therapy in the near future [121]. In 2010, the FDA has approved a clinical trial using human ES cell derived RPE cells for the treatment of dry AMD [125].

1.8 Outlook

Significant advances occurring across scientific disciplines now provide encouraging momentum for developing stem cell-based treatments for neurodegenerative disorders. Personalized therapies for intractable human diseases are no longer a distant and insurmountable scenario. It is conceivable that combined gene and cell therapy strategies might be designed and routinely offered to large patient cohorts with defined disorders that have been characterized with a number of diagnostic tools including high-throughput whole-genome sequencing, standardized cellular assays, and high-resolution functional imaging in the clinic. Integrating different cutting-edge technologies and data sharing will play crucial roles for leveraging personalized medicine. Comprehensive medical centers for cell therapy should be established in which collaboration and exchange between scientists and physicians

should speed up the efficient translation and application of breakthroughs in basic research into clinical trials. Currently, a number of challenges need to be solved as discussed and exemplified in this chapter using common neurodegenerative disorders (Fig. 1.2). For instance, to maximize the functional outcome of cell therapy it is critical to carefully select patients, generate enough numbers of relevant human cell types, and safely apply them in the appropriate time window. It is certain that the cell therapy field of the upcoming years will remain highly vibrant with regard to accumulating valuable data in clinical trials and opening up new diagnostic and therapeutic vistas.

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References

1. Bhardwaj RD, Curtis MA, Spalding KL, Buchholz BA, Fink D, Bjork-Eriksson T, Nordborg C, Gage FH, Druid H, Eriksson PS, Frisen J (2006) Neocortical neurogenesis in humans is restricted to development. Proc Natl Acad Sci USA 103:12564–12568
2. Geerts H (2009) Of mice and men: bridging the translational disconnect in CNS drug discovery. CNS Drugs 23:915–926
3. Dawson TM, Ko HS, Dawson VL (2010) Genetic animal models of Parkinson's disease. Neuron 66:646–661
4. Nestler EJ, Hyman SE (2010) Animal models of neuropsychiatric disorders. Nat Neurosci 13:1161–1169
5. Ramaswamy S, McBride JL, Kordower JH (2007) Animal models of Huntington's disease. ILAR J 48:356–373
6. Trancikova A, Ramonet D, Moore DJ (2011) Genetic mouse models of neurodegenerative diseases. Prog Mol Biol Transl Sci 100:419–482
7. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282: 1145–1147
8. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
9. Jain S, Heutink P (2010) From single genes to gene networks: high-throughput-high-content screening for neurological diseases. Neuron 68:207–217
10. Singec I, Knoth R, Meyer RP, Maciaczyk J, Volk B, Nikkhah G, Frotscher M, Snyder EY (2006) Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. Nat Methods 3:801–806
11. Singec I, Quiñones-Hinojosa A (2008) Neurospheres. In: Gage FH, Kempermann G, Song H (eds) Adult neurogenesis, vol 52, Cold Spring Harbor Laboratory Press. doi: 10.1101/087969784.52.119
12. Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, Smuga-Otto K, Howden SE, Diol NR, Propson NE, Wagner R, Lee GO, Antosiewicz-Bourget J, Teng JM, Thomson JA (2011) Chemically defined conditions for human iPSC derivation and culture. Nat Methods 8:424–429
13. Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, Crandall LJ, Daigh CA, Conard KR, Piekarczyk MS, Llanas RA, Thomson JA (2006) Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 24:185–187

14. Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA (2005) Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods 2:185–190
15. Meissner A (2010) Epigenetic modifications in pluripotent and differentiated cells. Nat Biotech 28:1079–1088
16. Pera MF, Tam PP (2010) Extrinsic regulation of pluripotent stem cells. Nature 465:713–720
17. Young RA (2011) Control of the embryonic stem cell state. Cell 144:940–954
18. Byrne JA, Pedersen DA, Clepper LL, Nelson M, Sanger WG, Gokhale S, Wolf DP, Mitalipov SM (2007) Producing primate embryonic stem cells by somatic cell nuclear transfer. Nature 450:497–502
19. Cowan CA, Atienza J, Melton DA, Eggan K (2005) Nuclear reprogramming of somatic cells after fusion with human embryonic stem cell. Science 309:1369–1373
20. Egli D, Rosains J, Birkhoff G, Eggan K (2007) Developmental reprogramming after chromosome transfer into mitotic mouse zygotes. Nature 447:679–685
21. Hochedlinger K, Jaenisch R (2002) Monoclonal mice generated by nuclear transfer from mature B and T donor cells. Nature 415:1035–1038
22. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
23. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920
24. Zhao XY, Li W, Lv Z, Liu L, Tong M, Hai T, Hao J, Guo CL, Ma QW, Wang L, Zeng F, Zhou Q (2009) iPS cells produce viable mice through tetraploid complementation. Nature 461:86–90
25. Bock C, Kiskinis E, Verstappen G, Gu H, Boulting G, Smith ZD, Ziller M, Croft GF, Amoroso MW, Oakley DH, Gnirke A, Eggan K, Meissner A (2011) Reference Maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. Cell 144:439–452
26. Zhao T, Zhang ZN, Rong Z, Xu Y (2011) Immunogenicity of induced pluripotent stem cell. Nature 474:212–215
27. Gaulden J, Reiter JF (2008) Neur-ons and neur-offs: regulators of neural induction in vertebrate embryos and embryonic stem cells. Hum Mol Genet 17(R1):R60–66
28. Zhang SC, Wernig M, Duncan ID, Brüstle O, Thomson JA (2001) In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechol 19: 1129–1133
29. Pruszak J, Sonntag KC, Aung MH, Sanchez-Pernaute R, Isacson O (2007) Markers and methods for cell sorting of human embryonic stem cell-derived neural cell populations. Stem Cells 25:2257–2268
30. Perrier AL, Tabar V, Barberi T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L (2004) Derivation of midbrain dopamine neurons from human embryonic stem cells. Proc Natl Acad Sci USA 101:12543–12548
31. Pera MF, Andrade J, Houssami S, Reubinoff B, Trounson A, Stanley EG, Ward-van Oostwaard D, Mummery C (2004) Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. J Cell Sci 117:1269–1280
32. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L (2009) Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol 27:275–280
33. Kriks S, Shim, JW, Piao J, Ganat YM, Wakeman DR, Xie Z, Carrillo-Reid L, Auyeung G, Antonacci C, Buch A, Yang L, Beal MF, Surmeier DJ, Kordower JH, Tabar V, Studer L (2011) Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. Nature 480(7378):547–551 doi: 10.1038/nature10648
34. Smith JR, Vallier L, Lupo G, Alexander M, Harris WA, Pedersen RA (2008) Inhibition of activin/nodal signaling promotes specification of human embryonic stem cells into neuroectoderm. Dev Biol 313:107–117

35. Sharp J, Hatch M, Nistor G, Keirstead H (2011) Derivation of oligodendrocyte progenitor cells from human embryonic stem cells. Methods Mol Biol 767:399–409
36. Krencik R, Weick JP, Liu Y, Zhang ZJ, Zhang SC (2011) Specification of transplantable astroglial subtypes from human pluripotent stem cells. Nat Biotechnol 29:528–534
37. Scadden DT (2006) The stem-cell niche as an entity of action. Nature 441:1075–1079
38. Vunjak-Novakovic G, Scadden DT (2011) Biomimetic platforms for human stem cell research. Cell Stem Cell 8:252–261
39. Gobaa S, Hoehnel S, Roccio M, Negro A, Kobel S, Lutolf MP (2011) Artificial niche microarrays for probing single stem cell fate in high throughput. Nat Methods 8:949–955
40. Park KI, Teng YD, Snyder EY (2002) The injured brain interacts reciprocally with neural stem cells supported by scaffolds to reconstitute lost tissue. Nat Biotechnol 20:1111–1117
41. Carvajal-Vergara X, Sevilla A, D'Souza SL, Ang YS, Schaniel C, Lee DF, Yang L, Kaplan AD, Adler ED, Rozov R, Ge Y, Cohen N, Edelmann LJ, Chang B, Waghray A, Su J, Pardo S, Lichtenbelt KD, Tartaglia M, Gelb BD, Lemischka IR (2010) Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome. Nature 465:808–812
42. Ebert AD, Yu J, Rose FF Jr, Mattis VB, Lorson CL, Thomson JA, Svendsen CN (2009) Induced pluripotent stem cells from a spinal muscular atrophy patient. Nature 457:277–280
43. Lee G, Papapetrou EP, Kim H, Chambers SM, Tomishima MJ, Fasano CA, Ganat YM, Menon J, Shimizu F, Viale A, Tabar V, Sadelain M, Studer L (2009) Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. Nature 461:402–406
44. Liu GH, Barkho BZ, Ruiz S, Diep D, Qu J, Yang SL, Panopoulos AD, Suzuki K, Kurian L, Walsh C, Thompson J, Boue S, Fung HL, Sancho-Martinez I, Zhang K, Yates J 3rd (2011) Izpisua Belmonte, J.C.: Recapitulation of premature ageing with iPSCs from Hutchinson-Gilford progeria syndrome. Nature 472:221–225
45. Yazawa M, Hsueh B, Jia X, Pasca AM, Bernstein JA, Hallmayer J, Dolmetsch RE (2011) Using induced pluripotent stem cells to investigate cardiac phenotypes in Timothy syndrome. Nature 471:230–234
46. Hockemeyer D, Jaenisch R (2010) Gene targeting in human pluripotent cells. Cold Spring Harb Symp Quant Biol 75:201–209
47. Hockemeyer D, Wang H, Kiani S, Lai CS, Gao Q, Cassady JP, Cost GJ, Zhang L, Santiago Y, Miller JC, Zeitler B, Cherone JM, Meng X, Hinkley SJ, Rebar EJ, Gregory PD, Urnov FD, Jaenisch R (2011) Genetic engineering of human pluripotent cells using TALE nucleases. Nat Biotechnol 29:731–734
48. Soldner F, Laganière J, Cheng AW, Hockemeyer D, Gao Q, Alagappan R, Khurana V, Golbe LI, Myers RH, Lindquist S, Zhang L, Guschin D, Fong LK, Vu BJ, Meng X, Urnov FD, Rebar EJ, Gregory PD, Zhang HS, Jaenisch R (2011) Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. Cell 146: 318–331
49. Nguyen HN, Byers B, Cord B, Shcheglovitov A, Byrne J, Gujar P, Kee P, Schule B, Dolmetsch RE, Langston W, Palmer TD, Pera RR (2011) LRRK2 mutant iPSC-derived DA neurons demonstrate increased susceptibility to oxidative stress. Cell Stem Cell 8:267–280
50. Saha K, Jaenisch R (2009) Technical challenges in using human induced pluripotent stem cells to model disease. Cell Stem Cell 5:584–595
51. Renfranz PJ, Cunningham MG, McKay RD (1991) Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. Cell 66:713–729
52. Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billinghurst LL, Jendoubi M, Sidman RL, Wolfe JH, Kim SU, Snyder EY (1998) Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. Nat Biotechnol 16: 1033–1039
53. Englund U, Bjorklund A, Wictorin K, Lindvall O, Kokaia M (2002) Grafted neural stem cells develop into functional pyramidal neurons and integrate into host cortical circuitry. Proc Natl Acad Sci USA 99:17089–17094

54. Lee JP, Jeyakumar M, Gonzalez R, Takahashi H, Lee PJ, Baek RC, Clark D, Rose H, Fu G, Clarke J, McKercher S, Meerloo J, Muller FJ, Park KI, Butters TD, Dwek RA, Schwartz P, Tong G, Wenger D, Lipton SA, Seyfried TN, Platt FM, Snyder EY (2007) Stem cells act through multiple mechanisms to benefit mice with neurodegenerative metabolic disease. Nat Med 13:439–447
55. Windrem MS, Schanz SJ, Guo M, Tian GF, Washco V, Stanwood N, Rasband M, Roy NS, Nedergaard M, Havton LA, Wang S, Goldman SA (2008) Neonatal chimerization with human glial progenitor cells can both remyelinate and rescue the otherwise lethally hypomyelinated shiverer mouse. Cell Stem Cell 2:553–565
56. Aboody KS, Brown A, Rainov NG, Bower KA, Liu S, Yang W, Small JE, Herrlinger U, Ourednik V, Black PM, Breakefield XO, Sndyer EY (2000) Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. Proc Natl Acad Sci USA 97:12846–12851
57. Glass R, Synowitz M, Kronenberg G, Walzlein JH, Markovic DS, Wang LP, Gast D, Kiwit J, Kempermann G, Kettenmann H (2005) Glioblastoma-induced attraction of endogenous neural precursor cells is associated with improved survival. J Neurosci 25:2637–2646
58. Imitola J, Raddassi K, Park KI, Mueller FJ, Nieto M, Teng YD, Frenkel D, Li J, Sidman RL, Walsh CA, Snyder EY, Khoury SJ (2004) Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor1alpha/CXC chemokine receptor 4 pathway. Proc Natl Acad Sci USA 101:18117–18122
59. Tamaki SJ, Jacobs Y, Dohse M, Capela A, Cooper JD, Reitsma M, He D, Tushinski R, Belichenko PV, Salehi A, Mobley W, Gage FH, Huhn S, Tsukamoto AS, Weissman IL, Uchida N (2009) Neuroprotection of host cells by human central nervous system stem cells in a mouse model of infantile neuronal ceroid lipofuscinosis. Cell Stem Cell 5:310–319
60. Jandial R, Singec I, Ames CP, Snyder EY (2008) Genetic modification of neural stem cells. Mol Ther 16:450–457
61. Aboody K, Capela A, Niazi N, Stern JH, Temple S (2011) Translating stem cell studies to the clinic for CNS repair: current state of the art and the need for a Rosetta Stone. Neuron 70:597–613
62. Nikkhah G, Falkenstein G, Rosenthal C (2001) Restorative plasticity of dopamine neuronal transplants depends on the degree of hemispheric dominance. J Neurosci 21:6252–6263
63. Alterman RL, Tagliati M, Olanow CW (2011) Open-label surgical trials for Parkinson disease: time for reconsideration. Ann Neurol 70:5–8
64. Roy NS, Cleren C, Singh SK, Yang L, Beal MF, Goldman SA (2006) Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes. Nat Med 12:1259–1268
65. Lane EL, Björklund A, Dunnett SB, Winkler C (2010) Neural grafting in Parkinson's disease unraveling the mechanisms underlying graft-induced dyskinesia. Prog Brain Res 184:295–309
66. Barker RA, Kuan WL (2010) Graft-induced dyskinesias in Parkinson's disease: what is it all about? Cell Stem Cell 7:148–149
67. Lindvall O, Björklund A (2011) Cell therapeutics in Parkinson's disease. Neurotherapeutics 8:539–548
68. Steiner B, Winter C, Blumensath S, Paul G, Harnack D, Nikkhah G, Kupsch A (2008) Survival and functional recovery of transplanted human dopaminergic neurons into hemiparkinsonian rats depend on the cannula size of the implantation instrument. J Neurosci Methods 169:128–134
69. Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH (2010) Mechanisms underlying inflammation in neurodegeneration. Cell 140:918–934
70. Hansen C, Angot E, Bergström AL, Steiner JA, Pieri L, Paul G, Outeiro TF, Melki R, Kallunki P, Fog K, Li JY, Brundin P (2011) α-Synuclein propagates from mouse brain to grafted dopaminergic neurons and seeds aggregation in cultured human cells. J Clin Invest 121:715–725

71. Kordower JH, Chu Y, Hauser RA, Freeman TB, Olanow CW (2008) Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease. Nat Med 14:504–506
72. Mendez I, Viñuela A, Astradsson A, Mukhida K, Hallet P, Robertson H, Tierney T, Holness R, Dagher A, Trojanowski JQ, Isacson O (2008) Dopamine neurons implanted into people with Parkinson's disease survive without pathology for 14 years. Nat Med 14:507–509
73. Berridge MJ (2010) Calcium hypothesis of Alzheimer's disease. Pflugers Arch 459:441–449
74. Nakamura T, Lipton SA (2010) Redox regulation of mitochondrial fission, protein misfolding, synaptic damage, and neuronal cell death: potential implications for Alzheimer's and Parkinson's diseases. Apoptosis 15:1354–1363
75. Sulzer D (2007) Multiple hit hypothesis for dopamine neuron loss in Parkinson's disease. Trends Neurosci 30:244–250
76. Lunn JS, Sakowski SA, Hur J, Feldman EL (2011) Stem cell technology for neurodegenerative diseases. Ann Neurol 70:353–361
77. Lott IT, Head E (2005) Alzheimer disease and Down syndrome: factors in pathogenesis. Neurobiol Aging 26:383–389
78. Knoth R, Singec I, Ditter M, Pantazis G, Capetian P, Meyer RP, Horvat V, Volk B, Kempermann G (2010) Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. PLoS ONE 5:e8809
79. Lazarov O, Mattson MP, Peterson DA, Pimplikar SW, van Praag H (2010) When neurogenesis encounters aging and disease. Trends Neurosci 33:569–579
80. Nagahara AH, Tuszynski MH (2011) Potential therapeutic uses of BDNF in neurological and psychiatric disorders. Nat Rev Drug Discov 10:209–219
81. Singec I, Jandial R, Crain A, Nikkhah G, Snyder EY (2007) The leading edge of stem cell therapeutics. Annu Rev Med 58:313–328
82. Tuszynski MH, Thal L, Pay M, Salmon DP, Salmon DP, U HS, Bakay R, Patel P, Blesch A, Vahlsing HL, Ho G, Tong G, Potkin SG, Fallon J, Hansen L, Mufson EJ, Kordower JH, Gall C, Conner J (2005) A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. Nat Med 11:551–555
83. Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, Lensch MW, Cowan C, Hochedlinger K, Daley GQ (2008) Disease-specific induced pluripotent stem cells. Cell 134:877–886
84. Berninger B, Costa MR, Koch U, Schroeder T, Sutor B, Grothe B, Götz M (2007) Functional properties of neurons derived from in vitro reprogrammed postnatal astroglia. J Neurosci 27:8654–8664
85. Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Südhof TC, Wernig M (2010) Direct conversion of fibroblasts to functional neurons by defined factors. Nature 463:1035–1041
86. Qiang L, Fujita R, Yamashita T, Angulo S, Rhinn H, Rhee D, Doege C, Chau L, Aubry L, Vanti WB, Moreno H, Abeliovich A (2011) Directed conversion of Alzheimer's disease patient skin fibroblasts into functional neurons. Cell 146:359–371
87. Joers VL, Emborg ME (2009) Preclinical assessment of stem cell therapies for neurological diseases. ILAR J 51:24–41
88. Björklund A, Dunnett SB (2007) Dopamine neuron systems in the brain: an update. Trends Neurosci 30:194–202
89. Braak H, Del Tredici K (2009) Neuroanatomy and pathology of sporadic Parkinson's disease. Adv Anat Embryol Cell Biol 201:1–119
90. Halliday G, Lees A, Stern M (2011) Milestones in Parkinson's disease—clinical and pathological features. Mov Disord 26:1015–1021
91. Vesper J, Haak S, Ostertag C, Nikkhah G (2007) Subthalamic nucleus deep brain stimulation in elderly patients–analysis of outcome and complications. BMC Neurol 7:7
92. Iravani MM, Jenner P (2011) Mechanisms underlying the onset and expression of levodopa-induced dyskinesia and their pharmacological manipulation. J Neural Transm 118:1661–1690
93. Kim JH, Auerbach JM, Rodríguez-Gómez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sánchez-Pernaute R, Bankiewicz K, McKay R (2002) Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 418:50–56

94. Hargus G, Cooper O, Deleidi M, Levy A, Lee K, Marlow E, Yow A, Soldner F, Hockemeyer D, Hallet PJ, Osborn T, Jaenisch R, Isacson O (2010) Differentiated Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and reduce motor asymmetry in Parkinsonian rats. Proc Natl Acad Sci USA 107:15921–15926
95. Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, Hargus G, Blak A, Cooper O, Mitalipova M, Isacson O, Jaenisch R (2009) Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell 136:964–977
96. Wernig M, Zhao JP, Pruszak J, Hedlund E, Fu D, Soldner F, Broccoli V, Constantine-Paton M, Isacson O, Jaenisch R (2008) Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. Proc Natl Acad Sci USA 105:5856–5861
97. Caiazzo M, Caiazzo M, Dell'Anno MT, Dvoretskova E, Lazarevic D, Taverna S, Leo D, Sotnikova TD, Menegon A, Roncaglia P, Colciago G, Russo G, Carninci P, Pezzoli G, Gainetdinov RR, Gustincich S, Dityatev A, Broccoli V (2011) Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. Nature 476:224–227
98. Pfisterer U, Kirkeby A, Torper O, Wood J, Nelander J, Dufour A, Björklund A, Lindvall O, Jacobsson J, Parmar M (2011) Direct conversion of human fibroblasts to dopaminergic neurons. Proc Natl Acad Sci USA 108:10343–10348
99. Benraiss A, Goldman SA (2011) Cellular therapy and induced neuronal replacement for Huntington's disease. Neurotherapeutics 8:577–590
100. Zuccato C, Cattaneo E (2009) Brain-derived neurotrophic factor in neurodegenerative diseases. Nat Rev Neurol 5:311–322
101. Dunnett SB, Rosser AE (2011) Cell-based treatments for huntington's disease. Int Rev Neurobiol 98:483–508
102. Nicoleau C, Viegas P, Peschanski M, Perrier AL (2011) Human pluripotent stem cell therapy for Huntington's disease: technical, immunological, and safety challenges. Neurotherapeutics 8:562–576
103. Bachoud-Lévi AC, Gaura V, Brugières P, Lefaucheur JP, Boissé MF, Maison P, Baudic S, Ribeiro MJ, Bourdet C, Remy P, Cesaro P, Hantraye P, Peschanski M (2006) Effect of fetal neural transplants in patients with Huntington's disease 6 years after surgery: a long-term follow-up study. Lancet Neurol 5:303–309
104. Capetian P, Knoth R, Maciaczyk J, Pantazis G, Ditter M, Bokla L, Landwehrmeyer GB, Volk B, Nikkhah G (2009) Histological findings on fetal striatal grafts in a Huntington's disease patient early after transplantation. Neuroscience 160:661–675
105. Ferraiuolo L, Kirby J, Grierson AJ, Sendtner M, Shaw PJ (2011) Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis. Nat Rev Neurol 7:616–630
106. Ilieva H, Polymenidou M, Cleveland DW (2009) Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J Cell Biol 187:761–772
107. Di Giorgio FP, Carrasco MA, Siao MC, Maniatis T, Eggan K (2007) Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. Nat Neurosci 10:608–614
108. Haidet-Phillips AM, Hester ME, Miranda CJ, Meyer K, Braun L, Frakes A, Song S, Likhite S, Murtha MJ, Foust KD, Rao M, Eagle A, Kammesheidt A, Christensen A, Mendell JR, Burghes AH, Kaspar BK (2011) Astrocytes from familial and sporadic ALS patients are toxic to motor neurons. Nat Biotechnol 29:824–828
109. Marchetto MC, Muotri AR, Mu Y, Smith AM, Cezar GG, Gage FH (2008) Non-cell-autonomous effect of human SOD1 G37R astrocytes on motor neurons derived from human embryonic stem cells. Cell Stem Cell 3:649–657
110. Peljto M, Dasen JS, Mazzoni EO, Jessell TM, Wichterle H (2010) Functional diversity of ESC-derived motor neuron subtypes revealed through intraspinal transplantation. Cell Stem Cell 7:355–366
111. Wichterle H, Lieberam I, Porter JA, Jessell TM (2002) Directed differentiation of embryonic stem cells into motor neurons. Cell 110:385–397
112. Dimos JT, Rodolfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, Croft GF, Saphier G, Leibel R, Goland R, Wichterle H, Henderson CE, Eggan K (2008) Induced

pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science 321:1218–1221
113. Son EY, Ichida JK, Wainger BJ, Toma JS, Rafuse VF, Woolf CJ, Eggan K (2011) Conversion of mouse and human fibroblasts into functional spinal motor neurons. Cell Stem Cell 9:205–218
114. Schwab ME (2010) Functions of Nogo proteins and their receptors in the nervous system. Nat Rev Neurosci 11:799–811
115. Deshpande DM, Kim YS, Martinez T, Carmen J, Dike S, Shats I, Rubin LL, Drummond J, Krishnan C, Hoke A, Maragakis N, Shefner J, Rothstein JD, Kerr DA (2006) Recovery from paralysis in adult rats using embryonic stem cells. Ann Neurol 60:32–44
116. Gaillard A, Prestoz L, Dumartin B, Cantereau A, Morel F, Roger M, Jaber M (2007) Reestablishment of damaged adult motor pathways by grafted embryonic cortical neurons. Nat Neurosci 10:1294–1299
117. Singec I, Snyder EY (2007) Quo vadis brain repair? A long axonal journey in the adult CNS. Cell Stem Cell 1:355–356
118. Gowing G, Svendsen CN (2011) Stem cell transplantation for motor neuron disease: current approaches and future perspectives. Neurotherapeutics 8:591–606
119. Eiraku M, Takata N, Ishibashi H, Kawada M, Sakakura E, Okuda S, Sekiguchi K, Adachi T, Sasai Y (2011) Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 472:51–56
120. Idelson M, Alper R, Obolensky A, Ben-Shushan E, Hemo I, Yachimovich-Cohen N, Khaner H, Smith Y, Wiser O, Gropp M, Cohen MA, Even-Ram S, Berman-Zaken Y, Matzrafi L, Rechavi G, Banin E, Reubinoff B (2009) Directed differentiation of human embryonic stem cells into functional retinal pigment epithelium cells. Cell Stem Cell 5:396–408
121. Marchetti V, Krohne TU, Friedlander DF, Friedlander M (2010) Stemming vision loss with stem cells. J Clin Investi 120:3012–3021
122. Meyer JS, Shearer RL, Capowski EE, Wright LS, Wallace KA, McMillan EL, Zhang SC, Gamm DM (2009) Modeling early retinal development with human embryonic and induced pluripotent stem cells. Proc Natl Acad Sci USA 106:16698–16703
123. Osakada F, Ikeda H, Sasai Y, Takahashi M (2009) Stepwise differentiation of pluripotent stem cells into retinal cells. Nat Protoc 4:811–824
124. Reh TA, Lamba D, Gust J (2010) Directing human embryonic stem cells to a retinal fate. Methods Mol Biol 636:139–153
125. Wong IY, Poon MW, Pang RT, Lian Q, Wong D (2011) Promises of stem cell therapy for retinal degenerative diseases. Graefes Arch Clin Exp Opthalmol 249:1439–1448

Chapter 2
New Prospects for Neural Stem Cell Therapies of Nervous System Diseases Toward the Establishment of Atypical Ectopic Perivascular Stem Cell Niches

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Abstract Compelling evidence exists that neural stem/precursor cell (NPC) transplants protect the central nervous system (CNS) from inflammation-driven degeneration, such as that occurring in multiple sclerosis (MS) and in the subacute stages after brain stroke and spinal cord injury (SCI). In immune-mediated experimental demyelination and stroke, transplanted NPCs display a constitutive and inducible ability to mediate efficient bystander myelin repair and axonal rescue. This is dependent on multiple '*chaperone*'-like capacities that NPCs exhibit within specific in vivo microenvironments after transplantation. Yet, a comprehensive understanding of the mechanisms by which NPCs exert their therapeutic impact is lacking. Here will review some of these bystander capacities, while pointing at the formation of the *atypical ectopic perivascular niches*, as the most challenging example of cross talk between the inflamed microenvironment(s) and transplanted therapeutic NPCs.

2.1 Introduction

During development, neural stem cells give rise to lineages leading to the formation of neurons or glial cells. Differentiated cells can be also generated through one or multiple stages of amplification by various types of more restricted progenitors [1, 2]. In the adult mammalian central nervous system (CNS), neural stem cells persist in the subventricular zone (SVZ) of the lateral ventricles, and in the dentate gyrus (DG) of the hippocampus. The largest neurogenic region is the SVZ, where stem cells give

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rise to immature neuroblasts that migrate towards the olfactory bulb and differentiate into interneurons [3] and to a small number of oligodendrocytes [4, 5]. In vitro, neural stem/precursor cells (NPCs) proliferate forming neurospheres, which are self-renewing and multipotent, and can be differentiated into neurons, oligodendrocytes, and astrocytes [6, 7]. The adult brain does not show significant regeneration in response to disease and injury [8]; hence, the possibility to isolate and propagate NPCs promptly raised the optimism about their therapeutic potential [9].

In the last decade, efficient procedures have been developed to safely expand and preserve these cells, making large numbers of NPCs available for transplantation. Also NPCs derived from embryonic stem (ES) cells, as well as more recently from induced pluripotent stem (iPS) cells might represent valuable cell sources for transplantation [10]. Nevertheless, there are crucial issues to consider before translating preclinical results to the humans. We need to understand which cell sources can be successful, but also the ideal administration route and time point for each pathological condition. Moreover, the specific mechanisms underlying both the cell replacement and tissue healing capabilities of NPCs after transplantation have been not completely unraveled [11].

The transplantation of NPCs could, in principle, restore neurological function through direct cell replacement or remyelination of damaged axons [12]. However, in practice, other tissue healing mechanisms such as promotion of tissue regeneration, neuroprotection, or immunomodulation have been described [11].

Accumulating evidence, in fact, supports that transplanted NPCs are able to protect the disease or injury microenvironment by a number of '*bystander*' mechanisms that are alternative to the initially expected cell-replacement. The specific molecular and cellular mechanisms underlying this effect are though far from being fully understood [11]. New approaches are likely to take advantage of this functional NPC plasticity, which represents the capacity of transplanted stem cells to engage multiple mechanisms of action within specific microenvironments in vivo [13]. The therapeutic potential of NPCs has been assessed in both acute and chronic neurological disease models, and strong evidence shows that transplanted NPCs are capable of protecting the CNS from chronic inflammation-driven degeneration, such as that occurring in experimental autoimmune encephalomyelitis (EAE), as model of multiple sclerosis (MS), stroke, and spinal cord injury (SCI) [11, 14].

In this book chapter, we will review some of the most recent work showing that experimental therapies based on NPC transplants contribute to the healing of the injured CNS and discuss the potential mechanisms accounting for this effect, including the formation of atypical ectopic perivascular stem cell niches. We will finally consider past and current attempts to translate NPC-based therapeutics into the clinics.

2.2 Multiple Sclerosis

MS is a chronic and progressive demyelinating disease particularly affecting young adults that leads to irreversible disability [15]. In MS, CNS-restricted demyelination and axonal loss are a consequence of both the recurrence of several immunological

attacks to the neural tissue and an overall failure of the remyelination process driven by endogenous oligodendrocyte precursor cells (OPCs) undergoing activation upon myelin damage [16]. Immune modulation and remyelination are, therefore, the main aims of the newest therapeutic interventions for MS [17, 18].

Most of the licensed drug therapies focus their mechanism of action at the suppression of the immune response, as the induction of remyelination is particularly complicated because of the disseminated (in space and time) and recurrent nature of MS [19]. There is currently no treatment for the motor, sensory, and cognitive deficits of chronic, late stage, progressive MS cases [17, 18]. Hence, stem cells might represent a potentially valuable therapeutic option for MS patients in their progressive stages, based on their tissue trophic (e.g., neuroregenerative), and immune regulatory potential. A wide range of transplantation studies using NPCs have been performed in mice with both chronic and relapsing EAE, the prototypical animal model for MS.

As consequence of these studies, solid evidence has been provided that the systemic (either intravenous or intracerebroventricular) transplantation of NPCs into EAE rodents reduces the amount of astrogliosis, demyelination, and axonal loss without overt side effects [20–24]. Accumulation of systemically injected NPCs into inflamed CNS areas in EAE is shown to be dependent from the constitutive expression of functional adhesion molecules (e.g., CD44), integrins (e.g., $\alpha 4$, $\beta 1$), and chemokine receptors (e.g., CCR1, CCR2, CCR5, CXCR3, CXCR4) [21, 25] (*pathotropism*). This adhesion molecule/chemokine receptor-dependent *pathotropism* of NPCs is well conserved across species, and has been recently confirmed also on human NPCs [26–28] that have ameliorated the clinic-pathological features of EAE nonhuman primates, after systemic NPC injection [27].

Remarkably, the functional recovery observed after NPC transplantation—both in rodents and nonhuman primates—has always scarcely correlated with the absolute numbers of derived terminally differentiated neural cells in vivo, thus suggesting a mechanism of action clearly distinct from the initially prospected replacement of damaged endogenous neural cells [12]. As such, NPCs accumulating and surviving at the level of the CNS inflammatory areas exert a number of tissue trophis and immune modulatory effects, and in turn decrease CNS inflammation and prevent the accumulation of secondary tissue damage [8].

NPCs may enhance intrinsic endogenous repair mechanisms e.g., by exerting neurotrophic activities that increase the number of OPCs recruited at the lesion level. As such, systemically injected NPCs in EAE mice increase the number of endogenous OPCs at the lesion site, thus improving spontaneous remyelination [21]. This effect on OPCs is paralleled by a decrease of the pro (astro)gliotic factors transforming growth factor (TGF)-β and fibroblast growth factor (FGF)-2 [21]. In cuprizone-induced demyelination, intracerebroventricularly transplanted NPCs induced OPC proliferation and enhanced remyelination via the secretion of the platelet-derived growth factor (PDGF)-AA and FGF-2 [29]. NPCs also secrete leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF); while the former promotes mature oligodendrocyte survival in EAE [30, 31], the latter has been implicated in both OPC survival and differentiation [32, 33].

In addition to tissue trophic activities, NPCs have also been shown to possess remarkable immune regulatory functions that may account for some of their therapeutic effects. After intracerebroventricular NPC transplantation in acute EAE rats, NPCs attenuated the burden of inflammation in vivo, leading to a significant clinical recovery, likely by inhibiting lymphocyte functions (e.g., proliferation) [24].

Also in mice with chronic EAE, intravenously injected NPCs reduced chronic neural tissue loss and clinical disability by inducing apoptosis of infiltrating encephalitogenic T cells [25]. NPCs were shown to attenuate EAE symptoms by inhibiting the activation and proliferation of lymph node T cells in response to myelin oligodendrocyte glycoprotein (MOG) 35–55. Moreover, antigen-primed lymph node cells obtained from NPC-transplanted mice exhibited poor encephalitogenicity on disease transfer to naive mice, indicating a long-lasting inhibition instead of a specific in vivo environmental effect [22]. After these studies, more mechanistic approaches have shown that NPCs exert a direct effect on Th1 proinflammatory (but not Th2 anti-inflammatory) lymphocytes, by both inhibiting proliferation and activation, as well as inducing apoptosis [25]. Moreover, NPCs have shown a selective pro-apoptotic effect also on Th17 proinflammatory cells via a CD95L/FasL-dependent mechanism, in vitro [34]. NPCs also inhibit T cell activation and proliferation in response to T cell receptor (TCR)-mediated stimuli, ultimately leading to T cell apoptosis without active induction of cell death [35]. On the other hand, human NPCs show only a limited effect on T cell apoptosis, as CD95L is only barely detectable on their surface. However, inflammatory cytokine-exposed human NPCs show a much higher cytotoxic potential (e.g., *vs.* rodent NPCs) against phagocytes via a tumor necrosis factor (TNF)-α-mediated mechanism [36]. Several mechanisms by which NPCs may suppress the proliferation of T cells have been described. These include the inhibition of interleukin (IL)-2 and IL-6 signaling on T and B lymphocytes, respectively [24], and the release of nitric oxide (NO) and prostaglandin E2 (PGE2) [37]. Also human NPCs inhibit proliferation of xenogeneic marmoset antigen-specific, and allogeneic mitogen-activated T cells both through release of soluble mediators into the culture supernatant as well as through direct cellular contacts [27, 38].

However, when injected in EAE mice through a systemic route that minimize the CNS *pathotropism* (e.g., sub cutaneously), NPCs almost exclusively localize persist at the level of the draining lymph nodes, where they exert remarkable immune regulation, while still fostering clinic-pathological amelioration [22, 39, 40]. Subcutaneously injected NPCs restrain the maturation of dendritic cells (DCs), thus limiting the expansion of antigen-specific encephalitogenic T cells at sites of antigen presentation [41]. Also hNPCs are capable of hindering the differentiation of myeloid precursor cells into immature DC, and their following maturation into functional (antigen-presenting) DCs [27]. More recently, Cao and colleagues showed that intravenously administered NPCs, also primarily accumulating in the peripheral lymphoid tissue, ameliorate EAE by inhibiting encephalitogenic T helper 17 (Th17) cell differentiation through a LIF-dependent mechanism, in which LIF antagonizes interleukin (IL)-6. Interestingly, similar clinical outcome was obtained after administration of irradiated NPCs or NPC supernatants [40].

In summary, NPCs have been shown to exert not only trophic support at the EAE lesioned CNS microenvironment, but also to play a crucial role at peripheral lymphoid organs in the regulation of the immune responses to myelin antigens [42–44].

2.3 Brain Stroke

Stroke and other cerebrovascular diseases are the second leading cause of death in middle and high-income countries. Stroke attacks are mostly caused by thrombosis or embolism, while hemorrhagic strokes are less frequent. Patients can suffer form paralysis, spasticity, aphasia, or dementia, disabilities that have a significant impact on their quality of life [45]. Clinical recovery after stroke remains very poor despite advances in therapy, and stem cell treatments are greatly considered as a promising alternative [46]. Transplantation of NPCs with different delivery strategies, intraparenchymal, or intracerebroventricular injection, as well as systemic administration, has been shown to improve clinical signs in experimental stroke models [47, 48].

When injected into the ischemic brain, NPCs tend to migrate towards the infarct sit [49–52], where chemoattractive gradients of proinflammatory cytokines and chemokines are released [53], and a crucial role of CCL2/CCR2 and CXCL12/CXCR4 in transendothelial recruitment and intraparenchymal migration, respectively [54–56], is established. Once they reach the boundaries of the ischemic area, grafted NPCs are shown to interact with the inflammatory environment, as suggested by the increase in the gene expression levels of VEGF, CXCL12/SDF1-α, and TGF-β in the NPC-transplanted mouse middle cerebral artery (MCA) occlusion brain at 4 h after the insult [57]. Intracerebrally transplanted NPCs are able to differentiate into neurons and glia. NPC-derived neurons have been described to produce neurotransmitters, form dendrites and show electrophysiological properties characteristics of integrated functional neurons [58–60]. However, only a minor percentage of transplanted NPCs undergo terminal differentiation or mainly differentiate towards a glial phenotype [11].

On the other hand, NPCs injected systemically in MCAo mice mostly maintain an undifferentiated phenotype, while accumulating at the boundaries of the lesional area [61, 62]. Therefore, besides from the (limited) cell replacement, NPCs are believed to exert tissue trophic and immune modulatory effects [53] also in stroke models. In line with this, the subacute (*delayed*) NPC injection after MCAo has been shown to significantly down regulate multiple RNA species involved in inflammation, including IFN-γ, TNF-α, IL-1β, IL-6, and leptin receptor [62]. NPCs may exert an immune modulatory action, while in an undifferentiated state, causing a profound down regulation of inflammatory *lymphoid* (T cells) and *myeloid* cells (macrophages) within inflamed brain areas. While the inhibition of the T cell responses by NPCs is a quite established concept [63], the effects on microglia/macrophages at the ischemic injury remain controversial, as professional phagocytes can exert both

protective and deleterious effects after brain injuries, including stroke [64]. In addition to have a direct beneficial effect on axonal sprouting [65], NPC transplantation has promoted the infiltration by $CD11b^+$ myeloid cells in the brain of MCAo mice [57, 66], thus suggesting that some myeloid cell activation might be required for transplanted NPCs to exert part of their neuroprotective action [57]. Consistently with these controversies, MCAo mice selectively ablated of CD11b-positive microglia, or mineralocorticoid receptor (MR)-expressing macrophages, show exacerbation or reduction of the ischemia-dependent brain injury, respectively [67, 68]. However, other studies show a significant reduction of microglia/macrophages in the brain of mice with either ischemic or hemorrhagic stroke after NPC transplantation, with improved neuronal survival, and locomotor functions [62, 69]. Interestingly, when injected systemically into mice with the collagenase-induced intracerebral hemorrhage (ICH), only very few transplanted NPCs migrated into the brain, with the majority of them accumulating predominantly at the level of the spleen. In ICH mice, only the hyperacute (e.g., 2 h) NPC injection resulted in decreased brain oedema, inflammatory infiltration, and neurological deterioration. Consistently, splenectomy prior to ICH induction eliminated the positive effect on oedema and inflammation of transplanted NPCs [69].

2.4 Spinal Cord Injury

Traumatic spinal cord injury (SCI) is a damage or trauma to the spinal cord that results in a loss or impaired function causing reduced mobility or feeling. SCI is a devastating and debilitating condition affecting all regions of the world [70], which is characterized by the interruption of fibers connecting the spinal cord with supraspinal centers, resulting in the impairment of sensory motor, and autonomic functions (ref). The pathological events following acute SCIs are divided into two broad chronological phases: the primary and the secondary injuries [71]. The primary injury phase is characterized by the mechanical disruption of the cord anatomy, during which the failure of the spinal column integrity (e.g., upon vertebral fracture or dislocation) directly imparts a certain force to the cord, thus leading to axonal disruption, as well as laceration of blood vessels, and cell membranes. This initial insult then leads to a secondary injury phase, which progressively exacerbates the injury to the cord via the activation of a chain of pathophysiological mechanisms (e.g., excitotoxicity, inflammation, Wallerian degeneration) [71, 72]. This latter injury phase is a major impediment to functional recovery after SCIs, as it inevitably conducts to the destruction of those (descending *vs.* ascending) axonal paths eventually left intact by the initial trauma.

Despite important advances in the understanding of SCI pathophysiology, pharmacological therapies capable of completely restoring the loss of neurological functions are currently missing. For this reason, the potential of NPC transplantation to drive spinal cord repair has been widely investigated, as NPCs may provide an effective treatment by replacing lost or damaged cells, remyelinating

spared axons and/or releasing a milieu of neuroprotective and neurotrophic factors favoring endogenous cell plasticity and sprouting [73].

Adult or embryonic mammalian NPCs from different sources have been transplanted into a wide range of SCI models with significant clinical improvement. Although human embryonic or foetal cell sources also showed promising results, ethics, and availability issues make from the recently discovered iPS cells a promising alternative [74, 75]. Recent studies have developed safe nontumorigenic mouse [76] and human [77] iPS cell-derived NPCs which were able to improve locomotor function recovery after contusive SCI. Most of the studies have delivered NPCs focally to increase their viability at the injury site. However, also alternative routes of administration (e.g., systemic) have been investigated to avoid the damage to the spared cord tissue at the time of focal cell injection, as well as other procedure-related complications, with the final aim to improve the chances of translation into clinical practice. After injection in the tail vein of nude SCI mice, human NPCs were able to reach the injury site [78]; and led to a significantly better behavioral recovery, compared to SCI mice transplanted intraspinally with NPCs [79]. In almost all cases, both the focal and systemic NPC transplantation resulted in significant recovery of functions, which were highly specific for the treatment applied, as they were completely abolished in human NPC-transplanted SCI mice treated with diphtheria toxin (DT; as human cells are about 100,000 times more sensitive to DT, via the human DT receptor, than mouse cells) [80]. There is a growing belief that the severity (and type) of the injury, as well as the time after injury at which cells are transplanted, are two major key factors influencing the capability of grafted NPCs to affect the healing of the damaged spinal cord tissue. As such, rat spinal cord-derived somatic NPCs failed to induce any detectable functional recovery when transplanted hyperacutely at the level of injury in a severe (35 gr.) clip-induced SCI model [81], but were indeed significantly efficacious when transplanted as early as 9 days after injury in a milder (27 gr.) SCI model [82].

Interestingly, NPCs survive, migrate, and generate functional remyelinating oligodendrocytes, which promote functional recovery, when transplanted subacutely (namely 2 weeks), but not chronically (namely 8 weeks) after SCI [83]. The transplantation of NPCs in experimental SCI has yielded a general low degree of differentiation [79, 84], most of which biased towards a glial fate [85–87]. When more lineage restricted neural precursors have been transplanted, a much higher rate of neuronal differentiation has been achieved, presumably because these latter cells are less sensitive than NPCs to inhibitory signals coming from the environment [88, 89].

Strategies to overcome the observed poor differentiation potential of transplanted NPCs have include the combination with valproic acid [90] or neurotrophic growth factors [91, 92], which promoted neuronal differentiation and achieved the establishment of functional synapses between host axons and graft neurons at the injury site. Further, the use of engineered NPCs transduced with transcription factors or survival genes [93, 94], as well as the cotransplantation with '*scaffold*' cells, such as mesenchymal/stromal stem cells or olfactory

ensheathing cells, [95–97] or the use of polymeric scaffolds [98] has increased the survival, migration, and differentiation of transplanted NPCs as well as the final functional recovery observed.

Parallel strong evidence suggests that undifferentiated NPCs can influence the injury environment at the spinal cord also through a bystander effect. Transplantation of C17.2 mouse embryonic NPCs both provided a cellular substrate as well as secreted several neurotrophic factors [e.g., nerve growth factor (NGF), brain-derived neurotrophic growth factor (BDNF) and GDNF], leading to substantial host axonal regrowth [99]. Also adult NPCs themselves were shown to induce the up regulation of neurotrophic factors and chemokines such as BDNF, NGF and LIF, and inflammatory molecules such as TNF-α [79]. In line with this, NPCs engineered to express a human NT-3 capable of binding to both trkB and trkC and showed increased survival and enhanced myelin formation, while leading to only a modest improvement of locomotor function, when transplanted into a chronic SCI model [100]. Interestingly, when combined with a self-antigen (e.g., myelin)-specific T cell vaccination [101] in SCI mice, transplanted NPCs migrated to the injury site and instructed the local macrophage/microglial cells towards a neuroprotective phenotype, leading to improved tissue preservation, and functional recovery [102]. This immune regulatory effect was also present when NPCs were transplanted into and extremely severe (e.g., 200 kdynes) model of contusion SCI. Subacutely (but not chronically) transplanted NPCs remained undifferentiated and altered the inflammatory infiltrate of the injured spinal cord by reducing the proportion of 'classically-activated' (M1) inflammatory macrophages, and increasing that of regulatory T cells, in turn promoting the healing of the injured cord [103].

Thus, similarly to what observed in EAE and brain stroke, the fact that NPCs induce significant clinical amelioration—while substantially escaping from programs of differentiation in vivo—suggests that also in SCI, cell-instructed neuroprotection rather cell-dependent integration is the foremost healing mechanism [8].

2.5 Ectopic Perivascular Niches

After transplantation, NPCs have been shown to accumulate at the level of perivascular areas forming atypical germinal-like ectopic niches, where they sustain neuroprotective and immune modulatory actions [24, 25, 27, 39, 62, 83, 104]. In these perivascular niches, NPCs are likely involved in a highly refined cross talk with reactive astrocytes, inflamed endothelial cells, and infiltrating T cells. Sophisticated mechanisms of communication with endogenous cells may constitute a crucial mechanism by which NPCs exert their protective effect in vivo, overall leading to the benefit observed at clinical, and pathological levels [11, 105]. Different mechanisms allowing the horizontal exchange of information might, in principle, account for the therapeutic effect of NPCs. Described

cell–to–cell communication mechanisms include cellular contacts [106]; nanotubes [107]; and the secretion of molecules [108]; and membrane vesicles [109]. Direct evidence of stem cell communication with the host was provided when ES cell-derived astrocytes grafted onto hippocampal slices where shown to integrate in the endogenous glial network, establishing gap junctions with recipient glia [110]. In fact, astrocytes form extensive networks in vivo connected through gap junctions [111], so that in principle, the integration of grafted stem cells into this network structure might allow cell-mediated delivery of small molecules, and account for the modulation of the damaged host environment. Recently, NPCs were shown to rescue host neurons in animal models of Purkinje neurodegeneration directly influencing host network coordinated activity via Ca^{++} waves and through transcellular delivery of homeostasis-modulating molecules [112]. In addition, environmental cues had a marked effect on stem cells plasticity, as hypoxic preconditioning of NSCs before the engraftment increased connexin 43 expression improving subsequent graft and cellular communication with host cells [113]. The injured or degenerating environment in vivo is critical to allow the establishment of intercellular communication between transplanted and host cells. NPCs injected subcutaneously into mice with PLP-induced EAE did not graft into the CNS or express major neural antigens, but instead accumulated at secondary lymphoid organs, establishing different types of cellular interactions with lymph node cells [39]. Moreover, in the severely (i.e., 200 kdynes) contused spinal cord, NPCs survive and preferentially localize in small clusters at the level of perivascular niches outside the injury cyst. The analysis of cell–to–cell interactions at these niches revealed the presence of tight contacts, up to structural junctional coupling, between NPCs and macrophages, but not B lymphocytes that were established via connexin 43^{+} gap junctions [103].

Also systemic delivery of nonneural somatic stem/precursor cells leads to their accumulation in inflamed CNS areas [102, 114–117] where direct communication with host cells can be postulated as the underlying mechanism for their therapeutic plasticity.

The precise knowledge of how communication of grafted NPCs with host cells modulates pathological processes promoting healing mechanisms can eventually lead to the development of more efficacious therapeutic alternatives.

2.6 Conclusions and Perspectives

Almost two decades of work on pathophysiologically relevant animal models of MS, brain stroke, and spinal injuries have taught us that neural stem cells—and the experimental therapies developed after their prospective isolated from the adult mammalian brain—are indeed much more complex than what naively envisaged at the very beginning [12]. Current phase I/II clinical studies using NPCs in the CNS are the forefront for the much-awaited next generation regenerative versus restorative therapies [118], and two mechanisms of action, the direct replacement

of, and the bystander support to endogenous cells, are being extensively proposed as major outcome measure facilitating the choice of the disease target [118]. Current potential CNS disease targets include brain stroke, traumatic brain injury (TBI), SCIs, MS, Alzheimer's disease (AD), Parkinson's disease (PD), and, perhaps further in the future, mental disorders such as depression, autism, and schizophrenia [118]. Based on the discoveries made at the bench side, these clinical studies fall into two broad categories: (1) Regeneration/restoration versus cell replacement, to promote host tissue repair and/or replace missing or damaged cells, and (2) Therapeutic delivery, to provide therapeutic molecules for neuroprotection and/or stimulation of repair. And MS, brain stroke and (in part) also SCI are all diseases in which the stem cell-instructed bystander effects are more likely desirable.

A big challenge for the future will be the identification of a reliable source of NPCs. Somatic NPCs have showed some limitations, which include their instrinsic allogeneicity in clinical settings, a relatively slow growth rate, a progressive shortening of telomeres and decrease of telomerase activity under scale-up conditions, and consequent limited (phenotypic and genotypic) instability upon continuous expansion [10, 118]. On the other hand, both pluripotent stem cell- and pluripotent induced stem cell-derived NPCs represent a highly valuable alternative whose credibility is under continuous challenge by the development of more and more reliable protocols of expansion, (neural) induction, and (neural vs. glial) differentiation. Therefore, while the possibility of having large amounts of *in principle* autologous NPCs for clinical applications is indeed work in progress, the scientific community must not stop our attempts toward the identification of the mechanisms regulating the NPC-instructed bystander effects, if we want to keep the rights to properly tailor future NPC therapies and avoid unwanted side effects.

Having established the atypical perivascular (stem cell) niche as surrogate marker of the capacity of NPCs to find in vivo the way(s) towards certain microenvironments in vivo where acting as therapeutic weapons, we envisage that understanding the modalities by which NPCs communicate horizontally to the cells of the microenvironment has the chances to reveal us a number of basal (*vs.* reactive) stem cell functions to be translated into innovative highly clinical impact therapeutics.

References

1. McKay R (1997) Stem cells in the central nervous system. Science 276(5309):66–71
2. Kriegstein A, Alvarez-Buylla A (2009) The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 32:149–184
3. Lois C, Alvarez-Buylla A (1994) Long-distance neuronal migration in the adult mammalian brain. Science 264(5162):1145–1148
4. Menn B, Garcia-Verdugo JM, Yaschine C, Gonzalez-Perez O, Rowitch D, Alvarez-Buylla A (2006) Origin of oligodendrocytes in the subventricular zone of the adult brain. J Neurosci 26(30):7907–7918

5. Nait-Oumesmar B, Decker L, Lachapelle F, Avellana-Adalid V, Bachelin C, Van Evercooren AB (1999) Progenitor cells of the adult mouse subventricular zone proliferate, migrate and differentiate into oligodendrocytes after demyelination. Eur J Neurosci 11(12):4357–4366
6. Anderson DJ (2001) Stem cells and pattern formation in the nervous system: the possible versus the actual. Neuron 30(1):19–35
7. Gage FH (1998) Stem cells of the central nervous system. Curr Opin Neurobiol 8(5): 671–676
8. Martino G, Pluchino S, Bonfanti L, Schwartz M (2011) Brain regeneration in physiology and pathology: the immune signature driving therapeutic plasticity of neural stem cells. Physiol Rev 91(4):1281–1304
9. Lindvall O, Kokaia Z (2006) Stem cells for the treatment of neurological disorders. Nature 441(7097):1094–1096
10. Martino G, Franklin RJ, Van Evercooren AB, Kerr DA (2010) Stem cell transplantation in multiple sclerosis: current status and future prospects. Nat Rev Neurol 6(5):247–255
11. Martino G, Pluchino S (2006) The therapeutic potential of neural stem cells. Nat Rev Neurosci 7(5):395–406
12. Rossi F, Cattaneo E (2002) Opinion: neural stem cell therapy for neurological diseases: dreams and reality. Nat Rev Neurosci 3(5):401–409
13. Pluchino S, Martino G (2008) The therapeutic plasticity of neural stem/precursor cells in multiple sclerosis. J Neurol Sci 265(1–2):105–110
14. Pluchino S, Zanotti L, Deleidi M, Martino G (2005) Neural stem cells and their use as therapeutic tool in neurological disorders. Brain Res Brain Res Rev 48(2):211–219
15. Compston A, Coles A (2002) Multiple sclerosis. Lancet 359(9313):1221–1231
16. Franklin RJ, Ffrench-Constant C (2008) Remyelination in the CNS: from biology to therapy. Nat Rev Neurosci 9(11):839–855
17. Fernandez–Fernandez O, Alvarez-Cermeno JC, Arbizu-Urdiain T, Arroyo-Gonzalez R, Arnal-Garcia C, Casanova-Estruch B, Calles-Hernandez MC, Coret-Ferrer F, Comabella M, de Garcia-Merino JA (2011) Review of the novelties presented at the 26th Congress of the European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS) (II). Revista de neurologia 52(5):289–299
18. Killestein J, Rudick RA, Polman CH (2011) Oral treatment for multiple sclerosis. Lancet Neurology 10(11):1026–1034
19. Kilsdonk ID, Barkhof F, Wattjes MP (2011) 2010 revisions to McDonald criteria for diagnosis of multiple sclerosis: impact of 3-Tesla magnetic resonance imaging. Ann Neurology 70(1):182–183
20. Giannakopoulou A, Grigoriadis N, Polyzoidou E, Lourbopoulos A, Michaloudi E, Papadopoulos GC (2011) Time-dependent fate of transplanted neural precursor cells in experimental autoimmune encephalomyelitis mice. Exp Neurol 230(1):16–26
21. Pluchino S, Quattrini A, Brambilla E, Gritti A, Salani G, Dina G, Galli R, Del Carro U, Amadio S, Bergami A, Furlan R, Comi G, Vescovi AL, Martino G (2003) Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. Nature 422(6933):688–694
22. Einstein O, Fainstein N, Vaknin I, Mizrachi-Kol R, Reihartz E, Grigoriadis N, Lavon I, Baniyash M, Lassmann H, Ben-Hur T (2007) Neural precursors attenuate autoimmune encephalomyelitis by peripheral immunosuppression. Ann Neurol 61(3):209–218
23. Einstein O, Grigoriadis N, Mizrachi-Kol R, Reinhartz E, Polyzoidou E, Lavon I, Milonas I, Karussis D, Abramsky O, Ben-Hur T (2006) Transplanted neural precursor cells reduce brain inflammation to attenuate chronic experimental autoimmune encephalomyelitis. Exp Neurol 198(2):275–284
24. Einstein O, Karussis D, Grigoriadis N, Mizrachi-Kol R, Reinhartz E, Abramsky O, Ben-Hur T (2003) Intraventricular transplantation of neural precursor cell spheres attenuates acute experimental allergic encephalomyelitis. Mol Cell Neurosci 24(4):1074–1082

25. Pluchino S, Zanotti L, Rossi B, Brambilla E, Ottoboni L, Salani G, Martinello M, Cattalini A, Bergami A, Furlan R, Comi G, Constantin G, Martino G (2005) Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. Nature 436(7048):266–271
26. Rampon C, Weiss N, Deboux C, Chaverot N, Miller F, Buchet D, Tricoire-Leignel H, Cazaubon S, Baron-Van Evercooren A, Couraud PO (2008) Molecular mechanism of systemic delivery of neural precursor cells to the brain: assembly of brain endothelial apical cups and control of transmigration by CD44. Stem Cells 26(7):1673–1682
27. Pluchino S, Gritti A, Blezer E, Amadio S, Brambilla E, Borsellino G, Cossetti C, Del Carro U, Comi G, t Hart B, Vescovi A, Martino G (2009) Human neural stem cells ameliorate autoimmune encephalomyelitis in non-human primates. Ann Neurol 66(3):343–354
28. Mueller FJ, Serobyan N, Schraufstatter IU, DiScipio R, Wakeman D, Loring JF, Snyder EY, Khaldoyanidi SK (2006) Adhesive interactions between human neural stem cells and inflamed human vascular endothelium are mediated by integrins. Stem Cells 24(11): 2367–2372
29. Einstein O, Friedman-Levi Y, Grigoriadis N, Ben-Hur T (2009) Transplanted neural precursors enhance host brain-derived myelin regeneration. J Neurosci 29(50):15694–15702
30. Butzkueven H, Zhang JG, Soilu-Hanninen M, Hochrein H, Chionh F, Shipham KA, Emery B, Turnley AM, Petratos S, Ernst M, Bartlett PF, Kilpatrick TJ (2002) LIF receptor signaling limits immune-mediated demyelination by enhancing oligodendrocyte survival. Nat Med 8(6):613–619
31. Marriott MP, Emery B, Cate HS, Binder MD, Kemper D, Wu Q, Kolbe S, Gordon IR, Wang H, Egan G, Murray S, Butzkueven H, Kilpatrick TJ (2008) Leukemia inhibitory factor signaling modulates both central nervous system demyelination and myelin repair. Glia 56(6):686–698
32. Talbott JF, Cao Q, Bertram J, Nkansah M, Benton RL, Lavik E, Whittemore SR (2007) CNTF promotes the survival and differentiation of adult spinal cord-derived oligodendrocyte precursor cells in vitro but fails to promote remyelination in vivo. Exp Neurol 204(1):485–489
33. Cagnon L, Braissant O (2009) CNTF protects oligodendrocytes from ammonia toxicity: intracellular signaling pathways involved. Neurobiol Dis 33(1):133–142
34. Knight JC, Scharf EL, Mao-Draayer Y (2010) Fas activation increases neural progenitor cell survival. J Neurosci Res 88(4):746–757
35. Fainstein N, Vaknin I, Einstein O, Zisman P, Ben Sasson SZ, Baniyash M, Ben-Hur T (2008) Neural precursor cells inhibit multiple inflammatory signals. Mol Cell Neurosci 39(3):335–341
36. Ricci-Vitiani L, Lombardi DG, Signore M, Biffoni M, Pallini R, Parati E, Peschle C, De Maria R (2007) Human neural progenitor cells display limited cytotoxicity and increased oligodendrogenesis during inflammation. Cell Death Differ 14(4):876–878
37. Wang L, Shi J, van Ginkel FW, Lan L, Niemeyer G, Martin DR, Snyder EY, Cox NR (2009) Neural stem/progenitor cells modulate immune responses by suppressing T lymphocytes with nitric oxide and prostaglandin E2. Exp Neurol 216(1):177–183
38. Kim SY, Cho HS, Yang SH, Shin JY, Kim JS, Lee ST, Chu K, Roh JK, Kim SU, Park CG (2009) Soluble mediators from human neural stem cells play a critical role in suppression of T-cell activation and proliferation. J Neurosci Res 87(10):2264–2272
39. Pluchino S, Zanotti L, Brambilla E, Rovere-Querini P, Capobianco A, Alfaro-Cervello C, Salani G, Cossetti C, Borsellino G, Battistini L, Ponzoni M, Doglioni C, Garcia-Verdugo JM, Comi G, Manfredi AA, Martino G (2009) Immune regulatory neural stem/precursor cells protect from central nervous system autoimmunity by restraining dendritic cell function. PLoS ONE 4(6):e5959
40. Cao W, Yang Y, Wang Z, Liu A, Fang L, Wu F, Hong J, Shi Y, Leung S, Dong C, Zhang JZ (2011) Leukemia inhibitory factor inhibits T helper 17 cell differentiation and confers treatment effects of neural progenitor cell therapy in autoimmune disease. Immunity 35(2):273–284
41. Pluchino S, Zanotti L, Brini E, Ferrari S, Martino G (2009) Regeneration and repair in multiple sclerosis: the role of cell transplantation. Neurosci Lett 456(3):101–106

42. Mohindru M, Kang B, Kim BS (2004) Functional maturation of proteolipid protein(139–151)-specific Th1 cells in the central nervous system in experimental autoimmune encephalomyelitis. J Neuroimmunol 155(1–2):127–135
43. Flugel A, Berkowicz T, Ritter T, Labeur M, Jenne DE, Li Z, Ellwart JW, Willem M, Lassmann H, Wekerle H (2001) Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. Immunity 14(5):547–560
44. de Vos AF, van Meurs M, Brok HP, Boven LA, Hintzen RQ, van der Valk P, Ravid R, Rensing S, Boon L, t Hart BA, Laman JD (2002) Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. J Immunol 169(10):5415–5423
45. Bright FA, Kayes NM, McCann CM, McPherson KM (2011) Understanding hope after stroke: a systematic review of the literature using concept analysis. Top Stroke Rehabil 18(5):490–508
46. Lindvall O, Kokaia Z (2011) Stem cell research in stroke: how far from the clinic? Stroke J Cereb Circ 42(8):2369–2375
47. Liu YP, Lang BT, Baskaya MK, Dempsey RJ, Vemuganti R (2009) The potential of neural stem cells to repair stroke-induced brain damage. Acta Neuropathol 117(5):469–480
48. Pluchino S, Cusimano M, Bacigaluppi M, Martino G (2010) Remodelling the injured CNS through the establishment of atypical ectopic perivascular neural stem cell niches. Arch Ital Biol 148(2):173–183
49. Lee HJ, Kim KS, Kim EJ, Choi HB, Lee KH, Park IH, Ko Y, Jeong SW, Kim SU (2007) Brain transplantation of immortalized human neural stem cells promotes functional recovery in mouse intracerebral hemorrhage stroke model. Stem Cells 25(5):1204–1212
50. Kelly S, Bliss TM, Shah AK, Sun GH, Ma M, Foo WC, Masel J, Yenari MA, Weissman IL, Uchida N, Palmer T, Steinberg GK (2004) Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. Proc Natl Acad Sci U S A 101(32):11839–11844
51. Hicks AU, Lappalainen RS, Narkilahti S, Suuronen R, Corbett D, Sivenius J, Hovatta O, Jolkkonen J (2009) Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: cell survival and functional recovery. Eur J Neurosci 29(3):562–574
52. Andres RH, Horie N, Slikker W, Keren-Gill H, Zhan K, Sun G, Manley NC, Pereira MP, Sheikh LA, McMillan EL, Schaar BT, Svendsen CN, Bliss TM, Steinberg GK (2011) Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. Brain 134(Pt 6):1777–1789
53. Bacigaluppi M, Pluchino S, Martino G, Kilic E, Hermann DM (2008) Neural stem/precursor cells for the treatment of ischemic stroke. J Neurol Sci 265(1–2):73–77
54. Imitola J, Raddassi K, Park KI, Mueller FJ, Nieto M, Teng YD, Frenkel D, Li J, Sidman RL, Walsh CA, Snyder EY, Khoury SJ (2004) Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. Proc Natl Acad Sci U S A 101(52):18117–18122
55. Darsalia V, Kallur T, Kokaia Z (2007) Survival, migration and neuronal differentiation of human fetal striatal and cortical neural stem cells grafted in stroke-damaged rat striatum. Eur J Neurosci 26(3):605–614
56. Andres RH, Choi R, Pendharkar AV, Gaeta X, Wang N, Nathan JK, Chua JY, Lee SW, Palmer TD, Steinberg GK, Guzman R (2011) The CCR2/CCL2 Interaction mediates the transendothelial recruitment of intravascularly delivered neural stem cells to the ischemic brain. Stroke 42(10):2923–2931
57. Capone C, Frigerio S, Fumagalli S, Gelati M, Principato MC, Storini C, Montinaro M, Kraftsik R, De Curtis M, Parati E, De Simoni MG (2007) Neurosphere-derived cells exert a neuroprotective action by changing the ischemic microenvironment. PLoS ONE 2(4):e373
58. Englund U, Bjorklund A, Wictorin K, Lindvall O, Kokaia M (2002) Grafted neural stem cells develop into functional pyramidal neurons and integrate into host cortical circuitry. Proc Natl Acad Sci U S A 99(26):17089–17094

59. Park KI, Teng YD, Snyder EY (2002) The injured brain interacts reciprocally with neural stem cells supported by scaffolds to reconstitute lost tissue. Nat Biotechnol 20(11):1111–1117
60. Buhnemann C, Scholz A, Bernreuther C, Malik CY, Braun H, Schachner M, Reymann KG, Dihne M (2006) Neuronal differentiation of transplanted embryonic stem cell-derived precursors in stroke lesions of adult rats. Brain 129(Pt 12):3238–3248
61. Sun C, Zhang H, Li J, Huang H, Cheng H, Wang Y, Li P, An Y (2010) Modulation of the major histocompatibility complex by neural stem cell-derived neurotrophic factors used for regenerative therapy in a rat model of stroke. J Translational Med 8:77
62. Bacigaluppi M, Pluchino S, Peruzzotti-Jametti L, Kilic E, Kilic U, Salani G, Brambilla E, West MJ, Comi G, Martino G, Hermann DM (2009) Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. Brain 132(Pt 8):2239–2251
63. Ben-Hur T (2008) Immunomodulation by neural stem cells. J Neurol Sci 265(1–2):102–104
64. Iadecola C, Anrather J (2011) The immunology of stroke: from mechanisms to translation. Nat Med 17(7):796–808
65. Daadi MM, Davis AS, Arac A, Li Z, Maag AL, Bhatnagar R, Jiang K, Sun G, Wu JC, Steinberg GK (2010) Human neural stem cell grafts modify microglial response and enhance axonal sprouting in neonatal hypoxic-ischemic brain injury. Stroke J Cereb Circ 41(3):516–523
66. Daadi MM, Davis AS, Arac A, Li Z, Maag AL, Bhatnagar R, Jiang K, Sun G, Wu JC, Steinberg GK (2010) Human neural stem cell grafts modify microglial response and enhance axonal sprouting in neonatal hypoxic-ischemic brain injury. Stroke 41(3):516–523
67. Lalancette-Hebert M, Gowing G, Simard A, Weng YC, Kriz J (2007) Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. J Neurosci 27(10):2596–2605
68. Frieler RA, Meng H, Duan SZ, Berger S, Schutz G, He Y, Xi G, Wang MM, Mortensen RM (2011) Myeloid-specific deletion of the mineralocorticoid receptor reduces infarct volume and alters inflammation during cerebral ischemia. Stroke J Cereb Circ 42(1):179–185
69. Lee ST, Chu K, Jung KH, Kim SJ, Kim DH, Kang KM, Hong NH, Kim JH, Ban JJ, Park HK, Kim SU, Park CG, Lee SK, Kim M, Roh JK (2008) Anti-inflammatory mechanism of intravascular neural stem cell transplantation in haemorrhagic stroke. Brain 131(Pt 3):616–629
70. Sekhon LH, Fehlings MG (2001) Epidemiology, demographics, and pathophysiology of acute spinal cord injury. Spine (Phila Pa 1976) 26(24 Suppl): S2–12
71. Rowland JW, Hawryluk GW, Kwon B, Fehlings MG (2008) Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. Neurosurg Focus 25(5):E2
72. Hagg T, Oudega M (2006) Degenerative and spontaneous regenerative processes after spinal cord injury. J Neurotrauma 23(3–4):264–280
73. Hernandeza J, Torres-Espina A, Navarro X (2011) Adult stem cell transplants for spinal cord injury repair: current state in preclinical research. Curr Stem Cell Res Ther 6(3):273–287
74. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
75. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872
76. Tsuji O, Miura K, Okada Y, Fujiyoshi K, Mukaino M, Nagoshi N, Kitamura K, Kumagai G, Nishino M, Tomisato S, Higashi H, Nagai T, Katoh H, Kohda K, Matsuzaki Y, Yuzaki M, Ikeda E, Toyama Y, Nakamura M, Yamanaka S, Okano H (2010) Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. Proc Natl Acad Sci U S A 107(28):12704–12709
77. Nori S, Okada Y, Yasuda A, Tsuji O, Takahashi Y, Kobayashi Y, Fujiyoshi K, Koike M, Uchiyama Y, Ikeda E, Toyama Y, Yamanaka S, Nakamura M, Okano H (2011) Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. Proc Natl Acad Sci U S A 108(40):16825–16830

78. Takeuchi H, Natsume A, Wakabayashi T, Aoshima C, Shimato S, Ito M, Ishii J, Maeda Y, Hara M, Kim SU, Yoshida J (2007) Intravenously transplanted human neural stem cells migrate to the injured spinal cord in adult mice in an SDF-1- and HGF-dependent manner. Neurosci Lett 426(2):69–74
79. Bottai D, Madaschi L, Di Giulio AM, Gorio A (2008) Viability-dependent promoting action of adult neural precursors in spinal cord injury. Mol Med Cambridge, Mass 14(9–10): 634–644
80. Cummings BJ, Uchida N, Tamaki SJ, Salazar DL, Hooshmand M, Summers R, Gage FH, Anderson AJ (2005) Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proc Natl Acad Sci U S A 102(39):14069–14074
81. Parr AM, Kulbatski I, Tator CH (2007) Transplantation of adult rat spinal cord stem/progenitor cells for spinal cord injury. J Neurotrauma 24(5):835–845
82. Parr AM, Kulbatski I, Zahir T, Wang X, Yue C, Keating A, Tator CH (2008) Transplanted adult spinal cord-derived neural stem/progenitor cells promote early functional recovery after rat spinal cord injury. Neuroscience 155(3):760–770
83. Karimi-Abdolrezaee S, Eftekharpour E, Wang J, Morshead CM, Fehlings MG (2006) Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury. J Neurosci 26(13):3377–3389
84. Cao QL, Howard RM, Dennison JB, Whittemore SR (2002) Differentiation of engrafted neuronal-restricted precursor cells is inhibited in the traumatically injured spinal cord. Exp Neurol 177(2):349–359
85. Cao QL, Zhang YP, Howard RM, Walters WM, Tsoulfas P, Whittemore SR (2001) Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage. Exp Neurol 167(1):48–58
86. Vroemen M, Aigner L, Winkler J, Weidner N (2003) Adult neural progenitor cell grafts survive after acute spinal cord injury and integrate along axonal pathways. Eur J Neurosci 18(4):743–751
87. Pfeifer K, Vroemen M, Blesch A, Weidner N (2004) Adult neural progenitor cells provide a permissive guiding substrate for corticospinal axon growth following spinal cord injury. Eur J Neurosci 20(7):1695–1704
88. Yan J, Xu L, Welsh AM, Hatfield G, Hazel T, Johe K, Koliatsos VE (2007) Extensive neuronal differentiation of human neural stem cell grafts in adult rat spinal cord. PLoS Med 4(2):e39
89. Han SS, Kang DY, Mujtaba T, Rao MS, Fischer I (2002) Grafted lineage-restricted precursors differentiate exclusively into neurons in the adult spinal cord. Exp Neurol 177(2):360–375
90. Abematsu M, Tsujimura K, Yamano M, Saito M, Kohno K, Kohyama J, Namihira M, Komiya S, Nakashima K (2010) Neurons derived from transplanted neural stem cells restore disrupted neuronal circuitry in a mouse model of spinal cord injury. J Clin Invest 120(9):3255–3266
91. Bonner JF, Blesch A, Neuhuber B, Fischer I (2010) Promoting directional axon growth from neural progenitors grafted into the injured spinal cord. J Neurosci Res 88(6):1182–1192
92. Bonner JF, Connors TM, Silverman WF, Kowalski DP, Lemay MA, Fischer I (2011) Grafted neural progenitors integrate and restore synaptic connectivity across the injured spinal cord. J Neurosci 31(12):4675–4686
93. Hwang DH, Kim BG, Kim EJ, Lee SI, Joo IS, Suh-Kim H, Sohn S, Kim SU (2009) Transplantation of human neural stem cells transduced with Olig2 transcription factor improves locomotor recovery and enhances myelination in the white matter of rat spinal cord following contusive injury. BMC Neurosci 10:117
94. Lee SI, Kim BG, Hwang DH, Kim HM, Kim SU (2009) Overexpression of Bcl-XL in human neural stem cells promotes graft survival and functional recovery following transplantation in spinal cord injury. J Neurosci Res 87(14):3186–3197
95. Oh JS, Kim KN, An SS, Pennant WA, Kim HJ, Gwak SJ, Yoon DH, Lim MH, Choi BH, Ha Y (2011) Co-transplantation of mouse neural stem sells (mNSCs) with adipose tissue-derived mesenchymal stem sells improves mNSC survival in a rat spinal cord injury model. Cell Transplant 20(6):837–849

96. Oh JS, Ha Y, An SS, Khan M, Pennant WA, Kim HJ, Yoon do H, Lee M, Kim KN (2010) Hypoxia-preconditioned adipose tissue-derived mesenchymal stem cell increase the survival and gene expression of engineered neural stem cells in a spinal cord injury model. Neurosci Lett 472(3):215–219
97. Wang G, Ao Q, Gong K, Zuo H, Gong Y, Zhang X (2010) Synergistic effect of neural stem cells and olfactory ensheathing cells on repair of adult rat spinal cord injury. Cell Transplant 19(10):1325–1337
98. Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, Langer R, Snyder EY (2002) Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. Proc Natl Acad Sci U S A 99(5):3024–3029
99. Lu P, Jones LL, Snyder EY, Tuszynski MH (2003) Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Exp Neurol 181(2):115–129
100. Kusano K, Enomoto M, Hirai T, Tsoulfas P, Sotome S, Shinomiya K, Okawa A (2010) Transplanted neural progenitor cells expressing mutant NT3 promote myelination and partial hindlimb recovery in the chronic phase after spinal cord injury. Biochem Biophys Res Commun 393(4):812–817
101. Schwartz M, Ziv Y (2008) Immunity to self and self-maintenance: a unified theory of brain pathologies. Trends Immunol 29(5):211–219
102. Ziv Y, Avidan H, Pluchino S, Martino G, Schwartz M (2006) Synergy between immune cells and adult neural stem/progenitor cells promotes functional recovery from spinal cord injury. Proc Natl Acad Sci U S A 103(35):13174–13179
103. Cusimano M, Biziato D, Brambilla E, Donegà M, Alfaro-Cervello C, Snider S, Salani G, Pucci F, Comi G, Garcia-Verdugo JM, De Palma M, Martino G, Pluchino S (2012) Transplanted neural stem/precursor cells instruct phagocytes and reduce secondary tissue damage in the injured spinal cord. Brain 35(Pt 2):447–460
104. Aharonowiz M, Einstein O, Fainstein N, Lassmann H, Reubinoff B, Ben-Hur T (2008) Neuroprotective effect of transplanted human embryonic stem cell-derived neural precursors in an animal model of multiple sclerosis. PLoS ONE 3(9):e3145
105. Uccelli A, Moretta L, Pistoia V (2008) Mesenchymal stem cells in health and disease. Nat Rev Immunol 8(9):726–736
106. Vultur A, Cao J, Arulanandam R, Turkson J, Jove R, Greer P, Craig A, Elliott B, Raptis L (2004) Cell-to-cell adhesion modulates Stat3 activity in normal and breast carcinoma cells. Oncogene 23(15):2600–2616
107. Gerdes HH, Bukoreshtliev NV, Barroso JF (2007) Tunneling nanotubes: a new route for the exchange of components between animal cells. FEBS Lett 581(11):2194–2201
108. Gnecchi M, Zhang Z, Ni A, Dzau VJ (2008) Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 103(11):1204–1219
109. Dinger ME, Mercer TR, Mattick JS (2008) RNAs as extracellular signaling molecules. J Mol Endocrinol 40(4):151–159
110. Scheffler B, Schmandt T, Schroder W, Steinfarz B, Husseini L, Wellmer J, Seifert G, Karram K, Beck H, Blumcke I, Wiestler OD, Steinhauser C, Brustle O (2003) Functional network integration of embryonic stem cell-derived astrocytes in hippocampal slice cultures. Development 130(22):5533–5541
111. Giaume C, McCarthy KD (1996) Control of gap-junctional communication in astrocytic networks. Trends Neurosci 19(8):319–325
112. Jaderstad J, Jaderstad LM, Li J, Chintawar S, Salto C, Pandolfo M, Ourednik V, Teng YD, Sidman RL, Arenas E, Snyder EY, Herlenius E (2010) Communication via gap junctions underlies early functional and beneficial interactions between grafted neural stem cells and the host. Proc Natl Acad Sci U S A 107(11):5184–5189
113. Jaderstad J, Brismar H, Herlenius E (2010) Hypoxic preconditioning increases gap-junctional graft and host communication. NeuroReport 21(17):1126–1132
114. Garbuzova-Davis S, Willing AE, Zigova T, Saporta S, Justen EB, Lane JC, Hudson JE, Chen N, Davis CD, Sanberg PR (2003) Intravenous administration of human umbilical cord

blood cells in a mouse model of amyotrophic lateral sclerosis: distribution, migration, and differentiation. J Hematother Stem Cell Res 12(3):255–270
115. Liu H, Honmou O, Harada K, Nakamura K, Houkin K, Hamada H, Kocsis JD (2006) Neuroprotection by PlGF gene-modified human mesenchymal stem cells after cerebral ischaemia. Brain 129(Pt 10):2734–2745
116. Xiao J, Nan Z, Motooka Y, Low WC (2005) Transplantation of a novel cell line population of umbilical cord blood stem cells ameliorates neurological deficits associated with ischemic brain injury. Stem Cells Dev 14(6):722–733
117. Chu K, Kim M, Park KI, Jeong SW, Park HK, Jung KH, Lee ST, Kang L, Lee K, Park DK, Kim SU, Roh JK (2004) Human neural stem cells improve sensorimotor deficits in the adult rat brain with experimental focal ischemia. Brain Res 1016(2):145–153
118. Aboody K, Capela A, Niazi N, Stern JH, Temple S (2011) Translating stem cell studies to the clinic for CNS repair: current state of the art and the need for a Rosetta Stone. Neuron 70(4):597–613

Chapter 3
Cultured Limbal Epithelial Stem Cell Therapy for Ocular Surface Diseases

Hannah J. Levis, Julie T. Daniels and Sajjad Ahmad

Abstract The cornea is our transparent window to the world and its integrity and transparency are essential for proper functioning of the eye. The corneal epithelium is a multilayered, renewable barrier that is maintained in health by epithelial stem cells from the limbus. A recent discovery leads us to believe that limbal epithelial stem cells (LESCs) reside in specialised protective niche structures identified as the limbal crypts. The pathology of limbal epithelial stem cell deficiency occurs when there is an insufficiency of LESCs to replace lost surface epithelial cells leading to persistent epithelial defects, conjunctivalisation and ultimately, loss of vision. Researchers continue to discover new ways to harness the power of epithelial stem cells for the treatment of limbal stem cell deficiency and one such way is with an expanded limbal epithelial stem cell graft. Advances in the field of tissue engineering are contributing to the optimisation of these transplantation techniques by providing new and innovative biomaterials for use as substrates.

3.1 The Cornea

The cornea is our transparent window to the world, said to be set in its sclerotic coat as a watch glass is set in its frame. It is the primary refractive element of the eye responsible for two-thirds of the eye's total optical power, much more than the

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lens. However, its curvature is fixed, unlike the lens that can be adjusted to focus when required. Together the cornea and the sclera are a roughly spherical, fibrous envelope whose important optical properties—curvature and axial length, are maintained by pressure from the intraocular fluids. Although both the cornea and the sclera are alike in consisting mostly of collagen, they are structurally very different. The transparency of the cornea sets it apart from the opaque sclera and maintenance of its integrity and transparency is essential for the proper functioning of the whole eye.

The cornea consists of 5 different layers (Fig. 3.1a); an outermost stratified, squamous epithelial layer, limited posteriorly by the Bowman's layer—an acellular layer 8–12 μm thick lying next to the basal lamina of the epithelium. Next is the corneal stroma, which accounts for around 90 % of corneal thickness and comprises aligned collagen fibrils interspersed with keratocytes. It is this precise arrangement of collagen fibrils in the cornea that are responsible for corneal transparency and what sets it apart from the disorganised sclera. The stroma is separated from the anterior most endothelial layer by the Descemet's membrane. Descemet's is considered the basement membrane of the endothelium consisting of several different collagen types that are secreted by the endothelial cells. In young eyes, the membrane is 3–4 μm thick but this can increase to 10–15 μm with age. The endothelial layer is a single layer of metabolically active cells that act as pumps to maintain the normal stromal water content, which in turn maintains the transparency of the cornea. Corneal endothelial cells are postmitotic and divide rarely, if at all, in the adult human cornea. Instead, wounding of the layer causes cells to enlarge to fill the gaps left by lost cells. If cell numbers fall below a certain threshold then corneal edema can occur, leading to disruption of the collagen fibril spacing, creating light scatter and ultimately, impaired vision.

3.2 The Corneal Epithelium

The ocular surface epithelium is a multilayered, renewable barrier consisting of 6–8 cell layers with a thickness of about 50 μm (Fig. 3.1b). The epithelial layer is stratified and cells differ in morphology depending on their location within the epithelium. The basal cells are a single layer of cells that rest on the basement membrane, adjacent to the Bowman's layer, are columnar in shape and are the newest mitotically active cells. Lying above this layer are the middle zone wing cells, so called because of their lateral wing-like side projections. This layer can be 1–3 cells thick containing cells with convex anterior surfaces, concave posterior surfaces and oval or round nuclei. This layer is then topped by several layers of flattened, squamous cells with horizontal nuclei. These cells form many tight junctions to act as a barrier to fluid loss and their superficial surfaces present numerous microvilli and microplicae, which support and stabilise the tear film on the ocular surface. The anterior most of this layer are the cells that are sloughed off first as virtually all the cells in the epithelium are born, transformed into

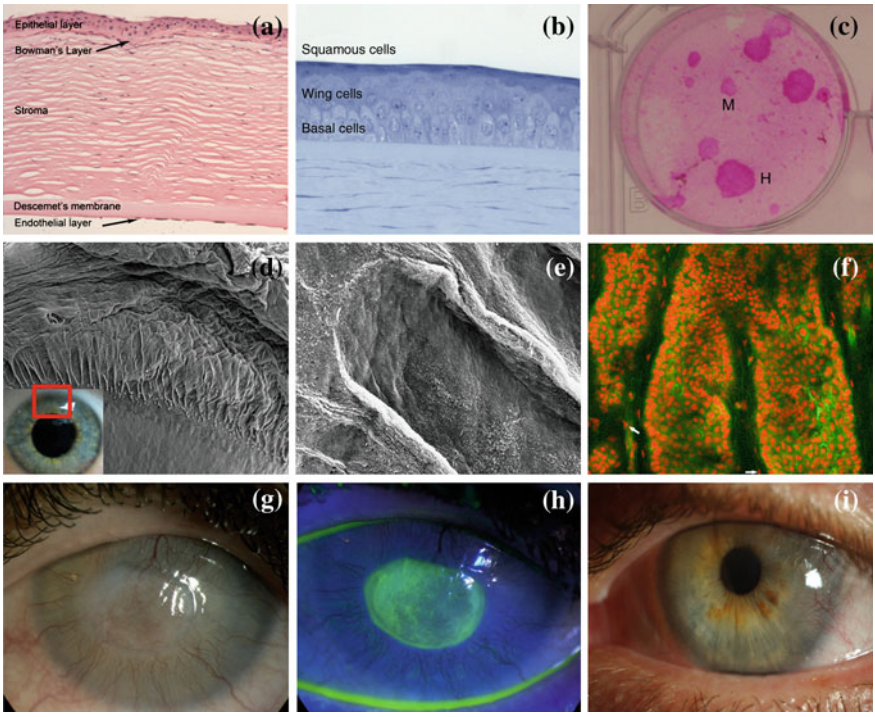

Fig. 3.1 The characteristics of human cornea and limbus. **a** The human cornea in histological cross-section stained with haematoxylin and eosin showing all five layers. **b** The human corneal epithelium showing stratification into three different layers. **c** The *CFE* assay with examples of holoclones (*H*) and meroclones (*M*). **d** Scanning electron microscope image of a decellularised human limbus showing the limbal crypts (image taken from the area indicated in the inset). **e** High magnification scanning electron microscope image of a single limbal crypt. **f** Confocal image of cells in the limbal crypts and closely associated keratocytes (*white arrows*) stained with propidium iodide (*red*) to label nuclei and fluorescein isothiocyanate—phalloidin (*green*) to label cell cytoskeleton. **g** Clinical signs of limbal epithelial stem cell deficiency resulting in neovascularization, epithelial surface breakdown and corneal opacity. **h** Fluorescein staining showing a large central epithelial defect of the cornea following unilateral alkali injury. **i** After receiving a cultured limbal epithelial autograft with cells harvested from his healthy eye, 3 years post-op the patient has a clear cornea with a healthy epithelium and limited neovascularization

differentiated epithelial cells and then shed within about 10 days in a healthy cornea. This concept of cell loss and replacement of the corneal epithelium was first described by Thoft in his *X*, *Y*, *Z* hypothesis [1]. He suggested that with *X* representing the proliferation of the basal cells, *Y* the proliferation and centripetal migration of the cells from the corneal periphery, an area known as the limbus, and *Z* the loss of cells from the surface, for healthy corneal equilibrium, $X + Y$ must equal *Z*. This theory has later been substantiated by additional evidence that the limbus is the source of cells that replace the lost squamous epithelial cells.

3.3 The Limbus

The limbus is described as an annulus of approximately 1.5 mm of tissue at the vascularised junction between the transparent cornea and opaque sclera. The limbal epithelial stem cells (LESCs) that reside in the basal layer of the limbus are thought to represent the population "*Y*" previously described by Thoft. Davanger and Everson first described this population with evidence that upon removal of the epithelium of the whole corneal surface, a normal epithelial cover redevelops after some days [2]. In addition they noted that pigment from the rabbit limbus could be visualised migrating in a centripetal formation to replace the lost central epithelium. These observations suggested that the basal cells of the central epithelium could not be solely responsible for the maintenance of the ocular surface. Further evidence suggesting the LESCs are responsible for the maintenance of the epithelium include the fact that limbal basal cells retain tritiated thymidine for prolonged periods suggesting cells with a long cell cycle, a characteristic common to all stem cells [3], LESCs have higher proliferative potential in vitro than central and peripheral epithelial cells [4] and surgical removal of the limbus in a rabbit model has been shown to delay re-epithelialisation [5].

3.4 Limbal Epithelial Stem Cells and Their Niche

A stem cell can be defined as a cell with the capacity to self-renew and differentiate along specified molecular pathways throughout life. Adult stem cells are present in many different areas of the body and can vary in their potential to produce different cell types. LESCs differ from differentiated cells as they are small in size with high nuclear to cytoplasm ratio, possess a high capacity for error free self-renewal over a long life span and are slow cycling during homeostasis [6]. This is in contrast to the transit-amplifying cells that they give rise to, which are less primitive than the LESCs, dividing more frequently but with a limited proliferative potential and the ability to ultimately transform into postmitotic, terminally differentiated cells. The LESCs within the basal layers of the limbal epithelium are thought to divide asymmetrically to give rise to a stem cell to maintain the pool and a daughter transit amplifying cell, which then migrates towards the central corneal epithelium to eventually differentiate to form the mature epithelium.

Unfortunately, a single LESC marker that is specific to these cells has yet to be determined, however, a number of general stem cell markers have been identified, which together with the absence of specific differentiated cell markers can help to identify this population. The ATP binding cassette transporter protein ABCG2 has been proposed as a universal marker of stem cells [7], and also has been found to be expressed in human limbal basal cells but not in most suprabasal or central corneal epithelial cells [8, 9]. p63 is a transcription factor that is expressed in the

nuclei of keratinocytes in areas including the skin, cervix, prostate and cornea [10]. p63 was found to be highly expressed by limbal basal cells but also by young transit amplifying cells [11]. It is therefore not regarded as a truly specific stem cell marker but a marker of cells with proliferative potential. Many other markers have been suggested and include integrins $\alpha 9$ and $\beta 1$, importin 13, OCT4, cytokeratin 15, N-cadherin [9, 12–16], however, all are disputed as true limbal stem cell markers. The absence of cornea specific differentiation markers cytokeratins 3 and 12 [17, 18] from the limbal basal epithelium indicates the basal cells are the least differentiated of the corneal epithelial cells [12]. LESCs can also be identified by their functional ability to form colonies, colony forming efficiency (CFE), when plated at a low density on a mouse embryonic 3T3 fibroblast feeder layer (Fig. 3.1c). Colony forming cells are heterogeneous in their capacity for growth and the growth potential of cells can be estimated from the colony type that results [19]. Holoclones are large with a smooth perimeter and are usually formed of small cells, which may concentrate at its perimeter. These cells have the highest growth potential and are likely to represent the LESCs. Paraclones are small, highly irregular and terminal and meroclones are typically in transition between the two with a wrinkled appearance suggesting a level of heterogeneity within the colony. Meroclones will eventually become terminal but reach a larger size than the paraclones. The number of holoclones within a given population of cells can be used to assess the percentage of stem-like cells within a population. As no definitive marker or test for LESCs exists, a battery of stem and differentiated markers and tests like the CFE assay must be used to characterise the phenotype of any corneal and limbal cells under investigation, until a suitable marker is found.

Stem cell populations throughout the body have been found to reside in specialised protective microenvironments, termed stem cell niches (reviewed in [20–22]). Although stem cells exhibit intrinsic properties that influence their behaviour, evidence suggests that cellular and extracellular components of the niche contribute to the regulation of a stem cell population to sustain their proliferative potential while at the same time preventing over-exuberant proliferation. The niches are not only home to stem cells but also a diverse range of differentiated cell types which secrete extracellular matrix components and soluble factors to contribute to the maintenance of the neighbouring stem cell population.

The LESC niche specifically has distinct anatomy that is thought to provide a physically protective environment for the stem cells. The limbal palisades of Vogt are a series of radially orientated fibrovascular ridges that were presumed to be the niche structures in the limbus [23]. However, it is now clear that the palisades are only one element of a more complex niche system as two novel niche structures have recently been described by Shortt et al. namely the limbal crypts and the focal stromal projections [24]. The limbal crypts are distinct invaginations of epithelial cells into the underlying stroma that extend from the peripheral cornea into the limbus. Scanning electron microscopy reveals the crypts are polarised, opening onto the central cornea as opposed to the conjunctival surface (Fig. 3.1d and e). Additionally, the surrounding stroma is highly cellularised with keratocytes and there is a distinct vascular supply closely associated with the crypts. Dua and

colleagues have described another limbal epithelial crypt structure as distinct anatomical extensions from the peripheral aspect of the limbal palisades [25]. These structures are thought to be larger in size but fewer in number than the limbal crypts described by Shortt. The focal stromal projections are finger-like projections of stroma extending upward into the corneal limbal epithelium. They are surrounded by small, tightly packed cells and contain a central blood vessel. There is regional variability in the distribution of the limbal crypts as they are predominant at the superior and inferior aspects of the corneal circumference, where the eyelids may offer added physical protection from ultraviolet light. The close proximity of the limbal cells in crypts to a vascular supply suggests an important niche feature likely to be providing nutrients and survival factors. Equally, the juxtaposition of keratocytes to LESCs is in keeping with the concept of cell–cell interactions playing an important role in maintenance of a stem cell population (Fig. 3.1f). This theory is supported by recent research using an in vitro 3D tissue model of the ocular surface and underlying stroma which suggests that if limbal epithelial cells are closely associated with keratocytes, as in vivo, the newly seeded epithelial layer becomes more organised and the production of basement membrane proteins is increased [26].

3.5 Limbal Stem Cell Deficiency

With regards to maintenance of a normal corneal epithelium, the main functions of the limbus are to harbour stem cells that replenish the corneal epithelium, and to act as a physical barrier separating the corneal epithelium and the phenotypically very different conjunctival epithelium and its underlying blood vessels [27, 28]. When these two functions of the limbus fail, the disease of LESC deficiency results. In this disease, due to a deficiency of the LESCs, the corneal epithelium cannot be renewed normally, and persistent and recurrent corneal epithelial defects occur [29]. In addition, the limbal barrier effect fails and the conjunctival epithelium and its underlying blood vessels encroach on to the normally avascular corneal surface to replace the failing or deficient corneal epithelium (Fig. 3.1g). The result is a disease, which is both painful and visually debilitating.

3.6 Causes of Limbal Stem Cell Deficiency

There are many causes of LESC deficiency [30]. In all however, the result is an unstable and conjunctivalised corneal surface. LESC deficiency essentially results from a primary loss of the numbers of limbal stem cells or a dysfunction of the limbal stem cells, either as a result of the stem cell itself being dysfunctional or from the microenvironment being abnormal which causes the stem cell dysfunction. The causes of LESC deficiency can be separated into primary or hereditary

causes, secondary or acquired cause, and idiopathic cases where no identifiable cause can be determined [28, 31]. Hereditary causes include aniridia, which results from a mutation in PAX6, and ectodermal dysplasia [32]. Acquired causes are by far the most common. They include injuries to the surface of the eye such as chemical or thermal burns; inflammatory diseases affecting the ocular surface such as ocular cicatricial pemphigoid, Stevens–Johnson syndrome and graft versus host disease; contact lens related keratitis which occurs either as a result of mechanical injury to the limbal epithelium, or contact lens solution related toxicity, or indeed from chronic low-grade ocular surface inflammation; and iatrogenic causes such as ocular radiation, the use of topical antiproliferative drugs for ocular surface pre-neoplastic and neoplastic conditions, and extensive limbal surgery or cryotherapy.

3.7 Signs and Symptoms of Limbal Stem Cell Deficiency

The main signs of LESC deficiency include the presence of corneal epithelial defects, which fail to heal. In addition, conjunctival epithelium can be seen on the corneal surface. Conjunctival epithelium is hazier in appearance as compared to corneal epithelium, which is clear. The presence of conjunctival epithelium on the corneal surface also results in delayed fluorescein dye staining (Fig. 3.1h). The corneal epithelium has strong tight junctions, which prevent the permeation of fluorescein dye when it is applied to the surface of the eye. Conjunctival epithelium on the other hand has fewer tight junctions and is more permeable, and so it stains slowly with fluorescein and retains the dye for longer. This is known as delayed fluorescein staining. The conjunctival epithelium has a blood vessel rich stroma unlike that of the cornea. The presence of blood vessels on the corneal surface can be indicative of conjunctivalisation. In addition to changes on the corneal surface, the clear and avascular stroma, which underlies the corneal epithelium, can also become scarred and vascularised.

Due to a conjunctivalised, vascularised and unstable corneal surface, the main symptoms of LESC deficiency are impaired vision as a result of loss of corneal clarity and chronic ocular surface irritation and pain. This makes LESC deficiency both a painful and blinding disease.

3.8 Investigations in Limbal Stem Cell Deficiency

The diagnosis of LESC deficiency is very much based on clinical examination. One useful and widely used investigative tool, which can be employed in LESC deficiency, is corneal impression cytology [30, 33]. This involves placing and then removing a piece of nitrocellulose filter paper on the surface of an anaesthetised cornea. By doing so, a sample of the superficial epithelial cells on the corneal surface can be removed for cytological analysis. The test looks specifically for the

presence of conjunctival epithelial cells in the sample by staining for goblet cell mucins or for conjunctival specific cytokeratins. If present, this confirms corneal surface conjunctivalisation and therefore, LESC deficiency.

Other tools being investigated for diagnosis of LESC deficiency include imaging modalities such as in vivo confocal microscopy and high resolution optical coherence tomography [34]. At present, however, these are not routinely used to diagnose LESC deficiency in the clinic.

3.9 The Management of Limbal Stem Cell Deficiency

The management of LESC deficiency can often be difficult and complex. The management options depend very much upon the extent of the disease and whether or not one or both eyes of the patient are affected [29]. LESC deficiency can be described as partial or total depending upon the extent of the limbal barrier broached and the amount of the corneal surface conjunctivalised. Unilateral LESC deficiency, where one eye remains normal, first means that the patient still has one seeing eye and second that the healthy eye can be used as a source of limbal tissue for any surgical options which may be considered.

Conservative management is often employed for the treatment of LESC deficiency. This includes the use of topical agents such as lubricants, anti-inflammatories, antibiotics and autologous serum. Bandage contact lenses can also be used to stabilise the ocular surface and promote epithelial healing.

Partial LESC deficiency is managed depending upon whether the patient is symptomatic from the disease. If the visual axis is not affected and the corneal surface is stable, conservative management or even simple observation may be employed. If the disease is progressive and the visual axis is affected or threatened, then surgical options may need to be employed. These include removing the encroaching conjunctival epithelium up to the limbus and allowing the limbal barrier to be restored [35]. This can be performed with or without amniotic membrane transplantation. Human amniotic membrane is often transplanted to the surface of the eye as it has a stabilising effect on the epithelium by providing an extracellular matrix scaffold, anti-inflammatory effects and anti-angiogenic properties [36]. In addition, amniotic membrane is relatively immune privileged and the recipient does not require immune suppression.

Total LESC deficiency requires the replacement of healthy limbal tissue. This can be in the form of whole tissue limbal grafts or cultured limbal epithelial grafts [37–42] (Fig. 3.1i). Corneal transplantation is not a primary treatment modality for LESC deficiency. Replacement of the central cornea means that when the corneal epithelium is shed from the grafted cornea, the LESC deficient host cannot replace the epithelium on the graft. The graft then either becomes conjunctivalised or is rejected. In LESC deficiency, corneal epithelial regeneration has to be normalised in the first instance. If despite this, the cornea remains hazy, for example as a result

of corneal stromal scarring, then corneal transplantation can be employed as a secondary treatment if the corneal epithelium is normal [43].

In unilateral total LESC deficiency, the other eye remains healthy and so that eye can be used as a source of healthy limbal tissue for transplantation. In order to reduce the risk of damage to the unaffected eye, the amount of tissue that can be removed is limited. In most cases, not enough whole limbal tissue can be removed for a whole tissue limbal transplant to the LESC deficient eye. For this reason, a small amount of limbal tissue can be removed (approximately 1–2 by 1–2 mm) and then this can be expanded in culture. The cultured limbal epithelial graft can then be transplanted to the LESC deficient eye after removing the conjunctival tissue on the corneal surface (superficial keratectomy). There are two major variables in the culture methods used to expand limbal epithelial tissue [44]. The first is whether the tissue is used intact (the explant method) or whether the epithelial cells are removed from the limbal tissue using enzymatic digestion (the suspension method). The second variable is whether mitotically inactivated 3T3 mouse fibroblasts are used as a feeder layer to grow the epithelium (the 3T3 co-culture method) or whether human amniotic membrane is used as a substrate to grow the limbal epithelium (the amniotic membrane method). Various other methods are also used, the other most common one being the use of a fibrin substrate.

In bilateral LESC deficiency, there is most often no available healthy limbus to procure limbal tissue for transplantation or culture from. Therefore, other sources have to be sought. Allogeneic limbal tissue can be procured from the eyes of healthy donors or from cadaveric sources and then transplanted as whole limbal tissue grafts, or indeed small pieces of limbal tissue can be removed, cultured and then expanded [45]. The latter may be the safest option if donor tissue is sourced from a living donor as it will be safer to remove a small amount of limbus from the donor eye. These allogeneic approaches will obviously require host immune suppression. If in bilateral disease there is some portion of the limbus which remains healthy in either eye, then this can be used to culture expand the healthy limbal epithelium and then transplant to one or both eyes. Another autologous treatment option is to culture oral mucosal epithelium and then transplant this to the eye with LESC deficiency [46]. This technique is known as cultured oral mucosal epithelial transplantation (COMET). As this tissue is not native to the cornea, it may not be as effective as limbal transplantation; however, it does mean that immune suppression and its associated risks are avoided.

Other sources of tissue for transplantation in LESC deficiency include stem cells from the hair follicle and differentiated human embryonic stem cells [47, 48]. These are still being investigated at the research stage at present and are not currently employed clinically. Mesenchymal stem cells sourced from the bone marrow or other tissues such as adipose tissue have also been suggested as a treatment option [49].

3.10 New Biomaterials in Transplanted LESC Therapy

The field of tissue engineering in general has rapidly expanded in recent times combining materials design and engineering with cell therapy and LESC therapy is no exception. As mentioned previously, the favoured carrier material for LESC transplantation has been amniotic membrane. Its use as a topical bandage for surgical wounds, ulcers or burns has exploited its natural anti-inflammatory, anti-angiogenic, anti-microbial and anti-viral properties in general medicine as well as ophthalmology (reviewed in [50]). However, as growing numbers of new materials with tuneable properties become available, the limitations of amniotic membrane as a substrate become increasingly apparent. As expected with any biological material there is considerable variation between different donors, often dependent on donor age, but also in the case of amniotic membrane there is even variation within the same donor depending on whether the material is taken from a distal or proximal location [51]. The processing of amniotic membrane by tissue banks is not optimal for LESC therapy and the extensive donor screening processes that are required are costly. Even if you do manage to obtain a suitable piece of amniotic membrane for use clinically, its mechanical properties make the material difficult to handle in surgery and its transparency is suboptimal, which is obviously a particularly important consideration when using this material for ocular surface repair. A worthy alternative to amniotic membrane must be optically transparent, mechanically strong in order to withstand manipulation in culture, irrigation and handling in surgery and also be able to conform to the concave surface of the eye. It must be cytocompatible and able to be produced with consistent quality preferably at high speed and low cost. These are the considerable challenges tissue engineers are currently facing in the field of LESC therapy and as a result, many innovative solutions have been proposed.

First, although the limitations of amniotic membrane have been highlighted, it has the potential to be enhanced with modifications—a worthwhile endeavour if its useful anti-inflammatory properties are retained. The shelf life of amniotic membrane can be enhanced by lyophilisation and this also allows sterilisation by gamma irradiation or peracetic acid [52, 53]. The freeze-drying process can affect some of the useful properties of amniotic membrane but treatment with a non-reducing disaccharide, trehalose, was found to significantly improve its quality [54]. Amniotic membrane can be combined with a polymer to create a hybrid, exploiting the biocompability of a natural material but enhancing its stability by cross-linking with substances such as polyvinyl alcohol (PVA) hydrogels or 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS). These mechanically strengthened materials are able to support stratified epithelia [55, 56].

Fibrin glue has historically been used in ocular surgery to aid sutureless attachment of amniotic membrane to the corneal surface but increasingly investigators are cutting out the middle man and using fibrin clots alone as a substrate for LESC culture for transplantation [57–59]. Fibrin glue is the product of fibrinogen

and thrombin which when combined form a natural, degradable clot. This system is particularly favourable because both of its constituent parts can be of human origin providing the potential for an autologous bioengineered tissue. Taking this approach one step further, some are attempting to remove the need for a biomaterial scaffold altogether. A highly innovative method to detach cell sheets from the tissue culture surface has been developed which allows whole sheets with no carrier substrate to be transplanted to the ocular surface [60]. A temperature responsive polymer, poly(N-isopropylacrylaminde), was covalently bonded to tissue culture plates so that under normal culture conditions of 37 °C the dish surface is hydrophobic and cells can attach. When the temperature is reduced below 32 °C the surface becomes hydrophobic forming a hydration later between the cells and the surface allowing spontaneous detachment of an intact cell layer. One advantage of this method is that extracellular matrix on the basal cell surface of the sheet is maintained, which would not be the case if the sheet was detached enzymatically, and can be transplanted directly onto the ocular surface. Additionally, the challenge of carrier material transparency is irrelevant as only cells are transplanted. Cell sheets cultured on these plates have been transplanted in a rabbit model of LESC deficiency and have been shown to successfully reconstruct the corneal surface [61], however, the increased fragility of the cell sheet in comparison to other substrates is still an important consideration and may not be suitable in all cases.

Natural materials such as keratin and silk fibroin have been proposed as suitable substrates for cellular scaffolds and their use in LESC therapy has been evaluated. Keratin films were produced from human hair using a multi-step process of extraction, neutral and alkaline dialysis, drying and curing [62]. The resultant films showed improved light transmission and biomechanical strength in comparison to amniotic membrane and a corneal epithelial cell line showed similar behaviour on the films as on amnion. Silk fibroin is a structural protein that can be harvested from the cocoon of the *Bombyx mori* silkworm. It displays a non-imunogenic response on implantation in vivo with a controlled degradation rate and is also mechanically robust, transparent and easy to handle [63]. For these reasons, it has been considered as suitable substrate for LESC culture and indeed it has been shown that it can support the growth of limbal epithelial cells once attached, although in some cases the initial cell attachment rate was much lower than the attachment to amniotic membrane [64]. The major limitation of using natural materials such as silk fibroin or keratin films is their cost and lengthy production time, which is considerably higher than most synthetic materials.

As previously described, the corneal stroma predominantly consists of collagen so the use of biopolymers such as collagen as a carrier material for LESCs that will ultimately remain on the stromal surface appears a wise choice. Furthermore, collagen as a cellular substrate has a number of favourable properties including low toxicity, low immunogenicity, biodegradability and a relatively low isolation cost. Collagen hydrogels are an early example of the use of collagen as a 3D cellular scaffold but simple hydrogels have a low starting collagen concentration and exhibit very poor mechanical properties that preclude their use as a carrier for transplantation. Steps have been taken to enhance the mechanical properties of

hydrogels by cross-linking with substances such as glutaraldehyde or carbodiimide, the latter being preferable, as it is not incorporated into the final product [65, 66]. The enhancement of mechanical strength comes with a tradeoff in reduction of the biomimetic qualities and prevention of cell-based scaffold remodelling. Another simple yet effective way to enhance the mechanical properties of collagen type I hydrogels, but without compromising their biomimetic qualities, is using a process called plastic compression. The process negates the need for slow and expensive production of tissues that rely on the ability of cells to fabricate new tissue around a scaffold. Instead, a cellular construct can be engineered in minutes by rapid removal of fluid from a hyperhydrated collagen gel [67]. Reconstituted collagen gels comprise a random mesh of collagen fibrils retaining 99 % excess fluid that can be expelled in an unconfined manner after application of a load and that fluid does not return on removal of the load, i.e. the gel undergoes plastic compression. The resulting stable collagen constructs resemble a thin, transparent membrane with enhanced mechanical strength. One distinct advantage that this process offers over others such as the cross-linked hydrogels is that cells can be seeded directly into the scaffold before compression—there is no lengthy wait for cells to populate the scaffold. Plastic compressed collagen has been shown to be a suitable substrate for human limbal epithelial cell culture. Cells grown on the substrate produced an epithelium with many features comparable to the central cornea such as a stratified epithelium, expression of putative stem cell marker, differentiated cell marker and basement membrane proteins as well as ultrastructural features akin to a functional corneal epithelium [26].

Additional collagen based scaffolds include collagen vitrigels and electrospun collagen mats but both processes do not allow the direct seeding of cells into the scaffold. A vitrigel is produced in a three-stage process of gelation, vitrification and rehydration resulting in a transparent, rigid, glass like material. A mixed population of both stem and differentiated cells can be maintained on the surface of the vitrigels which are approximately 20–50 μm thick [68]. Electro spinning is a process that is commonly used to produce synthetic polymer nano fibres but more recently it has been investigated as a potential method for creating an electron spun collagen substrate. The process uses an electric charge to draw very fine fibres from a liquid that is usually a combination of collagen and synthetic polymers. The resultant fibres can be aligned to promote corneal fibroblast cell elongation in response to these microstructural changes [69]. However, it remains to be seen whether electospun collagen matrices will be suitable carriers for limbal epithelial cells for transplantation.

3.11 Concluding Remarks

Since the discovery of LESCs and the classification of many ocular surface diseases as being a result of LESC failure, investigators have been seeking ways to harness the corneal epithelium's regenerative power for clinical therapy.

The advancement in cell culture techniques for limbal epithelial cells has allowed expansion of cells from limited starting material and now cultured stem cell therapy is used to treat ocular surface damage in many centres across the globe. Clinical therapies of the future will undoubtedly benefit from the expanding field of tissue engineering, which will provide new and innovative biomaterials for tissue transplantation carriers and cell culture substrates.

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References

1. Thoft RA, Friend J (1983) The X, Y, Z hypothesis of corneal epithelial maintenance. Invest Ophthalmol Vis Sci 24(10):1442–1443
2. Davanger M, Evensen A (1971) Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature 229(5286):560–561
3. Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM (1989) Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell 57(2):201–209
4. Pellegrini G, Golisano O, Paterna P, Lambiase A, Bonini S, Rama P, De Luca M (1999) Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. J Cell Biol 145(4):769–782
5. Chen JJ, Tseng SC (1991) Abnormal corneal epithelial wound healing in partial-thickness removal of limbal epithelium. Invest Ophthalmol Vis Sci 32(8):2219–2233
6. Li W, Hayashida Y, Chen YT, Tseng SC (2007) Niche regulation of corneal epithelial stem cells at the limbus. Cell Res 17(1):26–36
7. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 7(9):1028–1034
8. de Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li D-Q (2005) ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. Stem Cells 23(1):63–73
9. Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li D-Q (2004) Characterization of putative stem cell phenotype in human limbal epithelia. Stem Cells 22(3):355–366
10. Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, Ponzin D, McKeon F, De Luca M (2001) p63 identifies keratinocyte stem cells. Proc Natl Acad Sci U S A 98(6):3156–3161
11. Dua HS, Joseph A, Shanmuganathan VA, Jones RE (2003) Stem cell differentiation and the effects of deficiency. Eye (Lond Engl) 17(8):877–885
12. Chee KY, Kicic A, Wiffen SJ (2006) Limbal stem cells: the search for a marker. Clin Exp Ophthalmol 34(1):64–73
13. Zhou SY, Zhang C, Baradaran E, Chuck RS (2010) Human corneal basal epithelial cells express an embryonic stem cell marker OCT4. Curr Eye Res 35(11):978–985
14. Wang H, Tao T, Tang J, Mao Y, Li W, Peng J, Tan G, Zhou Y, Zhong J, Tseng S, Kawakita T, Zhao Y, Liu Z (2009) Importin 13 serves as a potential marker for corneal epithelial progenitor cells. Stem Cells 27:2516–2526

15. Yoshida S, Shimmura S, Kawakita T, Miyashita H, Den S, Shimazaki J, Tsubota K (2006) Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. Invest Ophthalmol Vis Sci 47(11):4780–4786
16. Hayashi R, Yamato M, Sugiyama H, Sumide T, Yang J, Okano T, Tano Y, Nishida K (2007) N-Cadherin is expressed by putative stem/progenitor cells and melanocytes in the human limbal epithelial stem cell niche. Stem Cells 25(2):289–296
17. Kurpakus MA, Stock EL, Jones JC (1990) Expression of the 55/64-kD corneal keratins in ocular surface epithelium. Invest Ophthalmol Vis Sci 31(3):448–456
18. Schermer A, Galvin S, Sun TT (1986) Differentiation-related expression of a major 64 K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J Cell Biol 103(1):49–62
19. Barrandon Y, Green H (1987) Three clonal types of keratinocyte with different capacities for multiplication. Proc Natl Acad Sci U S A 84(8):2302–2306
20. Spradling A, Drummond-Barbosa D, Kai T (2001) Stem cells find their niche. Nature 414(6859):98–104
21. Watt FM, Hogan BL (2000) Out of Eden: stem cells and their niches. Science 287(5457):1427–1430
22. Fuchs E, Tumbar T, Guasch G (2004) Socializing with the neighbors: stem cells and their niche. Cell 116(6):769–778
23. Goldberg MF, Bron AJ (1982) Limbal palisades of Vogt. Trans Am Ophthalmol Soc 80:155–171
24. Shortt A, Secker G, Munro P, Khaw P, Tuft S, Daniels J (2007) Characterization of the limbal epithelial stem cell niche: novel imaging techniques permit in vivo observation and targeted biopsy of limbal epithelial stem cells. Stem Cells 25(6):1402–1409
25. Dua H, Shanmuganathan VA, Powell-Richards AO, Tighe PJ, Joseph A (2005) Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. Br J Ophthalmol 89(5):529–532
26. Levis HJ, Brown RA, Daniels JT (2010) Plastic compressed collagen as a biomimetic substrate for human limbal epithelial cell culture. Biomaterials 31:7726–7737
27. Ahmad S, Figueiredo F, Lako M (2006) Corneal epithelial stem cells: characterization, culture and transplantation. Regen Med 1(1):29–44
28. Ahmad S, Kolli S, Lako M, Figueiredo F, Daniels JT (2010) Stem cell therapies for ocular surface disease. Drug Discovery Today 15(7–8):306–313
29. Ahmad S, Osei-Bempong C, Dana R, Jurkunas U (2010) The culture and transplantation of human limbal stem cells. J Cell Physiol 225(1):15–19
30. Puangsricharern V, Tseng SC (1995) Cytologic evidence of corneal diseases with limbal stem cell deficiency. Ophthalmology 102(10):1476–1485
31. Espana EM, Grueterich M, Romano AC, Touhami A, Tseng SC (2002) Idiopathic limbal stem cell deficiency. Ophthalmology 109(11):2004–2010
32. Ramaesh K, Ramaesh T, Dutton GN, Dhillon B (2005) Evolving concepts on the pathogenic mechanisms of aniridia related keratopathy. Int J Biochem Cell Biol 37(3):547–557
33. Dart J (1997) Impression cytology of the ocular surface–research tool or routine clinical investigation? Br J Ophthalmol 81(11):930
34. Shortt AJ, Secker GA, Rajan MS, Meligonis G, Dart JK, Tuft SJ, Daniels JT (2008) Ex vivo expansion and transplantation of limbal epithelial stem cells. Ophthalmology 115(11):1989–1997
35. Dua HS, Gomes JA, Singh A (1994) Corneal epithelial wound healing. Br J Ophthalmol 78(5):401–408
36. Grueterich M, Espana EM, Tseng SC (2003) Ex vivo expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche. Surv Ophthalmol 48(6):631–646
37. Kenyon KR, Tseng SC (1989) Limbal autograft transplantation for ocular surface disorders. Ophthalmology 96(5):709–722 (discussion 722–703)

38. Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M (1997) Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet 349(9057):990–993
39. Tsai RJ, Li LM, Chen JK (2000) Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. N Engl J Med 343(2):86–93
40. Shortt AJ, Secker GA, Notara MD, Limb GA, Khaw PT, Tuft SJ, Daniels JT (2007) Transplantation of ex vivo cultured limbal epithelial stem cells: a review of techniques and clinical results. Surv Ophthalmol 52(5):483–502
41. Baylis O, Figueiredo F, Henein C, Lako M, Ahmad S (2011) 13 years of cultured limbal epithelial cell therapy: a review of the outcomes. J Cell Biochem 4:993–1002
42. Holland EJ (1996) Epithelial transplantation for the management of severe ocular surface disease. Trans Am Ophthalmol Soc 94:677–743
43. Kolli S, Ahmad S, Lako M, Figueiredo F (2010) Successful clinical implementation of corneal epithelial stem cell therapy for treatment of unilateral limbal stem cell deficiency. Stem Cells 28(3):597–610
44. Osei-Bempong C, Henein C, Ahmad S (2009) Culture conditions for primary human limbal epithelial cells. Regen Med 4(3):461–470
45. James SE, Rowe A, Ilari L, Daya S, Martin R (2001) The potential for eye bank limbal rings to generate cultured corneal epithelial allografts. Cornea 20(5):488–494
46. Nakamura T, Takeda K, Inatomi T, Sotozono C, Kinoshita S (2011) Long-term results of autologous cultivated oral mucosal epithelial transplantation in the scar phase of severe ocular surface disorders. Br J Ophthalmol 95(7):942–946
47. Meyer-Blazejewska EA, Call MK, Yamanaka O, Liu H, Schlotzer-Schrehardt U, Kruse FE, Kao WW (2011) From hair to cornea: toward the therapeutic use of hair follicle-derived stem cells in the treatment of limbal stem cell deficiency. Stem Cells 29(1):57–66
48. Ahmad S, Stewart R, Yung S, Kolli S, Armstrong L, Stojkovic M, Figueiredo F, Lako M (2007) Differentiation of human embryonic stem cells into corneal epithelial-like cells by in vitro replication of the corneal epithelial stem cell niche. Stem Cells 25(5):1145–1155
49. Reinshagen H, Auw-Haedrich C, Sorg RV, Boehringer D, Eberwein P, Schwartzkopff J, Sundmacher R, Reinhard T (2011) Corneal surface reconstruction using adult mesenchymal stem cells in experimental limbal stem cell deficiency in rabbits. Acta Ophthalmol 89(8):741–748
50. Gomes JA, Romano A, Santos MS, Dua HS (2005) Amniotic membrane use in ophthalmology. Curr Opin Ophthalmol 16(4):233–240
51. Dua HS, Rahman I, Miri A, Said DG (2010) Variations in amniotic membrane: relevance for clinical applications. Br J Ophthalmol 94(8):963–964
52. Jang I-K, Ahn J-I, Shin J-S, Kwon Y-S, Ryu Y-H, Lee J-K, Park J-K, Song K-Y, Yang E-K, Kim J-C (2006) Transplantation of reconstructed corneal layer composed of corneal epithelium and fibroblasts on a lyophilized amniotic membrane to severely alkali-burned cornea. Artif Organs 30(6):424–431
53. von Versen-Höynck F, Hesselbarth U, Möller DE (2004) Application of sterilised human amnion for reconstruction of the ocular surface. Cell Tissue Banking 5(1):57–65
54. Nakamura T, Sekiyama E, Takaoka M, Bentley AJ, Yokoi N, Fullwood NJ, Kinoshita S (2008) The use of trehalose-treated freeze-dried amniotic membrane for ocular surface reconstruction. Biomaterials 29(27):3729–3737
55. Uchino Y, Shimmura S, Miyashita H, Taguchi T, Kobayashi H, Shimazaki J, Tanaka J, Tsubota K (2007) Amniotic membrane immobilized poly(vinyl alcohol) hybrid polymer as an artificial cornea scaffold that supports a stratified and differentiated corneal epithelium. J Biomed Mater Res Part B Appl Biomater 81(1):201–206
56. Zajicova A, Pokorna K, Lencova A, Krulova M, Svobodova E, Kubinova S, Sykova E, Pradny M, Michalek J, Svobodova J, Munzarova M, Holan V (2010) Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. Cell Transp 19:1281–1290

57. Marchini G, Pedrotti E, Pedrotti M, Barbaro V, Di Iorio E, Ferrari S, Bertolin M, Ferrari B, Passilongo M, Fasolo A, Ponzin D (2011) Long-term effectiveness of autologous cultured limbal stem cell grafts in patients with limbal stem cell deficiency due to chemical burns. Clin Exp Ophthalmol 40:255–267
58. Rama P, Bonini S, Lambiase A, Golisano O, Paterna P, De Luca M, Pellegrini G (2001) Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. Transplantation 72(9):1478–1485
59. Han B, Schwab IR, Madsen TK, Isseroff RR (2002) A fibrin-based bioengineered ocular surface with human corneal epithelial stem cells. Cornea 21(5):505–510
60. Yang J, Yamato M, Nishida K, Hayashida Y, Shimizu T, Kikuchi A, Tano Y, Okano T (2006) Corneal epithelial stem cell delivery using cell sheet engineering: not lost in transplantation. J Drug Target 14(7):471–482
61. Nishida K, Yamato M, Hayashida Y, Watanabe K, Maeda N, Watanabe H, Yamamoto K, Nagai S, Kikuchi A, Tano Y, Okano T (2004) Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface. Transplantation 77(3):379–385
62. Reichl S, Borrelli M, Geerling G (2011) Keratin films for ocular surface reconstruction. Biomaterials 32(13):3375–3386
63. Lawrence BD, Cronin-Golomb M, Georgakoudi I, Kaplan DL, Omenetto FG (2008) Bioactive silk protein biomaterial systems for optical devices. Biomacromolecules 9(4):1214–1220
64. Bray LJ, George KA, Ainscough SL, Hutmacher DW, Chirila TV, Harkin DG (2011) Human corneal epithelial equivalents constructed on *Bombyx mori* silk fibroin membranes. Biomaterials 33(16):4110–4117
65. Griffith M, Osborne R, Munger R, Xiong X, Doillon CJ, Laycock NL, Hakim M, Song Y, Watsky MA (1999) Functional human corneal equivalents constructed from cell lines. Science 286(5447):2169–2172
66. Dravida S, Gaddipati S, Griffith M, Merrett K (2008) A biomimetic scaffold for culturing limbal stem cells: a promising alternative for clinical transplantation. J Tissue Eng Regen Med 2(5):263–271
67. Brown R, Wiseman M, Chuo C, Cheema U, Nazhat S (2005) Ultrarapid engineering of biomimetic materials and tissues: Fabrication of Nano-and Microstructures by Plastic Compression. Adv Funct Mater 15(11):1762–1770
68. McIntosh AW, Salahuddin A, So S, Ng S, Ponce MS, Takezawa T, Schein O, Elisseeff J (2009) Collagen vitrigel membranes for the in vitro reconstruction of separate corneal epithelial, stromal, and endothelial cell layers. J Biomed Mater Res Part B Appl Biomater 90B(2):818–831
69. Wray L, Orwin E (2009) Recreating the microenvironment of the native cornea for tissue engineering applications. Tissue Engineering Part A 15(7):1463–1472

Chapter 4
Keratinocyte Stem Cells: Biology and Clinical Applications

Carlo Pincelli and Alessandra Marconi

Abstract Human epidermis represents a large reservoir of stem cells that continue to self-renew throughout life. Stem cells are essential for skin regeneration and for repair after wounding. They allow long-term culture of keratinocytes that produce large sheets of epidermis to cover extensive burns, thus being lifesaving for these patients. Furthermore, stem cells can be expanded in culture, genetically modified to correct the gene deficiency in genetic skin diseases. This chapter will describe the most recent data on stem cell biology and the potential medical applications of these cells.

4.1 Introduction

Epidermis acts as a barrier against harmful microbes, water loss, physical, thermal, and mechanical injuries. In addition, the skin plays a critical role in thermoregulation and in the sensory perception of environmental stimuli. To support this role, epidermis constantly self-renew throughout life to maintain normal homeostasis and to repair damage after wounding. The regenerative capacities of epithelial populations have been confirmed for many years by the evidence that cultured keratinocytes produce autologous grafts that regenerate an epidermis over a full-thickness wound [1]. Epidermal homeostasis depends on a balance between proliferation and differentiation/apoptosis of keratinocytes [2]. The epidermis contains a basal layer of proliferative keratinocytes that adhere to the underlying basement membrane (BM). Periodically, these cells detach from the BM, withdraw from the cell cycle and

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initiate a program of terminal differentiation, moving upward to the skin surface. Upon commitment to terminal differentiation, keratinocytes progress through different stages and form three distinct layers: the spinous, the granular, and the stratum corneum. Eventually, keratinocytes loose the nuclei and are continuously sloughed off from the skin surface. The entire process takes about 4 weeks. The epidermis comprises pilo-sebaceous units containing one hair follicle and its sebaceous gland (SG), surrounded by the interfollicular epidermis (IFE).

The hair follicle (HF) is characterized by cycles of growth and degeneration. During the first hair cycle, HF stem cells (SC) proliferate actively to form the new hair (anagen phase). After reaching the final size, HF cells stop proliferating and undergo apoptosis (catagen phase). Following this stage, HF SC enter a quiescent phase (telogen). The mature HF is composed of various layers of differentiated cells, including the outer root sheath, the inner root sheath, and the hair shaft. The matrix cells at the base of the HF differentiate into the inner root sheath and hair shaft cells [3].

4.2 Stem Cells of the Epidermis and the Niche

Epidermal homeostasis is maintained by the presence of keratinocyte SC. SC self-renew and generate the differentiated lineages that form the mature tissue. There are several SC pools in the epidermis. The IF SC of the basal layer, the HF SC of the bulge and the SG SC that are located above the bulge, and below the hair shaft orifice. During physiological tissue renewal, the three skin cell populations are each believed to be maintained by their own SC. While under homeostatic conditions HF SC fail to contribute to IFE regeneration [4], upon wounding, HF SC participate actively in the renewal of the epidermis [5].

The bulge region of the HF contains a population of quiescent cells [6] characterized by the highest clonogenic potential and by the greatest proliferation rate in vitro [7, 8], indicating that HF SC are located in this area. When studying HF SC, it should be noted that major differences exist between rodent and human HF [9]. For example, as opposed to the mouse HF SC niche, the human bulge is less morphologically distinct. Another difference is that HF grow for years in humans and for weeks in rodents. Moreover, human and murine follicular epithelial SC have a different biomarker expression. Human bulge cells express CD200, K15, K19, as the most reliable markers, while CD34, K15 are preferentially expressed by mouse bulge cells. Two SC subpopulations have been detected in human HF based on different markers: $CD200^{+}CD34^{-}K15^{+bright}$ cells are located in the basal bulge layer, and form few but large-sized colonies, while $CD200^{+}CD34^{-}K15^{+low}$ cells are mostly found in suprabasal location and form smaller colonies [10]. In the mouse HF, SC can be isolated and purified using a combination of $CD34^{+}$ α_6 integrin antibodies [11]. Recently, $Lgr5^{+}$ [leucine-rich repeat containing G protein-cupled receptor 5] SC were identified in the mouse telogen bulge and in the lower outer root sheath of anagen HF [12]. $Lgr5^{+}$ population was strongly enriched for

bulge markers such as CD34 [13]. $Lgr5^+$ cells act as fully functional HF SC in vitro and in vivo, but do not contribute to the SG or IF epidermis [12].

Lineage tracing experiments show that IF SC are comprised in the epidermal proliferative unit (EPU), where clones of cells extend from the basal layer to the top cornified cells [14]. According to this model, a single stem cell divides infrequently to produce a stem cell daughter and a nonstem committed progenitor cell, named transit amplifying (TA) cell. TA cells divide a small number of times before withdrawing from the cell cycle and undergoing terminal differentiation in the suprabasal layers. The EPU model has been questioned recently by lineage tracing experiments demonstrating that normal adult epidermis is maintained by a single population of committed progenitor cells with different stochastic choices [15]. Clonogenicity assays identify IF SC as holoclones that found large colonies with the greatest regenerative capacity in long-term culture [16]. IF SC well correlates with high β_1 integrin expression [17], allowing to distinguish SC from TA and post-mitotic (PM) cells that express lower levels of β_1 integrin [18] (Fig. 4.1). IF SC can also be isolated by the combination of α_6 integrin ($\alpha_6{}^{bri}$) and CD 71 ($CD71^{dim}$) monoclonal antibodies [19]. Indeed, these cells are quiescent and exhibit long-term regenerative capacity [20]. Other proteins such as melanoma chondroitin sulphate proteoglycan (MCSP), and the EGF receptor antagonist Lrig1 have been recently proposed as markers of IF SC [21, 22]. Using a scaffold-based organotypic skin culture, it is now possible to mark keratinocytes with iododeoxyuridine and follow them for 8–10 weeks, when only less than 1 % of basal cells have retained the label. This work demonstrates that label retaining quiescent cells are individually dispersed throughout the basal layer in a random distribution [23]. As a similar pattern was also observed for the IFE of mouse skin [24], this is likely to be the kind of distribution of SC in the basal layer. As opposed to other theories indicating SC to be distributed as clusters in different locations, the random pattern of distribution seems to better fulfill the tasks of IF SC that must be ready to reconstitute epidermis wherever it is needed.

4.3 Clinical Applications

4.3.1 Wounds and Burns

Globally, nearly 10 million people suffer from cutaneous burns each year [25], while the skin loss from trauma, chronic ulceration from diabetes or from venous ulcers are about 2 milions, and 6,00,000 patients, respectively [26]. These wounds lead to a loss of integrity of the skin and are a major cause of morbidity and mortality. The primary goals in treating burns are rapid closure, restoration of function, and esthetically satisfactory scar development. In the past two decades, advances in cellular and molecular biology have expanded our understanding of wound repair and tissue regeneration. Wound healing encompasses a complex process that requires the

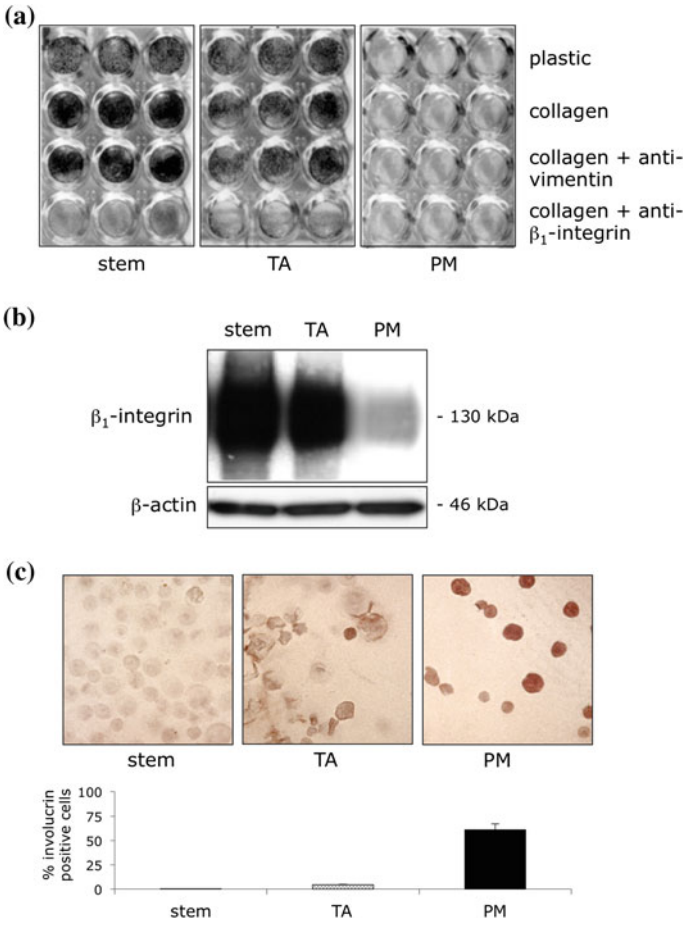

Fig. 4.1 β_1 integrin expression correlates with collagen IV-cell adhesion and differentiation in keratinocyte subpopulations **a** For cell adhesion assay, keratinocytes, with or without preincubation with anti-β_1 integrin or anti-vimentin antibody (used as control) for 1 h at 37 °C, were seeded onto microplate coated with or without collagen IV. The adhesion was blocked 2 h later. The adhered cells were fixed with formaldehyde and stained with 0.2 *% crystal violet*. **b** Protein extracts from keratinocyte subpopulations, isolated by their adhesiveness capacity to collagen IV, were analyzed by western blotting for β_1 integrin expression. β-actin expression was used as internal control. **c** Cells from KSC, TA and PM were fixed in situ in 4 % paraformaldehyde and cytospun onto slides. After removal endogenous peroxidase activity, slides were incubated with anti-involucrin antibody for 1 h. They were then incubated with secondary antibody for 1 h followed by incubation with ABC complex for 1 h. Finally, 3-amino-9-ethylcarbazole was used for visualization. Relative intensity of staining was quantified by ImageJ64 software

integration of cell migration and keratinocyte proliferation as well as extracellular matrix deposition, angiogenesis, and remodeling by fibroblasts that produce, among other substances, agents promoting keratinocyte growth [27, 28].

The tremendous ability of epidermal SC to continuously produce large quantitiy of human keratinocytes which undergo terminal differentiation [29] has made it possible to develop skin grafts from a small skin biopsy within 2–4 weeks [30]. These tissue engineered products have been used successfully in the treatment of chronic skin wounds and burns. While autologous skin grafting, consisting in the use of a piece of skin removed from the same patient and transplanted to the wound area, is the treatment of choice for limited skin injuries, it cannot be employed in extensive burns. In such cases, only few cells SC removed from the unaffected skin can be cultured and produce sufficient keratinocytes to cover in an autologous manner the entire skin surface, being thus lifesaving for the most severely burned patients. Considering that the number and size of HF have an impact on the number of SC present in the corresponding culture, it has been shown recently that biopsies taken from the periauricular skin allow a easier HF extraction and yield the highest number of SC [31]. All in all, few SC derived from a tiny piece of proper skin, possibly rich in HF, allows the regeneration of large sheets of skin that can successfully treat extensive wounds with good esthetical results.

4.4 Genetic Skin Diseases

There are a number of devastating genodermatoses that do not respond to any conventional cure. For monogenic diseases, gene therapy has the capability to ameliorate them and potentially be a decisive treatment. SC are the best choice for gene therapy because of their ability to self-renew indefinitely and their potential to produce differentiated cells. Adult skin is easy to access, allowing the recovery of a high number of SC that can be expanded in culture, genetically modified and transplanted into the diseased skin, where they allow the transgene to remain stable for a long time [32, 33]. Among genetic skin diseases, the family of epidermolysis bullosa (EB) includes severe conditions characterized by the disruption of dermal-epidermal junction. EB is classified into simplex, junctional, and dystrophic according to the site of defect of genetic defect. Junctional EB is caused by an autosomal recessive mutation in one chain of laminin-5, an essential component of the hemidesmosome. Laminin deficiency leads to various degree of severity, from early mortality (Herliz variant) to disfiguring and painful blisters, recurrent infections, and skin cancer [34]. Mavilio and co-workers were able to fix laminin-5 deficiency in junctional EB through transplantation of cultured skin derived from genetically modified epidermal SC, thus achieving a full functional protein and the amelioration of the disease [35]. Despite the successful therapy, major concerns were raised by the use of retroviral vectors and in particular, the Moloney leukemia virus-derived vectors were found to cause lymphoproliferative disorders during clinical trials. As an alternative strategy, Di Nunzio and co-workers have used a self-inactivating lentiviral vector in which transgene expression is under the control of keratin-14 that is restricted to the basal layer of the epidermis. These

vectors can effectively transduce SC derived from a skin biopsy of junctional EB, restore synthesis of laminin-5 in keratinocytes, and normal adhesion in vivo. Self-inactivating lentiviral vectors could become a good alternative for the gene therapy in junctional EB [36]. These studies clearly indicate the possibility of treating other genodermatoses with gene therapy and change/save life of a huge number of patients. To confirm this hope, a preclinical corrective gene transfer in SC from patients with Xeroderma pigmentosum (XP) has been recently reported. XP is a devastating skin disease associated with a high predisposition to skin cancer due to XP cells being deficient in nucleotide excision repair (NER) and causing genome instability. Wild-type XPC gene was transduced into SC and maintained long term in culture. In addition, corrected XP cells displayed normal NER capacity in skin equivalent cultures and murine model of human skin regeneration in vivo [37].

4.5 Vitiligo

Vitiligo is an acquired pigmentary disorder of unknown etiology that is clinically characterized by the development of white macules related to the selective disappearance or loss of function of melanocytes. Keratinocytes are essential for melanocyte function, in that they release a number of factors that contribute to cell differentiation and melanogenesis. In vitiligo, keratinocytes from involved skin contain a high proportion of apoptotic cells [38], have a shorter life span, and are unable to maintain melanocytes in vitro [39]. Keratinocyte and melanocyte suspensions have been recently used to improve vitiligo [40]. Similarly to grafting cultured keratinocytes enriched in SC in wounds, the transplantation of sheets from cultured keratinocytes could have a potential benefit in vitiligo, given the quality of the culture system which is based upon a high content in keratinocyte SC [41].

References

1. Coolen NA, Verkerk M, Reijnen L, Vlig M, van den Bogaerdt AJ, Breetveld M, Gibbs S, Middelkoop E, Ulrich MM (2007) Culture of keratinocytes for transplantation without the need of feeder layer cells. Cell Transplant 16(6):649–661
2. Pincelli C, Marconi A (2011) Keratinocyte stem cells: friends and foes. J Cell Physiol 225(2):310–315
3. Blanpain C, Fuchs E (2006) Epidermal stem cells of the skin. Annu Rev Cell Dev Biol 22:339–373
4. Levy V, Lindon C, Harfe BD, Morgan BA (2005) Distinct stem cell populations regenerate the follicle and interfollicular epidermis. Dev Cell 9(6):855–861
5. Jiang S, Zhao L, Teklemariam T, Hantash BM (2010) Small cutaneous wounds induce telogen to anagen transition of murine hair follicle stem cells. J Dermatol Sci 60(3):143–150
6. Cotsarelis G, Sun TT, Lavker RM (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61(7):1329–1337

7. Kobayashi K, Rochat A, Barrandon Y (1993) Segregation of keratinocyte colony-forming cells in the bulge of the rat vibrissa. Proc Natl Acad Sci USA 90(15):7391–7395
8. Zhang YV, White BS, Shalloway DI, Tumbar T (2010) Stem cell dynamics in mouse hair follicles: a story from cell division counting and single cell lineage tracing. Cell Cycle 9(8):1504–1510
9. Cotsarelis G (2006) Gene expression profiling gets to the root of human hair follicle stem cells. J Clin Invest 116:19–22
10. Inoue K, Aoi N, Sato T, Yamauchi Y, Suga H, Eto H, Kato H, Araki J, Yoshimura K (2009) Differential expression of stem-cell-associated markers in human hair follicle epithelial cells. Lab Invest 89:844–856
11. Blanpain C, Lowry WE, Geoghegan A, Polak L, Fuchs E (2004) Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell 118(5):635–648
12. Jaks V, Barker N, Kasper M, van Es JH, Snippert HJ, Clevers H, Toftgård R (2008) Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nat Genet 40:1291–1299
13. Snippert HJ, Haegebarth A, Kasper M, Jaks V, van Es JH, Barker N, van de Wetering M, van den Born M, Begthel H, Vries RG, Stange DE, Toftgård R, Clevers H (2010) Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. Science 327:1385–1389
14. Ghazizadeh S, Taichman LB (2001) Multiple classes of stem cells in cutaneous epithelium: a lineage analysis of adult mouse skin. EMBO J 20(6):1215–1222
15. Clayton E, Doupé DP, Klein AM, Winton DJ, Simons BD, Jones PH (2007) A single type of progenitor cell maintains normal epidermis. Nature 446:185–189
16. Barrandon Y, Morgan JR, Mulligan RC, Green H (1989) Restoration of growth potential in paraclones of human keratinocytes by a viral oncogene. Proc Natl Acad Sci USA 86(11):4102–4106
17. Jones PH, Watt FM (1993) Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 73(4):713–724
18. Tiberio R, Marconi A, Fila C, Fumelli C, Pignatti M, Krajewski S, Giannetti A, Reed JC, Pincelli C (2002) Keratinocytes enriched for stem cells are protected from anoikis via an integrin signaling pathway in a Bcl-2 dependent manner. FEBS Lett 524(1–3):139–144
19. Li A, Simmons PJ, Kaur P (1998) Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype. Proc Natl Acad Sci USA 95:3902–3907
20. Schlüter H, Paquet-Fifield S, Gangatirkar P, Li J, Kaur P (2011) Functional characterization of quiescent keratinocyte stem cells and their progeny reveals a hierarchical organization in human skin epidermis. Stem Cells 29(8):1256–1268
21. Legg J, Jensen UB, Broad S, Leigh I, Watt FM (2003) Role of melanoma chondroitin sulphate proteoglycan in patterning stem cells in human interfollicular epidermis. Dev 130:6049–6063
22. Jensen KB, Watt FM (2006) Single-cell expression profiling of human epidermal stem and transit-amplifying cells: Lrig1 is a regulator of stem cell quiescence. Proc Natl Acad Sci USA 103:11958–11963
23. Muffler S, Stark HJ, Amoros M, Falkowska-Hansen B, Boehnke K, Bühring HJ, Marmé A, Bickenbach JR, Boukamp P (2008) A stable niche supports long-term maintenance of human epidermal stem cells in organotypic cultures. Stem Cells 26:2506–2515
24. Braun KM, Niemann C, Jensen UB, Sundberg JP, Silva-Vargas V, Watt FM (2003) Manipulation of stem cell proliferation and lineage commitment: visualisation of label-retaining cells in wholemounts of mouse epidermis. Dev 130:5241–5255
25. Peck MD (2011) Epidemiology of burns throughout the world. Part I: distribution and risk factors. Burns 37(7):1087–1100
26. Clark RA, Ghosh K, Tonnesen MG (2007) Tissue engineering for cutaneous wounds. J Invest Dermatol 127(5):1018–1029
27. Singer AJ, Clark RA (1999) Cutaneous wound healing. N Engl J Med 341(10):738–746
28. Wu Y, Chen L, Scott PG, Tredget EE (2007) Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. Stem Cells 25(10):2648–2659

29. Green H, Kehinde O, Thomas J (1979) Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. Proc Natl Acad Sci USA 76(11):5665–5668
30. Masson-Gadais B, Fugere C, Paquet C, Leclerc S, Lefort NR, Germain L, Guérin SL (2006) The feeder layer-mediated extended lifetime of cultured human skin keratinocytes is associated with altered levels of the transcription factors Sp1 and Sp3. J Cell Physiol 206:831–842
31. Lavoie A, Fugère C, Fradette J, Larouche D, Paquet C, Beauparlant A, Gauvin R, Têtu FA, Roy A, Bouchard M, Genest H, Auger FA, Germain L (2001) Considerations in the choice of a skin donor site for harvesting keratinocytes containing a high proportion of stem cells for culture in vitro. Burns 37(3):440–447
32. Shi C, Zhu Y, Su Y, Cheng T (2006) Stem cells and their applications in skin-cell therapy. Trends Biotechnol 24(1):48–52
33. Lapouge G, Blanpain C (2008) Medical applications of epidermal stem cells. In: Research Community (ed) The stem cell, Stembook. Harvard Stem Cell Institute, Cambridge
34. Schneider H, Mühle C, Pacho F (2007) Biological function of laminin-5 and pathogenic impact of its deficiency. Eur J Cell Biol 86(11–12):701–717
35. Mavilio F, Pellegrini G, Ferrari S, Di Nunzio F, Di Iorio E, Recchia A, Maruggi G, Ferrari G, Provasi E, Bonini C, Capurro S, Conti A, Magnoni C, Giannetti A, De Luca M (2006) Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells. Nat Med 12(12):1397–1402
36. Di Nunzio F, Maruggi G, Ferrari S, Di Iorio E, Poletti V, Garcia M, Del Rio M, De Luca M, Larcher F, Pellegrini G, Mavilio F (2008) Correction of laminin-5 deficiency in human epidermal stem cells by transcriptionally targeted lentiviral vectors. Mol Ther 16(12):1977–1985
37. Warrick E, Garcia M, Chagnoleau C, Chevallier O, Bergoglio V, Sartori D, Mavilio F, Angulo JF, Avril MF, Sarasin A, Larcher F, Del Rio M, Bernerd F, Magnaldo T (2012) Preclinical corrective gene transfer in xeroderma pigmentosum human skin stem cells. Mol Ther 20(4):798–807
38. Moretti S, Fabbri P, Baroni G, Berti S, Bani D, Berti E, Nassini R, Lotti T, Massi D (2009) Keratinocyte dysfunction in vitiligo epidermis: cytokine microenvironment and correlation to keratinocyte apoptosis. Histol Histopathol 24(7):849–857
39. Bondanza S, Maurelli R, Paterna P, Migliore E, Giacomo FD, Primavera G, Paionni E, Dellambra E, Guerra L (2007) Keratinocyte cultures from involved skin in vitiligo patients show an impaired in vitro behaviour. Pigment Cell Res 20(4):288–300
40. Khodadadi L, Shafieyan S, Sotoudeh M, Dizaj AV, Shahverdi A, Aghdami N, Baharvand H (2010) Intraepidermal injection of dissociated epidermal cell suspension improves vitiligo. Arch Dermatol Res 302(8):593–599
41. Guerra L, Capurro S, Melchi F, Primavera G, Bondanza S, Cancedda R, Luci A, De Luca M, Pellegrini G (2000) Treatment of "stable" vitiligo by Timed surgery and transplantation of cultured epidermal autografts. Arch Dermatol 136(11):1380–1389

Chapter 5
Cardiac Regeneration with Stem Cells

Beatriz Pelacho, Manuel Mazo, Sheyla Montori, Ana Maria Simon-Yarza, Juan Jose Gavira, Maria J. Blanco-Prieto and Felipe Prósper

Abstract Cardiovascular diseases (CVD) are the main causes of morbidity and mortality worldwide. A huge effort has been made to improve current standard approaches for treating patients with ischemic heart disease. However, despite the greater efficacy of new drugs and clinical techniques, which have decreased the number of acute patients and prolonged the life of chronic ones, the classic treatments are still not able to regenerate the diseased heart. For this reason, alternative therapies based on the use of gene, protein, and stem cells have been developed in combination with bioengineering techniques, with the aim not only of protecting but also repairing the damaged heart. All these new therapies, especially stem cell therapy and the possibility of combining these cells with biomaterials in order to reinforce their potential or even create new tissues, are reviewed in this chapter.

Abbreviations

AAV	Adeno-associated virus
ADSC	Adipose-derived stem cells
AMI	Acute myocardial infarction
BMC	Bone marrow cells
BM-MNC	Bone marrow mononuclear cells

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b.p.m.	Beats per minute
CABG	Coronary artery bypass surgery
CD	Cell doses
CM	Cardiomyocytes
CPC	Cardiac progenitor cells
CVD	Cardiovascular diseases
DCM	Dilated cardiomyopathy
ECM	Extracellular matrix
EDV	End Diastolic Volume
EHT	Engineered heart tissue
EP	Epicardial
EPC	Endothelial progenitor cells
EPO	Erythropoietin
ESC	Embryonic Stem Cells
ESV	End Systolic Volume
EV	Endoventricular
HSC	Hematopoietic stem cells
IC	Intracoronary
IHD	Ischemic heart disease
IM	Intramyocardial
IV	Intravenous
iPS	Induced Pluripotent stem cells
LAD	Left anterior descending
LV	Left ventricle
LVSD	Left ventricular systolic dysfunction
MI	Myocardial infarction
MSC	Mesenchymal stem cells
PC	Percutaneous
PET	Positron emission tomography
PLGA	Poly(lactic-co-glycolic acid
SC	Stem cell
SkM	Skeletal Myoblasts
SIS	Small intestine submucose
SVF	Stromal vascular fraction
TC	Transcoronary
TEp	Transepicardial
TE	Tissue Engineering
TEc	Transendocardial
UCBC	Umbilical cord blood mononuclear cells

5.1 Introduction

According to the World Heart Organization, more people die annually from cardiovascular diseases (CVD) than from any other cause, since they represent 29 % of all deaths. By 2030, almost 23.6 million people will probably die from CVD, this being the first cause of death, representing 42 % of deaths [1]. The major modifiable risk factors associated with ischemic heart disease (IHD) are tobacco and alcohol use, hypertension, high cholesterol, obesity, diabetes, and physical inactivity. Other non-modifiable factors related to CVD include aging, family history of cardiovascular disease, gender, and ethnic origin.

IHD develops when deposits of cholesterol particles accumulate on the walls of heart blood vessels. These deposits, called plaques, narrow or block the arteries that supply blood to the heart. Myocardial infarction occurs when, due to lack of blood flow, there is not enough oxygen in the myocardium. Over time, damage becomes irreversible, and is accompanied by cell death and tissue necrosis (Fig. 5.1). In heart infarct, after cardiomyocyte death, the heart replaces these necrotic cells with a fibrotic scar mainly composed of activated fibroblasts and extracellular matrix components. Although cardiac remodeling is a compensatory mechanism that initially decreases wall stress and increases cardiac output and stroke volume, ultimately it becomes a maladaptive response leading to contractile dysfunction, arrhythmias, and heart failure.

Therapies driven to improve myocardial function in IHD include pharmacological treatment, percutaneous intervention, and surgery. Most of these are aimed at minimizing the symptoms and preventing progression of the disease, but are able neither to regenerate the tissue nor to restore the heart function in a maintained form. In fact, the last and only resort for severe cases is heart transplantation with the concomitant limitations of the donor waiting lists and the need for an immunosuppressive regimen to prevent rejection, which obviously has its own significant deleterious side effects. The failure of these therapies to rescue the damaged heart and the inconvenience of heart transplants have led to the emergence of alternative treatments, including gene (reviewed in [2, 3]), protein (reviewed in [4, 5]), and stem cell (reviewed in [6, 7]) therapies.

Importantly, these new approaches have gone a step further, aiming not only at the protection but also the regeneration of the damaged heart. Thus, overexpression of key genes or release of angiogenic and survival cytokines/growth factors could exert a significant therapeutic potential. Also, stem cell therapy has emerged as an up-and-coming strategy for obtaining new functional myocytes and vascular cells. This has led to a renewed interest in the main pathways leading to myocardium regeneration and identification of cardiovascular progenitors.

Furthermore, combination of these therapies with tissue engineering (TE) could boost their benefits, through strategies that could increase cell function, survival, and cell homing. Thus, cells, biomaterials and/or biologically active molecules could be applied with the main objective of restoring, maintaining and/or enhancing tissue and organ function [8] gathering engineering, medical, and biological applications.

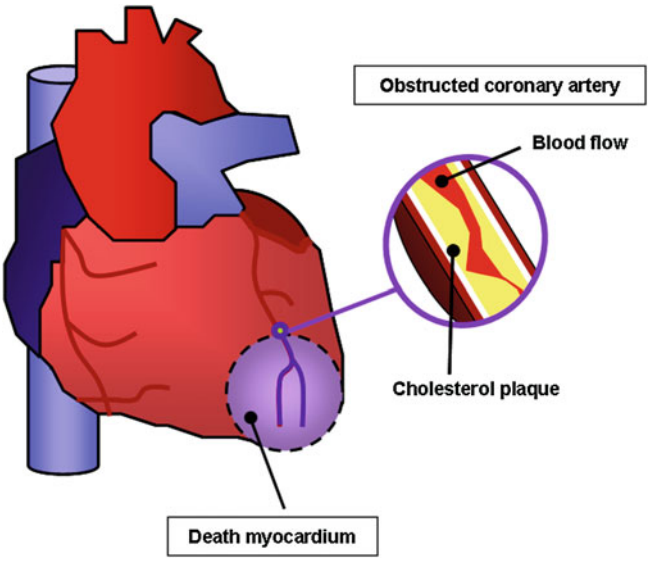

Fig. 5.1 Ischemic heart disease. Cholesterol plaques deposited in heart vessel walls result in narrowing and eventual blocking of blood flow. They may cause a heart attack when oxygen deprivation occurs, giving place to tissue death and subsequent pathological remodeling

Throughout this chapter, the major milestones and trials that have lately been conducted in the therapy of IHD are discussed, as well as the challenges that emerge with the new approaches to this widespread disease.

5.2 Gene and Protein Therapies for Cardiovascular Disease

5.2.1 Gene Therapy

The development of molecular biology techniques and our increasing knowledge of the genome over recent decades have contributed to the development of a new therapy concept with a more ambitious projection than the conventional one. Gene-based treatments seek an approach that would not only alleviate the negative effects of diseases, but also correct the causes at a genetic level. In heart disease, the aim of gene therapy is to restore dysfunctional myocytes and to prevent the non-diseased myocytes from becoming lost or diseased. It requires the introduction of DNA/RNA that targets specific defective processes including lack of blood flow in the ischemic tissue, cell death, fibrosis, etc. One of the main goals of gene therapy in IHD has been to increase the perfusion in the ischemic area. Numerous models of ischemic disease in animals have shown improvement after vector-mediated delivery of angiogenic factors including VEGF and FGF, prompting

several clinical trials. Some VEGF family members have been shown to induce angiogenesis in a rabbit model of hind limb ischemia [9]. Analogously, VEGF-165 gene therapy in rat [10, 11] and rabbit [12] has resulted in significant neovascularization after myocardial infarction. In like manner, swine models of myocardial infarction have been shown to enhance myocardial blood flow after VEGF-165 treatment [13, 14] and cardiac tissue viability and function were improved in dogs following Adeno-associated virus (AAV)-mediated transduction of this factor [15]. VEGF-121 gene therapy in rat [16] and swine [17] has also demonstrated its effectiveness as a neovascularizing agent. Growth factors belonging to FGF family such as FGF2 [18], FGF4 [19], and FGF5 [20] have also improved regional perfusion and function in porcine models.

Genetic manipulation of the β-adrenergic system has also been tested to improve heart function. Target genes in these studies including β2-AR, GRK2, and AC6 have a positive inotropic effect and some of them also improve ventricular remodeling [21–23]. However, none of these approaches have reached clinical trials. Regulation of calcium handling appears also as a potential pathway to treat IHD and its main objective is focused on increasing the activity of SERCA2a, which it has been shown is closely related to the failing heart when expressed at low levels [24]. Further potential gene therapy targets include oxidative stress, inflammation, apoptotic/prosurvival pathways, and homing of stem cells (reviewed in [25]).

Gene therapy has already been explored in clinical settings. The first cardiac gene therapy trial was directed toward the myocardium for the treatment of coronary disease. This pioneering study was performed in 21 patients by the group of Dr. Crystal and it involved direct intramyocardial injections of an adenovirus vector expressing VEGF121 cDNA [26]. From then until now, there have been conducted numerous clinical trials of gene therapy with angiogenic gene transfer of this growth factor and FGF (see Table 5.1). Nowadays, most clinical gene therapy trials carried out target the revascularization of the ischemic myocardium. Importantly, while preclinical models and gene therapy trials performed without controls that have tested angiogenic factors have shown positive effects, randomized clinical trials with placebo-control groups have led to inconclusive and clinically irrelevant results. The lack of efficacy in proangiogenic trials suggests that growth factor concentration does not reach the appropriate dose or does not exert its effect long enough to result in a significant angiogenic effect [27].

There are other factors in heart failure that cannot be managed by angiogenesis and that should be tackled. In this respect, new therapeutic targets in cardiac gene therapy have emerged and have been applied to clinical trials based on the large amount of data from animal models.

Studies of SERCA2a-related genes in animals have resulted in some current clinical trials [28, 29]. A gene therapy assay for advanced heart failure has recently been conducted consisting of gene transfer of the SERCA2a cDNA via a recombinant AAV vector. Although the results of this trial are preliminary and larger studies are needed, it seems that SERCA2a could be a critical target in the pathogenesis of heart failure since in 6 months this strategy has been shown to improve symptomatic, functional, biomarker, and left ventricular function/

remodeling parameters. At present, an ongoing study is recruiting patients to assess the safety and efficacy of gene transfer of adenovirus vector expressing AC6 (ClinicalTrials.gov Identifier: NCT00787059) and another incipient clinical trial will directly inject into the myocardium naked SDF-1 DNA to evaluate safety, tolerability, and preliminary efficacy 1 month post-injection (ClinicalTrials.gov Identifier: NCT01082094). Some of the most relevant clinical trials in cardiac gene therapy are shown in Table 5.1.

5.2.2 Protein Therapy

On the other hand, there have also been significant efforts to introduce novel therapeutic strategies in IHD pharmacology based on the use of growth factors, which are able to enhance the intrinsic capacity of the heart to repair itself or regenerate after damage. Angiogenic cytokine therapy has been widely regarded as an attractive, straightforward treatment for ischemic heart disease. The main goal of this therapeutic approach in myocardial ischemia is coronary collateral development by means of the administration of angiogenic cytokines. Research in preclinical models has screened the potential use of molecules such as FGF2, VEGF, PDGF, Neuregulin 1, or SHH.

One of the first angiogenic growth factors related to tumor vascularization to be discovered was FGF, which was later linked to angiogenesis and cardiac repair through its action on different cell types including endothelial cells, smooth muscle cell, and myoblasts that express FGF receptors [40, 41]. FGF1 or FGF2 treatments have resulted in hemodynamic recovery after ischemia-reperfusion in mouse [42] and after myocardial infarction in rats, rabbits, and dogs [43–45]. The most widely used protein to induce angiogenesis both in preclinical models and in clinical assays is VEGF, a factor that induces vascular hyperpermeability and acts as an endothelial cell-specific mitogen. PDGF participates in angiogenesis and vessel stabilization [46] and its angiogenic synergism in combination with FGF has already been proven in a myocardial infarction model in swine [47]. Regarding Neuregulin-1, it triggers multiple responses including proliferation and survival of cardiomyocytes, promotion of regeneration, and decrease of hypertrophy among others [48, 49]. Despite its complexity, some investigations in mice have elucidated the critical role of SHH signaling in the maintenance of adult coronary vasculature by promoting angiogenesis and cell survival [50]. Its therapeutical potential has also been proven in myocardial ischemia models both in mice and rats [51–53].

The first phase-I clinical trial was performed in 20 patients in which FGF-1 was intramyocardially injected in patients undergoing coronary artery bypass of the left anterior descending coronary artery [54]. The results of this study showed an improvement neither in ventricular function nor in coronary perfusion except for an increase in the capillary filling. Other phase I studies with FGF2 proved the safety of this compound and prompted some functional benefits [55, 56]. Nonetheless, a multicenter, randomized, double bind, placebo-controlled phase-II trial

Table 5.1 The most relevant clinical trials in gene therapy

Trial	Phase	N (Treated/ Control)	Agent	Vector	Delivery method	Patients	Outcomes
AGENT-1[30] Grines et al. 2002	I–II	60/19	FGF4	AdV	IC	Angina II–III patients	Improvement in exercise time
AGENT-2[31] Grines et al. 2003	II	35/17	FGF4	AdV	IC	Angina II–IV patients (no-option for revascularization)	↓ Infarct size
AGENT-4[32] Henry et al. 2007	III	78 (2CD)/ 38	FGF4	AdV	IC	Angina pectoris (no-option for revascularization)	No significant effect in exercise tolerance
AWARE[32] Henry et al. 2007	III	300	FGF4	AdV5	IC	Angina pectoris (no-option for revascularization)	Non provided
KAT301 Trial[33,34] Hedman et al. 2003 & 2009	II–III	28/37	VEGF165	AdV *vs* plasmid liposomes	IC	Angina II–III patients undergoing coronary angioplasty and stenting procedure on restenosis and myocardial perfusion	Improvement in LV function (No adverse effects 8 years later)
Euroinject One Study[35] Kastrup et al. 2005 [35]	II–III	40/40	VEGF165	Naked plasmid	IM	Angina III–IV patients (no-option for revascularization)	Improvement in wall motion and LV function.
NORTHERN[36] Stewart et al. 2009	II–III	48/45	VEGF121	AdV	PC	Angina III–IV patients (no-option for revascularization)	↑ Revascularization
REVASC[37] Stewart et al. 2006	II	32/35	VEGF121	AdV	EP	Patients with severe angina due to coronary artery disease (no-option for revascularization)	Non provided
CUPID Jaski et al. 2009[38]; Jessup et al, 2011[39]	II	25 (3CD)/ 14	SERCA2a	AAV1	PC	III–IV Angina patients	Improvements in symptomatic and LV function/remodeling parameters (No adverse effects)
NCT00787059[30] Grines et al. 2002	I–II	Ongoing (∼72)	AC6	AdV5	IC	Congestive Heart Failure	Non provided
NCT01082094[31] Grines et al. 2003	I	Ongoing (∼16)	ACRX-100 (SDF-1)	Naked plasmid	IC	Heart Failure	Non provided

IC intracoronary artery infusion; *PC* percutaneous; IM: intramyocardial; *EP* epicardial; *LV* left ventricle; *CD* cell doses

(FIRST) of FGF2 contradicted previous results. The first trial showed non-significant beneficial effects in any of the groups of patients who received different single doses of recombinant FGF2 at 180 days of treatment [57]. Phase I trials administering VEGF reported promising results such as improved exercise capacity or enhanced myocardial perfusion at rest [58]. With this angiogenic factor it was also found that a larger randomized, double blind, placebo-controlled phase II trial did not corroborate the previous encouraging results. The VIVA trial compared two doses of VEGF-A to placebo in 178 patients with coronary artery disease and failed to show differences between treatment and placebo groups [59]. Proteins that have lasted longer at the clinical-stage are those that belong to the family of FGF and VEGF. Nevertheless, other growth factors known to have a role in tissue repair and angiogenesis have been tested in myocardial clinical settings, including colony granulocyte stimulating factor, erythropoietin, and neuregulin among others (see Table 5.2).

To sum up, the major hurdle found in these angiogenic clinical trials is the short-lived effect of the administered molecules due to the high instability of proteins when injected as a bolus [60]. The evanescence of these compounds in heart tissue has led to unsatisfactory results and studies have failed to demonstrate significant amelioration in treated patients. To overcome these limitations, several technologies have been explored to allow the encapsulation of factors by developing drug delivery systems that permit a controlled and localized release of the growth factors for longer time. These systems can also protect proteins from degradation, preserving their bioactivity during release (reviewed in [61]).

To date, several of these carriers have been intensively assayed for their angiogenic potential in animal models of ischemic heart disease, hydrogels, liposomes, and micro and nanoparticles being the most used [62–64].

Regarding hydrogels, several natural polymers like collagen and gelatin have been used for delivering the cytokines and have been tested in animal ischemia models. Thus, it has been shown that intramyocardial administration of FGF-2 loaded gelatine hydrogels in rat and pig models of myocardial infarction induces significantly increased angiogenesis and an improved left ventricular function [65, 66]. Also, gelatin hydrogels were used to incorporate other factors such as angiopoietin-1 [67] and erythropoietin (EPO) [64] for cardiac repair, demonstrating that post-MI treatment with an EPO-gelatine hydrogel improves left ventricle (LV) remodeling and function by activating pro-survival signaling, anti-fibrosis, and angiogenesis, without causing any side effect. Moreover, alginate-based hydrogels have also been used as a localized delivery platform for angiogenic proteins, showing, for example, a significant angiogenic response in ischemic hindlimbs when treated with an injectable alginate hydrogel loaded with VEGF [68]. Also, injection of FGF-2 in a temperature-responsive chitosan hydrogel was performed in rat [69] and rabbit [70] models of myocardial infarction, resulting in positive cardiac repair. On the other hand, liposomes, a lipid-based system, have been also assayed as carriers for several cytokines. In an interesting study, anti-P-selectin-conjugated liposomes were prepared for targeted delivery of VEGF to the rat infarcted myocardium, resulting in a significant increase in fractional shortening and improved

Table 5.2 The most relevant clinical trials in protein therapy

Trial	Phase	N (Treated/Control)	Agent	Delivery method	Patients	Outcomes
Shumacher et al.1998[71]	I	20/20	FGF-1	IM	LAD coronary artery anastomosis	↑ capillary network sprouting No improved coronary perfusion or LV function
Laham et al.2000[46]	I	52 (8CD)	FGF-2	IC	Stable angina pectoris	Improvement in exercise tolerance and ↓ infarct size
FIRST Simons et al. 2002[57]	II	251 (3CD)/86	FGF-2	IC	Coronary artery disease	No long-term symptoms improvement and no improvement in myocardial perfusion
VIVA Henry et al. 2003[59]	II	125 (2CD)/63	VEGF-A	IV	Angina pectoris (no-option for revascularization)	Safe and feasible; Significant improvement in angina but no improvements in myocardial perfusion
THEANGIOGEN	II	Ongoing (~20)	VEGF-165	IM	Patients with advanced severe angina	Non provided
STEM-AMI Achilli et al. 2010[72]	II	29/27	G-CSF	PC	Patients with STEMI	No significant differences in LVEF or perfusion between groups
SITAGRAMI[73] Theiss et al. 2010	III	50/50	G-CSF + Sitagliptine	PC	Acute myocardial infarction patients	Safe and feasible
Gao et al.2010[74]	IV	32 (3CD)/11	NRG	IV	Cardiac heart failure patients	No significant improvement of cardiac function and anti-remodeling effect
Voors et al.2010[75]	II	263/266	EPO	IV	Patients with STEMI	No significant improvement of cardiac function

LVEF left ventricular ejection fraction; *LV* left ventricle; *LAD* left anterior descending; anterior *STEMI* ST-elevation myocardial infarction; *IM* intramyocardial; *IC* intracoronary artery infusion; *PC* percutaneous; *IV* intravenous injection; *CD* cell doses

systolic function [76]. Finally, reports have shown that angiogenic factors can promote localized angiogenesis in vivo when administered in a nano- or microparticulate depot [77, 78]. Particles prepared with the Poly(lactic-*co*-glycolic acid) (PLGA) copolymer have been widely used due to the excellent biocompatibility and biodegradability of the material [79–83]. Benefits of using PLGA particles for angiogenesis have been shown in hindlimb ischemia models, resulting in increased blood vessel formation [84–86]. Also, the effect of delivery of PLGA microparticles loaded with VEGF-A_{165} has been studied in a rat model of cardiac ischemia-reperfusion, demonstrating an increase in heart tissue angiogenesis and arteriogenesis, besides positive remodeling of the heart [63]. Moreover, PLGA has been also used to encapsulate heat shock protein 27 (HSP27), which exerts protective effects in cardiac cells under hypoxic conditions [87]. Finally, PLGA microparticles have also been combined with other delivery systems in order to optimize the patterns of growth factor controlled release. Alginate gel/PLGA microsphere combination system containing VEGF enhanced the angiogenic response after hind limb ischemia in rats [84] and mice [85]. This combination system also allowed a dual delivery strategy and improved the effects of single factors.

The different growth-factor delivery systems listed above constitute an important body of intensive efforts to overcome the limitations of protein-based therapy for therapeutic angiogenesis. The protein threshold concentration and its local exposure duration remain to be determined and still represent the paramount challenge.

Over the past years, many growth-factor delivery strategies have been tested in preclinical studies. However, little information on clinical settings using protein delivery systems is available. Controlled release of FGF-2 encapsulated in heparin-alginate pellets led to significant angiogenesis with low systemic effects in patients undergoing bypass surgery, but this approach did not alleviate operative risks [88]. Therefore, further clinical trials to evaluate the effects of treatment induced by controlled growth factors delivery methods may be necessary.

5.3 Stem Cell Therapy for Cardiovascular Disease

One of the main problems in cardiac disease is that the regenerative capacity of the heart is very limited compared with other high regenerative organs like the liver, skeletal muscle, or skin. Some mathematical models have suggested that only 0.4–1 % of the cardiomyocytes are renewed per year [89]. Thus, the heart tissue can hardly regenerate after an ischemia episode where up to 25 % of the cardiomyocytes of the human left ventricle can be wiped out in a few hours [90]. A similar problem occurs in other disorders such as hypertension or valvular heart disease [91].

Nowadays, there is no treatment able to restore the injured heart and stem cell therapy has become a new option to rebuild the damaged myocardium [92]. In the last two decades, many studies have been performed in order to identify and characterize many stem cell populations, also testing their potential to regenerate the infarcted heart. Different cell types derived from a wide variety of adult tissue sources like the bone

marrow, the blood, the umbilical cord, the skeletal and cardiac muscle, or the adipose tissue, among others, together with embryonic stem cells, have been identified. Their properties and regenerative cardiovascular potential are described below.

5.3.1 Stem Cell Populations

5.3.1.1 Myoblasts

One of the first candidates for use in cellular therapy was the myoblasts or satellite cells. These progenitors give rise to skeletal muscle, and initially, were expected also to derive toward cardiomyocytes. However, their exclusive contribution to the skeletal muscle cells was soon demonstrated [93–95]. On the other hand, their autologous origin, the possibility of in vitro expansion and their resistance to ischemia, made them good candidates for transplantation into the failed myocardium [96]. Preclinical animal studies demonstrated their ability to engraft, improving the cardiac function after transplantation into infarcted myocardium [97, 98]. In a dog model of chronic heart failure, autologous skeletal myoblast transplantation improved hemodynamics and left ventricular function [99]. An improvement in regional wall thickening and an attenuation of ventricular remodeling were also observed in a rabbit [100] and sheep models of ischemic heart failure [101]. Finally, an improvement in wall motion was perceived in a rabbit model of ventricular aneurysm [102]. These results showed that myoblasts act by attenuating left ventricular remodeling but not by generating new cardiomyocytes because of their strict commitment to a myogenic lineage [96]. In spite of their lack of differentiation capacity [103], myoblasts were able to act in a paracrine manner, secreting growth factors involved in angiogenesis (VEGF, PIGF, angiogenin, angiopoietin, HGF, and PDGF-BB) as well as proteases involved in matrix remodeling (MMP2, MMP9, and MMP10) and their inhibitors (TIMPs) [104]. This influence over extracellular matrix remodeling [105] explains, at least partially, the functional and histological benefits from transplanted cells [106–110]. Also, the paracrine effect was observed in a xeno-myoblast transplantation study in which engrafted cells acted as cytokine sinks releasing some of the factors involved in the key events (particularly, increased angiogenesis and decreased fibrosis) that contribute to tissue salvage. These cytokines could be detected for as long as 1 month after cell transplantation in spite of the low percentage of skeletal myoblast that was still engrafted at this remote time point [104].

5.3.1.2 Bone Marrow Cells

The most widely studied adult stem cell population has been the bone marrow-derived mononuclear cells (BMC) [98]. The BMC is home to a variety of cell populations, capable of migrating and differentiating into diverse cell types. Major subsets of these cells are hematopoietic stem cells (HSC), mesenchymal stem cells

(MSC), and endothelial progenitor cells (EPC). These cells can be sorted and categorized into subpopulations according to their cell-surface markers [92]. Also, the presence of cardiovascular progenitors with regenerative potential in vivo has been shown [111], although there is still some controversy regarding the real potential of that population [92]. Other groups have observed that bone marrow cell transplantation improves tissue vascularization and collagen content, which was tightly related to a functional improvement. Also, an infarct size reduction has been observed [112, 113]. Furthermore, it has been reported that the mesenchymal phenotype (MSC), had better engraftment than BMC, apart from pro-angiogenic, and anti-fibrotic capacities [114]. Moreover, Dr. Hatzistergos and colleagues [115] have shown that the administration of MSC to the pig infarcted heart stimulated endogenous cardiac progenitors cells (CPC) contributing to the repair of the infarcts . On the other hand, MSC could also modulate the immune response [116], stabilizing the transplanted cells, although their in vivo immunomodulatory properties are not yet clear. Taken together, the best current evidence indicates that BMC do not work by directly differentiating into new cardiomyocytes. Instead, the cells have been shown to produce signals that control the response of cells native to the myocardium, and thereby regulate healing. This phenomenon seems to fit under one heading: inflammation. The participation of marrow derivatives in cardiac repair has to be considered as part of the inflammatory response, which is known to regulate angiogenesis, cardiomyocyte survival, and left ventricular remodeling after infarction (reviewed in [89]).

5.3.1.3 Umbilical Cord Cells

A large number of non-hematopoietic stem cells have been detected in the cord blood. These cells are an attractive option for regenerative therapy because they rarely express HLA class II antigens, which make them immunologically naïve, thus reducing the risk of rejection [117]. In animal models of acute MI, the injection of human umbilical cord blood mononuclear cells (UCBC) has been associated with significant reductions in infarct size, particularly when given by the intramyocardial route [118]. Moreover, the functional improvement in contractility and the increased vascularization 2 months after UCBC transplantation explain the role of the angiogenic activity when using this cellular therapy [119].

5.3.1.4 Adipose Derived Stem Cells

The adipose tissue has been investigated as a source of adult progenitor/stem cells for the purpose of cardiac repair, as this tissue contains a rich mixture of progenitor cells and can be easily harvested by liposuction [98]. At the histological level, white adipose tissue is characterized by being composed of mature adipocytes and the so-called stromal vascular fraction (SVF) which is composed of mast cell precursors, hematopoietic and cardiovascular progenitors, and stromal cells [120–

122]. Interestingly, it has been demonstrated that the SVF can be isolated and cultured on methylcellulose dishes, giving rise to vascular and cardiac cells among others [123–127]. Studies in vivo with SVF show that this cardiomyogenic cells can survive and differentiate in rodent acute and chronic myocardial infarction models, avoiding remodeling and impairment of cardiac function, and promoting neo-vascularization in the ischemic heart [128, 129].

On the other hand, SVF cells can be cultured in vitro under normal conditions, deriving toward a much more homogenous population with mesenchymal phenotype and features that has been termed as adipose-derived stem cells (ADSC). Preclinical studies have shown that ADSC not only induce a benefit over cardiac function, but they can also improve tissue metabolism, vascularization, and infarct size reduction through a paracrine action [130], postulated as their main mechanism of action [131, 132], as their rate of differentiation is quite limited [131]. This has also been related to their in vitro expression of cytokines [128, 133] or to the in vivo decrease in the level of pro-fibrotic molecules [134]. Thus, it has been shown that ADSC transplantation in a chronic MI model elicited a significant benefit in cardiac function, which was related to the cell release of growth factors such as VEGF and HGF [135]. Also, in the acute MI (AMI) model, most studies have shown a consistent and significant benefit of transplanted cells upon cardiac function (reviewed in [130]).

5.3.1.5 Cardiac Progenitor Stem Cells

Despite the traditional dogma regarding the lack of endogenous renewal capacity of the heart, it has been shown that this organ possesses an intrinsic regenerative potential, which depends on the presence of cardiac progenitors (CPC) (reviewed in [136]). Importantly, these progenitors, which are localized in small clusters at the interstitium of the heart, can be isolated, grown, and made to differentiate in vitro toward mature cardiomyocytes and also, vascular cells. Moreover, an increase of the CPC pool of the heart has been demonstrated after acute myocardial infarction [137] and also, their cardiovascular contribution has been shown through an improvement of the cardiac function when they are transplanted into the ischemic heart [138].

This adult and autologous population without tumorigenic risk could represent an ideal source; however, the present description of the cardiac cell populations is still confusing, since they have been characterized by different markers. Thus, for example, the first reported cardiac progenitors were defined as a Sca-1$^-$cKit$^+$ population [138] whereas almost simultaneously, another study showed the existence of a Sca-1$^+$cKit$^-$ cell population in the heart [139]. Other groups have shown also the existence of Sca-1$^+$ CPC populations in the heart [140, 141] some of them also being defined by the expression of the transporter protein Abcg-2 [142]. On the other hand, the ability of some murine and human heart-derived cells to form clusters in vitro when cultured in suspension (named “cardiospheres”) has also been demonstrated [143]. These clusters contain clonally derived cells which

organize in a core composed by proliferating c-Kit positive cells and a surrounding layer of spontaneously differentiated cells that express markers characteristic of cardiac, endothelial and mesenchymal cells. Their transplantation into immunosuppressed infarcted mice, improved cardiac function [144]. Finally, a new population isolated from the embryo and adult atrium of the heart characterized by the expression of the transcription factor Islet-1, but negative for the c-Kit or the Sca1 markers, has been described [145]. As well as the other cell populations, these cells possess self-renewing, clonogenic and multipotent abilities, including cardiac differentiation potential.

In view of all this, it is of great relevance to better characterize these progenitors in order to be able to isolate them in a reproducible and consistent manner.

5.3.1.6 Fetal Cardiomyocytes

One of the first cell types to be investigated as potential candidates for cardiac repair were fetal cardiomyocytes. Animal studies have shown that transplanted fetal cardiomyocyte engraft into the heart tissue were electromechanically coupled with the host cardiomyocytes improving the function of ischemic and globally failing hearts [146, 147]. However, the use of fetal cardiomyocytes presents several concerns including availability, immunogenicity, and ethics, which explains why other cell types have surpassed them as likely candidates for use in cardiac repair [98].

5.3.1.7 Embryonic Stem Cells

The regenerative capacity of adult stem cells is quite limited, since they are able in the best cases to contribute to vascular tissue but, in general, not to cardiac tissue. Only a small population possesses robust cardiomyogenic potential, namely embryonic stem cells (ESC), induced pluripotent stem cells, and the cardiac progenitors present in the heart, as described above. Because both ESC and iPS cells can be propagated indefinitely while still retaining their pluripotency, they are a potentially inexhaustible supply of cardiomyocytes [89].

ESC are derived from the inner cell mass of mammalian blastocysts, and were first isolated in 1981 from mice [148, 149], and 17 years later from the human species [150]. ESC have the broadest developmental potential (pluripotent) since they can give rise to cells of all three embryonic germ layers. Furthermore, functionally intact cardiomyocytes have been generated from human ESC in vitro [151]. The ESC-derived cardiomyocytes injected into a mouse infarcted myocardium formed stable grafts and subsequently contracted in synchrony with adjacent cells [152]. In 2007 three different groups reported the formation of human myocardium in infarcted rodent hearts using human ESC-derived cardiomyocytes [153–155]. Studies with human ESC-derived cardiomyocytes have been shown to engraft in infarcted mouse, rat, guinea pig and swine hearts, forming

islands of nascent, proliferating human myocardium within the scar zone [153, 154, 156]. This partial remuscularization was accompanied by beneficial effects on regional and global cardiac function [153, 154], and the co-transplantation of ESC with MSC provides a better functional outcome than any of the single cell treatments [157]. However, some researchers have questioned whether these effects are sustained at later time points [158], because no functional benefit has been found by other groups [159]. Therefore, the mechanisms underlying the observed improvements in contractile function remain unclear. In the aforementioned rodent studies, most of the graft tissue was isolated from the host myocardium by means of scar tissue, which may prevent synchronous beating. Furthermore, these human cells, which fire in vitro at ~50–150 b.p.m. [160], may not keep pace with the rapid rate of rats (~400 b.p.m.) and mice (~600 b.p.m.). If they cannot, then the observed salutary effects probably resulted from an indirect, paracrine mechanism, like those described above for adult cells. This also indicates that further beneficial effects on cardiac function may be possible after transplantation to a slower-rated recipient, such as a canine or porcine infarct model [89]. On the other hand, we have to bear in mind the teratoma formation associated with the use of ESC in animal models [161] which raises concerns regarding their malignant potential [98]. Moreover, ESC-based therapies will be allogeneic and require immunosuppression. Finally, these cells are derived from the inner cell mass of preimplantation-stage blastocysts [150] which contributes to the ethical controversy surrounding their use. All these limitations have hampered the use of ESC in patients [98].

5.3.1.8 Inducible Pluripotent Stem Cells

An exciting alternative has emerged with the generation of inducible Pluripotent Stem (iPS) cells, adult cells that can be successfully reprogrammed back to an undifferentiated pluripotent state [162–164]. The iPS cells were first established in 2006 by Takahashi and Yamanaka [162] by the retrovirus-mediated transduction of four transcription factors (c-Myc, Oct3/4, SOX2, and Klf4) into mouse fibroblasts. Human iPS cells were established in 2007, by the transduction of either the same set or another set of transcription factors (Oct3/4, SOX2, Nanog, Lin28) into human fibroblasts [163, 165]. Human iPS cells are similar to human ESC in their morphology, gene expression, and the epigenetic status of pluripotent cell-specific genes, being also able to differentiate in vitro and in vivo into cell types of the three germ layers (reviewed in [166]). Advantages of iPS cells with regard to ESC are their derivation, which does not involve the destruction of embryos, and the fact that they could be used in autologous cell therapies. Nonetheless, first-generation iPS was problematic because the reprogramming factors were introduced using integrating viruses, raising concerns about neoplastic transformation. More recently, there have been a variety of refinements to iPS generation that should reduce or eliminate this risk, including the use of episomal gene delivery, excisable transgenes, cell-permeable recombinant proteins and synthetic messenger

RNA (reviewed in [89]). With respect to the use of these cells in cellular therapy, Nelson et al. [167] reported that the intramyocardial delivery of mouse iPS cells also achieved the in situ regeneration of cardiac tissue, while also improving the post-ischemic cardiac function . Other groups demonstrated that iPS cells can derive into spontaneously contracting cardiomyocytes [168, 169]. However, other results showed that iPS-derived cardiomyocytes have impaired capacity to form differentiated, functional cells [170], and also that iPS cells, like ESC, can form tumors [92]. To overcome this problem, the field needs to develop methods to enrich iPS cells derivatives for cardiomyocytes or other useful cell types (such as endothelial, smooth muscle and stromal cells), with strict methods for sorting out the remaining undifferentiated cells, like the use of fluorescent molecules (cDy1) that stain pluripotent cells in live conditions [171]. In any case, further work will be required to define more precisely the safety, phenotype and maturation potential of cardiomyocytes derived from iPS cells [89].

To sum up, the injection of many of the cell types described above seems to improve cardiac function in animal models of MI, suggesting that the simple short-term improvement in cardiac function cannot be taken as direct evidence of cardiac regeneration per se. Moreover, a portion of the effect may relate to effects of decreasing wall stress by increasing the tissue mass in a thinning myocardial wall, an anatomic effect that is independent of a real regenerative effect (reviewed in [172]). Up to now, results suggest that the benefit induced by stem cells in the treated hearts is due to paracrine mechanisms more than through cardiovascular differentiation of the transplanted cells, despite the reported ability of some adult stem cell populations to in vivo differentiate to the cardiovascular lineages (mainly to the vascular lineages) (reviewed in [173]). This hypothesis has been reinforced by the fact that injection of conditioned media recovered from cultured stem cells can also provoke a benefit in the injected hearts [174, 175] (See Fig. 5.2).

5.3.2 Clinical Assays

Although more basic studies are needed in order to better understand the mechanisms involved in cardiac repair, a number of early phase clinical as well as randomized trials have been performed to determine the feasibility and safety of stem cell transplantation. Also, several ongoing Phase III trials have now been initiated (reviewed in [176]) for efficacy testing. Based on the encouraging experimental results and due to their putative feasibility and safety, skeletal myoblasts and bone marrow derived stem cells have been the first populations tested (reviewed in [98]). Their autologous application (that avoids the need for immune-suppression), and innocuous tissue isolation and relatively easy cell culture procedure when needed, have been important factors taken into account for their choice. More recently, ADSC and CSC have also been introduced in the clinical arena (see Table 5.3).

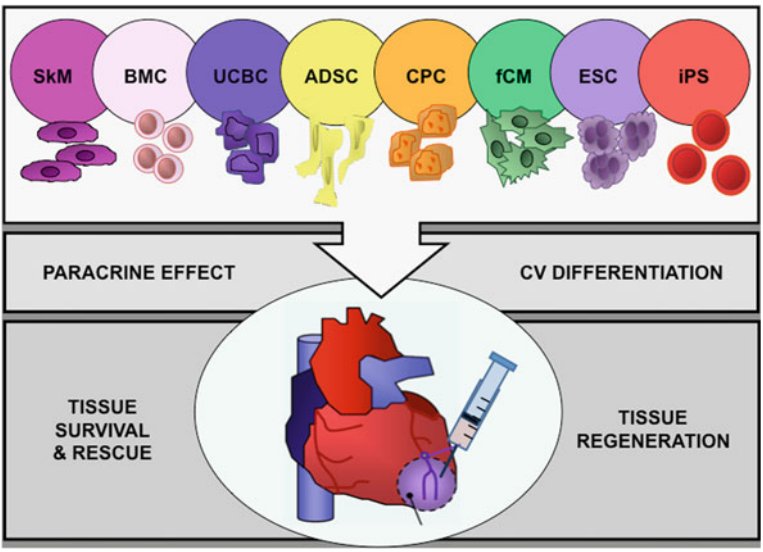

Fig. 5.2 Stem cell therapy for cardiovascular disease. Several stem cell populations have been assayed as a therapy for myocardial infarction, being their mechanisms of action analyzed. In general, a trophic effect has been described for them, secreting factors and cytokines responsible of the protection and rescue of the damaged tissue. Also, although with not such a relevant contribution, it has been shown the potential of some stem cell populations (like some endothelial progenitors present in the BM, the CSC or pluripotent cells like the ESC and iPS) to differentiate towards cardiovascular lineages, which could contribute to the regeneration of the heart tissue. (*SkM* skeletal myoblasts; *BMC* bone marrow cells; *UCBC* umbilical cord blood cells; *ADSC* adipose derived stem cells; *CPC* cardiac progenitor cells; *fCM*; fetal cardiomyocytes; *ESC* embryonic stem cells; *iPS* induced pluripotent stem cells; *CV* cardiovascular)

5.3.2.1 Myoblast Clinical Trials

Skeletal myoblast transplantation was initially investigated in patients undergoing open-heart surgery. The feasibility and safety of this approach was determined in a phase I, non-randomized, multi-center pilot study published in 2005. In this study, 30 patients with ischemic heart failure received autologous skeletal myoblasts (obtained from culture of a prior muscle biopsy) injected into the epicardium at the time of coronary artery bypass surgery (CABG) [97]. Myoblasts were successfully transplanted in all patients without any acute injection-related complications or significant long term, unexpected adverse events, apart from the arrhythmic events observed in a few patients. This study showed that epicardial injection of skeletal myoblasts is feasible with potential functional benefits. Thus, follow-up positron emission tomography (PET) scans showed new areas of glucose uptake within the infarct scar, suggestive of improved myocardial viability. Echocardiography measured an average improvement in the LVEF even 2 years after the surgery. Another relevant trial was the MAGIC trial, which was the first randomized placebo-controlled study of myoblast transplantation in patients with left ventricular systolic dysfunction (LVSD) secondary

Table 5.3 The most relevant clinical trials in stem cell therapy

Trial	N (Treated/ Control)	Cell type	Delivery method	Outcomes
Menasche et al. 2003[177]; Hagege et al. 2006 [178]	10/0	SkM	CABG	↑ regional wall motion; ↑ global LVEF
Smits et al. 2003 [179]	5/0	SkM	TEc	↑ regional wall motion; ↑ global LVEF
Ince et al. 2004 [180]	6/6	SkM	TEc	↑ global LVEF
Siminiak et al. 2004 [181]	10/0	SkM	TEp w/o CABG	↑ regional wall motion; ↑ global LVEF
Chachques et al. 2004 [182]	20/0	SkM	TEp w/o CABG	↑ regional wall motion; ↑ global LVEF; ↑ tissue viability
Dib et al. [97]	30/0	SkM	TEp w/o CABG	↑ regional wall motion; ↑ global LVEF; ↑ tissue viability; ↓ ESV; ↓ EDV
Siminiak et al. 2005 [183]	9/0	SkM	TC	Symptoms improved
Gavira et al. [110]	12/14	SkM	TEp + CABG	↑ regional wall motion; ↑ global LVEF; ↑ tissue viability; ↑ perfusion
Biagini et al. 2006 [184]	10/0	SkM	TEc	↑ regional wall motion; ↓ ESV; ≈ EDV
MAGIC/Menasche et al. [94]	67 (2CD)/30	SkM	TEp + CABG	≈ global LVEF; ↓ ESV
CAUSMIC/Dib et al. [185]	23	SkM	TEc	Improvement in heart failure symptoms;
SEISMIC/Duckers et al.[186]	40	SkM	TEc	≈ LVEF; improvement in patient symptoms
Strauer et al. 2002 [187]	10/10	BMC	IC	↑ regional wall motion; ↓ ESV; ≈ EDV; ↑ perfusion; ↓ infarct size
TOPCARE-AMI/ et al. [188]; Britten et al. 2003 [189]; Schachinger et al. 2004 [190]	19/11	BMC/ CPC	IC	↑ regional wall motion; ↓ ESV; ≈ EDV; ↓ infarct size; improvement in global LVEF
Fernandez-Aviles et al. 2004 [191]	20/13	BMC	IC	↑ thickness of infarct wall; ↓ ESV; ≈ EDV
Perin et al. 2004 [192]	11/9	BMC	TEc	Improvement in myocardial perfusion
Erbs et al. [193]	13/13	BMC	IC	↓ infarct size
Patel et al. 2005 [194]	10/10	BMC	dMI	Improvement in LV function
BOOST I/Wollert et al. [195]; Schaefer 2006 [196]	30/30	BMC	IC	↓ infarct size; improvement in global LVEF
LEUVEN-AMI/Jannsens et al. 2006 [197]	66	BMC	IC	↓ infarct size; ↑ tissue viability; ↑ perfusion; ↑ regional contractility; ≈ LVEF

(continued)

Table 5.3 (continued)

Trial	N (Treated/ Control)	Cell type	Delivery method	Outcomes
Hendrikx et al. 2006 [198]	10/10	BMC	dMI	Better recovery of LV function
Meyer et al. 2006 [199]	30/30	BMC	IC	Improvement in LV function
Mocini et al. 2006 [200]	18/18	BMC	dMI	Improvement in LV function
Fuchs et al. 2006a [201]	27/0	BMC	TEc	Safety and feasibility
Briguori et al. 2006 [202]	10/0	BMC	TEc	Safety and feasibility
ASTAMI/Lunde et al. [203], 2006	47/50	BMC	IC	≈ ESV; ≈ EDV; ≈ perfusion; ≈ infarct size; ≈ LVEF; improvement in exercise time
REPAIR-AMI/ Schachinger et al. [204]; Assmus et al. 2010 [205]	101/103	BMC	IC	≈ ESV; ≈ EDV; ≈ infarct size, acceleration of LV contractile recovery; improvement in LVEF
Merluzin et al. 2006 [206], 2008 [207]	44 (2CD)/22	BMC	IC	↓ ESV; ≈ EDV; ↑ perfusion
de la Fuente et al. 2007 [208]	10/0	BMC	TEc	Improvement in LV function
Tse et al. 2007 [209]	19/9	BMC	TEc	Safety and feasibility
Stamm et al. 2007 [210]	20/20	BMC	dMI	Improvement in LV function and perfusion
Zhao et al. 2008 [211]	18/18	BMC	dMI	Improvement in LV function and perfusion
Ang et al. 2008 [212]	63	BMC	TEp	No beneficial effect
FINCELL/Huikuri et al. [213]	77	BMC	IC	Improvement in LV function
Akar et al. 2009 [214]	25/25	BMC	dMI	Improvement in myocardial perfusion
van Ramshorts et al. 2009 [215]	25/25	BMC	IC	Symptoms improved; ↑ perfusion; ↑ global LVEF
Herbots et al. 2009 [216]	33/34	BMC	IC	Better recovery of LV function
Beitnes et al. [217]	50/50	BMC	IC	Improvement in exercise tolerance
Plewka et al. 2009 [218]	40/20	BMC	IC	Improvement in LV function
Tendera et al. 2009 [219]	80/40	BMC	IC	Longer delay between the symptoms and revascularization
STAR-Heart/Strauer et al. 2010 [220]	191/200	BMC	IC	Improvement in LV function
Traverse et al. 2010 [221]	30/10	BMC	IC	Favorable effect on LV remodeling
HEBE/Hirsch et al. 2010 [222]	200	BMC	IC	≈ LVEF
Chen et al. 2004 [223]	34/35	MSC	IC	↑ regional wall motion; ↓ ESV; ↑ perfusion

(continued)

Table 5.3 (continued)

Trial	N (Treated/ Control)	Cell type	Delivery method	Outcomes
Hare et al. [224]	53	MSC	IV	↑ LVEF led to reverse remodeling
Dib et al. 2009 [225]	20	MSC	EV	↑ LVEF
Viswanathan et al. 2010 [226]	15/15	MSC	dMI	Improvement in myocardial perfusion

CABG coronary artery bypass graft; *TC* transcoronary; *TEp* transepicardial; *TEc* transendocardial; *IC* intracoronary artery infusion; *IV* intravenous; *EV* endoventricular; *dMI* direct myocardial injection; *SkM* skeletal myoblasts; *BMC* bone marrow-derived cells; *CPC* circulating progenitor cells; *MSC* mesenchymal stem cells; *LVEF* left ventricular ejection fraction; *LV* left ventricle; *ESV* end systolic volume; *EDV* end diastolic volume; *CD* cell doses

to previous myocardial infarction that required coronary surgery [94]. Cells were injected into the epicardium within scarred areas during open-heart surgery, but myoblast transfer neither improved regional or global left ventricular function beyond that seen in patients receiving placebo, nor bettered echocardiographic heart function. Moreover it led to a higher number of arrhythmic events. More recently, percutaneous transcatheter intramyocardial injection of skeletal myoblasts (CAUSMIC study) into areas of viable myocardium in patients with severe ischemic heart failure has shown promise with improvement in NYHA functional class, quality-of-life and evidence of reverse ventricular remodeling when compared with controls after 1 year follow-up [185]. In a similar study design, the SEISMIC trial (presented at the 2008 American College of Cardiology Meeting), the safety and feasibility of catheter-based intramyocardial injection of skeletal myoblasts were confirmed. The study reported some improvement in patient symptoms but failed to show any significant improvement in the LVEF [186]. The results of these small studies have prompted the design of larger randomized controlled trials including the MARVEL Trial (ClinicalTrials.gov Identifier: NCT00526253) which is an ongoing randomized, double-blind, placebo-controlled, multi-center Phase II/III Trial involving 330 patients in North America and Europe. Enrolment in the MARVEL Trial began in October 2007, targeting patients who fall into Class II or III heart failure. This trial will further study the safety and efficacy of intramyocardial injection skeletal myoblasts in patients with chronic ischemic heart failure (reviewed in [98]). The results of these studies generally have suggested improved systolic performance and demonstrated the feasibility and safety of cellular therapy although special caution is needed regarding arrhythmic events, warranting further investigation [94].

5.3.2.2 Bone Marrow Cell Clinical Trials

Bone marrow has been extensively assayed in patients with cardiovascular disease, with Phase I trials already demonstrating the safety and feasibility of BMC transplantation. Currently, total mononuclear cells, MSC, and enriched progenitor

cells from bone marrow are being employed in many Phase II and/or III trials (for review see [176]). Thus, Hare et al. [224] observed that allogeneic MSC administered to patients intravenously within 10 days of infarction were well tolerated and were associated with decreased arrhythmias and an improvement in contractile function. The majority of the BMC-therapy studies have used intracoronary delivery of BMC following successful stenting of the infarct-related artery. Four main clinical trials have been published with positive findings so far. The TOPCARE-AMI trial was the first published study to demonstrate the potential beneficial effect of BMC therapy following acute MI (AMI) with improvement in the LVEF at 4 months [188–190]. Recently, the 5 year follow-up of the TOPCARE-AMI trial has been published, providing reassurance with respect to the long-term safety of intracoronary cell therapy and suggesting favorable effects on LV function [227]. In the BOOST trial [195], [196], global LVEF improvement was observed after 6 months, although this improvement was maintained only in the patients with larger infarcts at long-term follow-up (18 months). The third study with positive results is the REPAIR–AMI trial, the largest trial to date, in which BMC therapy was associated with an LVEF increase after 12 months [204] and a significant reduction of major adverse cardiovascular events. That functional improvement was maintained 2 years later [205]. Finally, the FINCELL trial [213] reported an improvement of global LVEF and no arrhythmia risk profile. In contrast, three other clinical trials did not show positive effects. Janssens et al. [197], from the LEUVEN-AMI study, reported no changes in global LVEF after BMC infusion, although sub-set analysis showed a reduction in the infarct size of patients who had suffered the largest infarcts. In the ASTAMI trial [203] BMC administration had no significant effect on the LVEF, LV volumes, or infarct size, and only the safety of the treatment in the long term [217] was the positive conclusion. Finally, in the recently published HEBE trial [222], no changes in global or regional LV systolic function were reported after BMC therapy. The reasons for the inconsistent findings from these clinical trials are unclear but possibilities include variations in the cell isolation protocol, cell dose, timing of delivery after AMI and type of patient. A meta-analysis performed to elucidate the impact of BMC transplantation in the AMI [228], showed the safety of the treatment together with a LVEF improvement of 2.99 % and a reduction in the myocardial scar of 3.51 %, compared to controls. A more recent meta-analysis has confirmed the feasibility and security of stem cell therapy together with a putative benefit in comparison with conventional treatments [229].

Although promising, these findings are much less than what would have been anticipated from the earlier animal results, which showed up to a LVEF 40 % improvement when BMC were delivered to the peri-infarct zone within 3–5 h of infarction [111]. In an attempt to replicate the experimental models as closely as possible, the REGENERATE-AMI study has been designed to assess the safety, feasibility and efficacy of BMC when delivered early in patients with AMI (ClinicalTrial.gov. Identifier: NCT00765453). This study is ongoing and specifically aims to deliver BMC to patients undergoing primary angioplasty for acute anterior myocardial infarction. Finally, newly initiated phase-I/II studies include

the Transendocardial Autologous Cells (hMSC or hBMC) in Ischemic Heart Failure Trial (TAC-HFT; ClinicalTrial.gov Identifier: NCT00768066), the Prospective Randomised study Of MSC THErapy in patients Undergoing cardiac Surgery (PROMETHEUS) trial (ClinicalTrial.gov Identifier: NCT00587990), and the Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis (POSEIDON) pilot study (ClinicalTrial.gov Identifier: NCT01087996) [224], among others.

5.3.2.3 Other Cell Type Clinical Trials

Besides the bone marrow, several other sources of stem cells are also being tested for their therapeutic potential. Phase I clinical studies are being planned/performed with the use of *hUCBC* for the treatment of patients with dilated cardiomyopathy (DCM) and refractory angina. On the other hand, *resident cardiac stem cells* are clearly an attractive option for cardiac repair, although a harvesting technique remains to be perfected and clinical trials for safety and efficacy are still awaited. The ongoing CArdiosphere-Derived aUtologous Stem CElls to Reverse ventricULar dySfunction (CADUCEUS) study, which is a Phase I study where 30 patients have been recruited to receive autologous cardiosphere-derived stem cells, will hopefully answer some of these questions (ClinicalTrials.gov Identifier: NCT00893360) (reviewed in [98]). The adipose tissue is another ideal source for immediate access to a patient's own stem cells. Two Phase I trials, the APOLLO Study (ClinicalTrials.gov Identifier: NCT00442806) and PRECISE Study (ClinicalTrials.gov Identifier: NCT00426868), are now underway to explore the safety, feasibility and efficacy of the freshly isolated stromal vascular fraction present in the adipose tissue, in both acute and chronic myocardial ischemia patients, respectively (reviewed in [98, 176]). Finally, to date, positive results have been observed in the clinical assays using circulating progenitor cells [193] transplanted into the infarcted myocardium of patients with ischemic heart disease.

5.4 Tissue Engineering

Despite the general benefit that stem cell therapy offers, some important limitations like the low degree of cell engraftment and survival in the heart have been evidenced in cell therapy. As an average, more than 70 % of the transplanted cells are lost during the first 48 h, progressively disappearing during the following days [230]. Cell injection implies that a great percentage of cells directly leak through capillaries [231] and also die through anoikis due to the lack of matrix anchorage-dependent survival signals [232]. Furthermore, MI imposes a hypoxic, pro-inflammatory and/or fibrotic environment that harms the transplanted cells [233]. Despite this aspect, a functional improvement has generally been demonstrated after cell treatment in the settings of AMI, chronic ischemic heart failure and

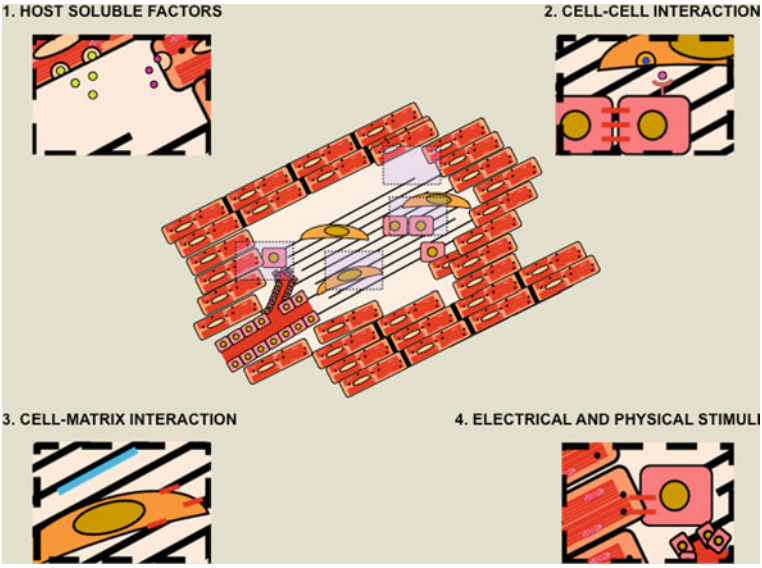

Fig. 5.3 Heart tissue and TE constructs interaction. TE constructs are subjected to several stimuli when transplanted to the diseased heart. *1*. Paracrine secretion of molecules by the surrounding tissue can affect seeded cells and the immunological response to the implanted material. *2*. Cells can interact either via secreted molecules or through direct contact with both the transplanted population and those of the host. 3. The interaction with the matrix also influences the behavior of cells (and thus the result of the treatment) by way of its stiffness, topology or geometry. 4. Finally, the peculiar characteristics of the heart subject cells to various physical forces, including, electrical stimulus, cyclic strain, shear stress and load, all of which have an impact upon cell biology

dilated cardiomyopathy (reviewed in [234]). Therefore, it has been hypothesized that an increase in the survival rate of the cells would improve their positive effects by reinforcing their trophic effect or even their in vivo differentiation. With this in mind, different scaffold-based approaches (which have received the general term of tissue engineering) have been tested in order to favor cell retention.

Tissue engineering (TE), has been defined as the process of creating living, functional tissues to repair or replace the tissue or organ function lost due to age, disease, damage, or congenital defects. Novel biomaterials are being designed to direct cell organization, growth and differentiation in the process of forming functional tissue by providing physical, mechanical and chemical cues. In the setting of cardiac regeneration, the ideal material should be biocompatible, biodegradable (at a rate coupled to cell proliferation and native-tissue deposition), allow cell proliferation, stimulate its differentiation and maturation, and present similar mechanical and physical properties to the healthy heart [235, 236]. Notably, this would include the capacity to sustain rhythmic contraction, variations in frequency and impulse propagation, which are key features of the cardiac tissue [236]. On the other hand, cellular TE constructs could be subject of heart tissue paracrine, matrix or electro-mechanical stimuli (Fig. 5.3).

One of the first attempts to provide a supportive scaffold for MI dates from 1937, when Dr. O'Shaughnessy used omental wrapping to promote neovascularization [237] of the ischemic organ. Since then, the field has experienced an incredible boost, especially during the last decade. In the following sections, we will describe some of the approaches developed and discuss their strengths and pitfalls.

5.4.1 Injectable Materials and Cell Microencapsulation

Initial studies focused on the use of injectable materials that could improve cell retention and provide structural anchorage [238] while allowing the use of less invasive ways of delivery like catheter-based injections.

Early experiments were performed by combining the cells with biomaterials derived from the extracellular matrix, like collagen, fibrin or gelatin. Also, matrigel or other factors that provided a favorable environment rich in cytokines and growth factors were tested. In general, an increased survival rate of the transplanted cells was shown and consequently, a greater improvement of the cardiac function of the treated hearts [239, 240].

Thanks to this relatively simple approach, the trophic effect exerted by the cells was boosted by increasing their survival and engraftment in the tissue. Moreover, importantly, it has been observed that some of the injected materials can exert a positive effect themselves, as has been shown, for example, for alginate. This material is liquid, but suffers a phase transition to hydrogel when injected into the desired tissue, as the local calcium concentration increases. Thus, the groups of Dr. Cohen and Dr. Leor have shown that when recent (7 days) or old (60 days) rat infarcts were treated with this alginate solution, wall thickness was significantly increased, while both systolic and diastolic dilatation and dysfunction were prevented. Interestingly, the effect was even superior to that of neonatal CM transplantation [241]. Moreover, this benefit was also confirmed in a pre-clinical model of myocardial infarction in swine, showing a positive left ventricular remodeling [242].

Furthermore, alginate also provides means for material modification, as demonstrated by the same group. The former approach was altered by linking IGF1 and HGF to the hydrogel, supplying these cytokines with proteolysis protection. When injected in a rat model of acute MI, modified alginate sequentially released the molecules, which preserved ventricle thickness, attenuated infarct expansion and fibrosis deposition, and also increased angiogenesis and induced CM-cycle re-entry [243]. In a different approach, the group led by Randall Lee conjugated the adhesion-promoting motif arginine-glycine-asparagine (RGD) sequence to alginate and showed its therapeutic capacity to treat a model of chronic MI in rat [244].

Self-assembling peptide nanofibers [245] have also proved their therapeutic potential for angiogenesis, growth factor-release and cell-delivery [246–248]. Lin et al. [249] compared the combination of nanofibers with BM-MNC in a pig preclinical model of MI with nanofiber or cell injection alone. It was found that, in

spite of material-injection being able to prevent geometry worsening in diseased hearts, the combination improved cell retention and systolic and diastolic function more, whereas cell-treatment was only able to ameliorate systolic function.

On the other hand, another aspect involved in the rapid cell clearance is the immune-rejection that the transplanted cells provoke, being phagocytized by the inflammatory cells present in the infarcted tissue. In order to avoid this aspect, another interesting approach is being assayed, which is the encapsulation of the cells to protect them. Thus, microcapsules allow the diffusion of nutrients and oxygen towards the cells to keep them alive as well as the cytokines and factors released by the cells diffuse in the opposite direction, mimicking in this way the cell paracrine secretion (reviewed in [250]). It has been shown for example, that when hMSCs were encapsulated in RGD-Alginate microbeads and administered in a model of rat MI, they successfully exerted a paracrine effect, responsible for an increase of angiogenesis and improvement of cell survival and in last instance, in the maintenance of LV geometry and preservation of LV function [251]. The most complex challenges of this approach include controlling the growth factor release rate, the cell survival/replication rate within the capsule and the successful prevention of immune rejection, which hampers its reproducibility [252–255]. Furthermore, a novel cellular delivery silicon-based platform forming the "nanoporous micromachined biocapsules" for cell encapsulation and immunoprotection is now under investigation, and represents a more recent approach using non-biodegradable polymers [256, 257].

In general, hydrogel combination or cell microencapsulation are interesting approaches that have shown good results, although some limitations still need to be solved, like the fact that they do not assure complete cell retention or adequate distribution of the cells. Techniques like the creation of cell sheets and patches and microtissues are now being developed in order to allow, together with greater cell survival, a more homogeneous, and organized distribution of the cells (reviewed in [256]).

5.4.2 Cell Patches

The in vitro construction of 3-D grafts and their epicardial implantation has been studied by several groups worldwide. In general, this approach provides cells with a structural support, which helps to increase their retention within the desired area, but also hinders remodeling processes that eventually end up in chamber dilatation.

The creation of cellular patches has been developed by using different materials characterized by their biocompatibility and/or biodegradability. Two types of materials in particular have been tested: porous biomaterials or hydrogel/extracellular matrix (ECM)-based matrices.

Regarding the first ones, for example, Leor and coworkers tested the putative benefit of treating infarcted rats with a porous 3-D alginate scaffold seeded with rat CM previously matured in vitro. Nine weeks after transplantation, graft-implanted animals showed a significant improvement of heart function and decrease in LV

dilatation, which was accompanied by extensive vascularization of the scaffold by host-derived vessels, not withstanding the fact that transplanted CM were mostly replaced by collagen, and no evidence of structural integration was found [258]. Similarly, Piao et al. [259] seeded BM-MNC on a poly-glycolide-co-coprolactone scaffold to treat MI in rats. The treatment prevented LV dysfunction and adverse remodeling, and stimulated BM-MNC to migrate towards the diseased tissue. Along similar lines, Jin and coworkers plated MSC on poly-lactide-co-coprolactone patches. Four weeks after transplantation on a cryoinjury model of MI, construct-treated animals showed a significant benefit on cardiac function and geometry [260]. Fitzpatrick et al. [261] employed a mesh of poly-glicolic acid seeded with human fibroblasts. Implanted in a rat model of MI, the patch preserved cardiac function through an increased wall thickness and a smaller infarct, mostly related to the paracrine action of the cells. Finally, in an interesting approach, the group of Dr. Levenberg reported the in vitro generation of a cardiac tissue by a tri-culture of hESC-derived CM, hESC-derived endothelial cells and embryonic fibroblasts on a sponge of PLGA. The engineered tissues showed high interaction between cell-types, with endothelial cells promoting CM proliferation, and fibroblasts stabilizing endothelial cell-derived capillaries. The constructs also proved their in vitro functionality, but have not been subjected to in vivo testing yet [262].

On the other hand, ECM-derived materials have also been extensively studied. In this approach, cells are usually embedded in soluble hydrogels matrices that can condense after temperature changes, thereby forming a cellularized patch that can be applied to the heart pericardium. Xiang et al. [263] employed type I collagen-glycosaminoglycan patches seeded with MSC to treat a rat model of ischemia–reperfusion, again demonstrating a benefit upon both cardiac function and geometry and CM-differentiation capacity of cells. In a recent report, the Joseph Wu's group used collagen in combination with SDF1-primed EPC. MI-subjected rats were treated with these constructs, showing a preserved function, mostly related to a prevention of ventricle dilatation and scar expansion, as well as neovascularization of the infarcted area, concomitant with elevated levels of the pro-angiogenic molecule VEGF [264]. In a sophisticated approach, the group of Dr. Zimmermann prepared heart tissue construct by combining neonatal rat CM and a mixture of nutrients plus matrix-derived molecules (collagen type I and Matrigel) that jellified after being pipetted into a mold. The resulting engineered heart tissue (EHT), showed spontaneous contractile activity and characteristics of neonatal heart tissue in force generation and response to chemicals [265]. The cardiac constructs were later modified to include, together with the neonatal rat heart cells, endothelial cells, cardiac fibroblasts, and smooth muscle cells [266]. This version resembled adult rather than immature cardiac tissue with formation of vascular structures [267]. Furthermore, application of EHT was tested in a rat model of MI, showing a significant improvement of the cardiac function along with prevention of the ventricle dilatation and wall thinning 1 month after transplantation [268]. Moreover, EHT were electrically coupled to native myocardium, showed undelayed transmission of impulse and, importantly, did not evidence any arrhythmia.

5.4.3 Cell Sheets

In year 2002, Shimizu and coworkers first published the application of the cell sheet technology for the treatment of MI [269]. In their work, they employed a temperature-responsive polymer poly(*N*isopropylacrylamide). When cells were cultured on this material at 37 °C, they were able to attach and grow to confluence. Then, when temperature was lowered below 32 °C, the polymer rapidly hydrated and swollen, allowing cells to detach, forming the so-called cell sheet. Furthermore, with a simple procedure, it was also possible to stack sheets, thus increasing the thickness of the graft or its composition [270]. Their first paper [269] showed the production of a four layer graft composed of neonatal rat CM which contracted in vitro. When it was subcutaneously transplanted in nude rats, it continued to beat and promote cell maturation. This group also proved that when transplanted into an injured myocardium, grafts integrated and transmitted impulse propagation [271] without evidence of arrhythmia. On the other hand, Sekine and coworkers showed that when CM were cultured in the same cell sheet as endothelial cells, there was a significant increase in the survival of the CMs and paracrine activity of the sheets. Moreover, when employed to treat a rat model of MI, the effect of the mixed-culture sheets was significantly better than the obtained with CM-only sheets [272]. Similarly, when co-cultures of fibroblast and endothelial cells were used, the effect was greater than that of cell sheets composed of endothelial cells alone [64].

Finally, the feasibility of this therapy was established in two preclinical relevant models. Bel et al. [273] employed a Rhesus monkey model of MI in which animals were treated with cell sheets composed of adipose-derived stromal cells and ESC-derived cardiac progenitors (SSEA1+). Cells showed a robust engraftment in diseased organs even 2 months post-grafting, inducing an increase of angiogenesis and what is more relevant, no evidence of teratoma formation. In a second paper, Miyagawa et al. [274] demonstrated that skeletal myoblast sheets induce a significant benefit upon cardiac function, fibrosis, and angiogenesis in a pig model of MI.

5.4.4 Decellularized Matrix and Hearts

Components of the ECM are usually conserved among species and well tolerated even by xenogenic recipients (reviewed in [275]). Thus, the use of decellularized matrices seems feasible, when deprived of cellular and nuclear material and maintaining composition, structural integrity, and biological activity.

Following this hypothesis, Tan and coworkers created a 1.5 × 1.5 cm patch of porcine small intestine submucose (SIS), an acellular tissue rich en ECM proteins and growth factors [276]. When seeded with MSC and transplanted in a rabbit model of MI, it showed a significant benefit upon cardiac function and histology,

as well as MSC migration towards injured tissue and their differentiation to cardiac and smooth muscle lineages. Dr. Badylak's group also studied the ability of SIS to regenerate a model of injured skeletal muscle, demonstrating that it induced a significantly enhanced recuperation when compared to a polypropylene mesh or autologous skeletal tissue [277]. Importantly, this same group has shown the great impact that age of donor animal can exert on SIS characteristics [278], with very different products rendered after decellularization protocols [279], so special care must be taken to address these issues.

In another interesting example, mixing cell sheet technology and a decellularized tissue, Dr Sung's group stacked layers of MSC sheets with porous acellular bovine pericardia. When transplanted in a syngenic model of rat chronic MI [280, 281], the combination of cells and acellular scaffold improved the recipient′s cardiac function and vascularization. Moreover, composite grafts were able to maintain the structure significantly better than acellular tissues transplanted in the same model, and were populated by host-derived vessels and connective fibrils. Additionally, grafts stimulated the release of cardioprotective cytokines such as bFGF, PDGF-B, IGF1 or HGF.

Thus, so far decellularized matrices have proven an interesting approach for cardiac regeneration. Nonetheless, the use of this method still imposes the need for open-chest surgery for successful treatment. Singelyn and coworkers proposed an intermediate solution by using porcine decellularized myocardial matrix, processed so that it can be injected and jellifies at 37 °C [282]. In their work, this product was employed in a rat model of MI, where it formed a nanofibrous structure that promoted vascular cell migration associated with an increase in tissue vascularization. Their material was also successfully pushed through a clinically employed catheter, demonstrating its potential for clinical applications. However, functional analysis has not been performed.

Despite the above-mentioned approaches and their positive effects, partial tissue substitution may not be sufficient for some individuals and organ transplantation is so far regarded as the best solution for the ischemic myocardium were it not for the immunological and availability issues. An alternative option to organ transplantation that may avoid the mentioned limitations might be the creation of bioartificial hearts. Here, cadaveric hearts could be employed to be decellularized, to be then recellularized with the patient-derived cells. This option has been proposed for the heart [283] but also for other organs like the lung [284] or the liver [285]. In their work, Ott et al. set up the decellularization of an intact heart by coronary perfusion with detergents. The resultant matrix was recellularized by injection and infusion of cardiac and endothelial cells. A week later, constructs could generate pump function equivalent to 2 % of the adult heart, with 34 % recellularization of cross sections. Thus, although these results are encouraging, many factors still need to be controlled, like the need for a cardiac cell source (by optimizing the isolation and expansion of cardiac progenitors or through differentiation of pluripotent cells) and the prevention of fatal arrythmias. In any case, this represents a great step forward for the treatment of cardiac diseases.

5.5 Conclusions and Final Remarks

In the last two decades, science has assayed new approaches for treating cardiovascular diseases. Among others, stem cell, gene, and protein therapies have been shown to present an enormous potential, although, of course, many aspects still need to be solved or better understood. In the case of stem cell therapy, hopes were initially directed toward the differentiation capacity of the cells, which ideally, could replace the injured heart with new cardiac and vascular tissue. However, the results have not been as positive as expected and data obtained from many in vivo and even clinical studies have shown that the main mechanisms of action of the cells are not through differentiation but through cytokine and factor secretion. There are several reasons for this lack of translation from in vitro to in vivo differentiation, including, together with the variable degree of real differentiation potential among the stem cell populations, the lack of an adequate microenvironment to host the cells and guide their differentiation. Thus, it has been shown that one of the main limitations that stem cell therapy has presented is the low level of engraftment and survival of the transplanted cells, which greatly diminish their efficacy. New strategies, like the combination of stem cells with the bioengineering or micro/nano-technologies, are intended to solve this problem and furthermore, allow to more complex tissues to be created, which can be transplanted into the tissue. Importantly, the employment of materials has proven useful to limit infarct expansion, maintain ventricle geometry, and compensate loss of functional capacity. Thus, although many aspects like the electro-mechanical properties of the cardiac cell/tissues will need to be strictly controlled and obtaining a real source of cardiac progenitor cells without tumor or immunological risks is still not straightforward, this new approach for treating cardiovascular disease appears to be a very promising alternative that will boost the established positive benefits of stem cell transplantation.

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References

1. In: http://www.who.int/mediacentre/factsheets//fs317/en/index.html
2. Gaffney MM, Hynes SO, Barry F, O'Brien T (2007) Cardiovascular gene therapy: current status and therapeutic potential. Br J Pharmacol 152(2):175–188
3. Rissanen TT, Yla-Herttuala S (2007) Current status of cardiovascular gene therapy. Mol Ther 15(7):1233–1247
4. Shah PB, Losordo DW (2005) Non-viral vectors for gene therapy: clinical trials in cardiovascular disease. Adv Genet 54:339–361
5. Maulik N, Thirunavukkarasu M (2008) Growth factors and cell therapy in myocardial regeneration. J Mol Cell Cardiol 44(2):219–227

6. Passier R, van Laake LW, Mummery CL (2008) Stem-cell-based therapy and lessons from the heart. Nature 453(7193):322–329
7. Segers VF, Lee RT (2008) Stem-cell therapy for cardiac disease. Nature 451(7181):937–942
8. Langer R, Vacanti JP (1993) Tissue engineering. Science 260(5110):920–926
9. Takeshita S, Weir L, Chen D, Zheng LP, Riessen R, Bauters C, Symes JF, Ferrara N, Isner JM (1996) Therapeutic angiogenesis following arterial gene transfer of vascular endothelial growth factor in a rabbit model of hindlimb ischemia. Biochem Biophys Res Commun 227(2):628–635
10. Ruixing Y, Jiaquan L, Jie C, Dezhai Y (2006) Intravenous administration of vascular endothelial growth factor improves cardiac performance and inhibits cardiomyocyte apoptosis. Growth Factors 24(3):209–217
11. Hao X, Mansson-Broberg A, Grinnemo KH, Siddiqui AJ, Dellgren G, Brodin LA, Sylven C (2007) Myocardial angiogenesis after plasmid or adenoviral VEGF-A(165) gene transfer in rat myocardial infarction model. Cardiovasc Res 73(3):481–487
12. Bull DA, Bailey SH, Rentz JJ, Zebrack JS, Lee M, Litwin SE, Kim SW (2003) Effect of Terplex/VEGF-165 gene therapy on left ventricular function and structure following myocardial infarction. VEGF gene therapy for myocardial infarction. J Control Release 93(2):175–181
13. Tio RA, Tkebuchava T, Scheuermann TH, Lebherz C, Magner M, Kearny M, Esakof DD, Isner JM, Symes JF (1999) Intramyocardial gene therapy with naked DNA encoding vascular endothelial growth factor improves collateral flow to ischemic myocardium. Hum Gene Ther 10(18):2953–2960
14. Rutanen J, Rissanen TT, Markkanen JE, Gruchala M, Silvennoinen P, Kivela A, Hedman A, Hedman M, Heikura T, Orden MR, Stacker SA, Achen MG, Hartikainen J, Yla-Herttuala S (2004) Adenoviral catheter-mediated intramyocardial gene transfer using the mature form of vascular endothelial growth factor-D induces transmural angiogenesis in porcine heart. Circulation 109(8):1029–1035
15. Ferrarini M, Arsic N, Recchia FA, Zentilin L, Zacchigna S, Xu X, Linke A, Giacca M, Hintze TH (2006) Adeno-associated virus-mediated transduction of VEGF165 improves cardiac tissue viability and functional recovery after permanent coronary occlusion in conscious dogs. Circ Res 98(7):954–961
16. Lee LY, Patel SR, Hackett NR, Mack CA, Polce DR, El-Sawy T, Hachamovitch R, Zanzonico P, Sanborn TA, Parikh M, Isom OW, Crystal RG, Rosengart TK (2000) Focal angiogen therapy using intramyocardial delivery of an adenovirus vector coding for vascular endothelial growth factor 121. Ann Thorac Surg 69(1):14–23 discussion 23–24
17. Mack CA, Patel SR, Schwarz EA, Zanzonico P, Hahn RT, Ilercil A, Devereux RB, Goldsmith SJ, Christian TF, Sanborn TA, Kovesdi I, Hackett N, Isom OW, Crystal RG, Rosengart TK (1998) Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for vascular endothelial growth factor 121 improves myocardial perfusion and function in the ischemic porcine heart. J Thorac Cardiovasc Surg 115(1):168–176 discussion 176–177
18. Heilmann C, von Samson P, Schlegel K, Attmann T, von Specht BU, Beyersdorf F, Lutter G (2002) Comparison of protein with DNA therapy for chronic myocardial ischemia using fibroblast growth factor-2. Eur J Cardiothorac Surg 22(6):957–964
19. Gao MH, Lai NC, McKirnan MD, Roth DA, Rubanyi GM, Dalton N, Roth DM, Hammond HK (2004) Increased regional function and perfusion after intracoronary delivery of adenovirus encoding fibroblast growth factor 4: report of preclinical data. Hum Gene Ther 15(6):574–587
20. Giordano FJ, Ping P, McKirnan MD, Nozaki S, DeMaria AN, Dillmann WH, Mathieu-Costello O, Hammond HK (1996) Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. Nat Med 2(5):534–539
21. Maurice JP, Hata JA, Shah AS, White DC, McDonald PH, Dolber PC, Wilson KH, Lefkowitz RJ, Glower DD, Koch WJ (1999) Enhancement of cardiac function after

adenoviral-mediated in vivo intracoronary beta2-adrenergic receptor gene delivery. J Clin Invest 104(1):21–29
22. Shah AS, White DC, Emani S, Kypson AP, Lilly RE, Wilson K, Glower DD, Lefkowitz RJ, Koch WJ (2001) In vivo ventricular gene delivery of a beta-adrenergic receptor kinase inhibitor to the failing heart reverses cardiac dysfunction. Circulation 103(9):1311–1316
23. Hammond HK (2006) Adenylyl cyclase gene transfer in heart failure. Ann N Y Acad Sci 1080:426–436
24. Houser SR, Margulies KB (2003) Is depressed myocyte contractility centrally involved in heart failure? Circ Res 92(4):350–358
25. Kawase Y, Ladage D, Hajjar RJ (2011) Rescuing the failing heart by targeted gene transfer. J Am Coll Cardiol 57(10):1169–1180
26. Rosengart TK, Lee LY, Patel SR, Sanborn TA, Parikh M, Bergman GW, Hachamovitch R, Szulc M, Kligfield PD, Okin PM, Hahn RT, Devereux RB, Post MR, Hackett NR, Foster T, Grasso TM, Lesser ML, Isom OW, Crystal RG (1999) Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. Circulation 100(5):468–474
27. Hedman M, Hartikainen J, Yla-Herttuala S (2011) Progress and prospects: hurdles to cardiovascular gene therapy clinical trials. Gene Ther 18(8):743–749
28. Hajjar RJ, Zsebo K, Deckelbaum L, Thompson C, Rudy J, Yaroshinsky A, Ly H, Kawase Y, Wagner K, Borow K, Jaski B, London B, Greenberg B, Pauly DF, Patten R, Starling R, Mancini D, Jessup M (2008) Design of a phase 1/2 trial of intracoronary administration of AAV1/SERCA2a in patients with heart failure. J Card Fail 14(5):355–367
29. Rapti K, Chaanine AH, Hajjar RJ (2011) Targeted gene therapy for the treatment of heart failure. Can J Cardiol 27(3):265–283
30. Grines CL, Watkins MW, Helmer G, Penny W, Brinker J, Marmur JD, West A, Rade JJ, Marrott P, Hammond HK, Engler RL (2002) Angiogenic Gene Therapy (AGENT) in trial patients with stable angina pectoris. Circulation 105(11):1291–1297
31. Grines CL, Watkins MW, Mahmarian JJ, Iskandrian AE, Rade JJ, Marrott P, Pratt C, Kleiman N (2003) Angiogene GENe Therapy (AGENT-2) Study Group.: A randomized, double-blind, placebo-controlled trial of Ad5FGF-4 gene therapy and its effect on myocardial perfusion in patients with stable angina. J Am Coll Cardiol 42(8):1339–1347
32. Henry TD, Grines CL, Watkins MW, Dib N, Barbeau G, Moreadith R, Andrasfay T, Engler RL (2007) Effects of Ad5FGF-4 in patients with angina: an analysis of pooled data from the AGENT-3 and AGENT-4 trials. J Am Coll Cardiol 50(11):1038–1046
33. Hedman M, Hartikainen J, Syvanne M, Stjernvall J, Hedman A, Kivela A, Vanninen E, Mussalo H, Kauppila E, Simula S, Narvanen O, Rantala A, Peuhkurinen K, Nieminen MS, Laakso M, Yla-Herttuala S (2003) Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT). Circulation 107(21):2677–2683
34. Hedman M, Muona K, Hedman A, Kivela A, Syvanne M, Eranen J, Rantala A, Stjernvall J, Nieminen MS, Hartikainen J, Yla-Herttuala S (2009) Eight-year safety follow-up of coronary artery disease patients after local intracoronary VEGF gene transfer. Gene Ther 16(5):629–634
35. Kastrup J, Jorgensen E, Ruck A, Tagil K, Glogar D, Ruzyllo W, Botker HE, Dudek D, Drvota V, Hesse B, Thuesen L, Blomberg P, Gyongyosi M, Sylven C (2005) Euroinject One Group.: Direct intramyocardial plasmid vascular endothelial growth factor-A165 gene therapy in patients with stable severe angina pectoris A randomized double-blind placebo-controlled study: the Euroinject One trial. J Am Coll Cardiol 45(7):982–988
36. Stewart DJ, Kutryk MJ, Fitchett D, Freeman M, Camack N, Su Y, Della Siega A, Bilodeau L, Burton JR, Proulx G, Radhakrishnan S (2009) NORTHERN Trial Investigators.: VEGF gene therapy fails to improve perfusion of ischemic myocardium in patients with advanced coronary disease: results of the NORTHERN trial. Mol Ther 17(6):1109–1115

37. Stewart DJ, Hilton JD, Arnold JM, Gregoire J, Rivard A, Archer SL, Charbonneau F, Cohen E, Curtis M, Buller CE, Mendelsohn FO, Dib N, Page P, Ducas J, Plante S, Sullivan J, Macko J, Rasmussen C, Kessler PD, Rasmussen HS (2006) Angiogenic gene therapy in patients with nonrevascularizable ischemic heart disease: a phase 2 randomized, controlled trial of AdVEGF(121) (AdVEGF121) versus maximum medical treatment. Gene Ther 13(21):1503–1511
38. Jaski BE, Jessup ML, Mancini DM, Cappola TP, Pauly DF, Greenberg B, Borow K, Dittrich H, Zsebo KM, Hajjar RJ (2009) Calcium Up-Regulation by Percutaneous Administration of Gene Therapy In Cardiac Disease (CUPID) Trial Investigators.: Calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID Trial), a first-in-human phase 1/2 clinical trial. J Card Fail 15(3):171–181
39. Jessup M, Greenberg B, Mancini D, Cappola T, Pauly DF, Jaski B, Yaroshinsky A, Zsebo KM, Dittrich H, Hajjar RJ (2011) Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) Investigators.: Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID): a phase 2 trial of intracoronary gene therapy of sarcoplasmic reticulum Ca2+- ATPase in patients with advanced heart failure. Circulation 124(3):304–313
40. Losordo DW, Dimmeler S (2004) Therapeutic angiogenesis and vasculogenesis for ischemic disease. Part I: angiogenic cytokines. Circulation 109(21):2487–2491
41. Kardami E, Detillieux K, Ma X, Jiang Z, Santiago JJ, Jimenez SK, Cattini PA (2007) Fibroblast growth factor-2 and cardioprotection. Heart Fail Rev 12(3–4):267–277
42. Palmen M, Daemen MJ, De Windt LJ, Willems J, Dassen WR, Heeneman S, Zimmermann R, Van Bilsen M, Doevendans PA (2004) Fibroblast growth factor-1 improves cardiac functional recovery and enhances cell survival after ischemia and reperfusion: a fibroblast growth factor receptor, protein kinase C, and tyrosine kinase-dependent mechanism. J Am Coll Cardiol 44(5):1113–1123
43. Kawasuji M, Nagamine H, Ikeda M, Sakakibara N, Takemura H, Fujii S, Watanabe Y (2000) Therapeutic angiogenesis with intramyocardial administration of basic fibroblast growth factor. Ann Thorac Surg 69(4):1155–1161
44. Engel FB, Hsieh PC, Lee RT, Keating MT (2006) FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues function after myocardial infarction. Proc Natl Acad Sci USA 103(42):15546–15551
45. Bougioukas I, Didilis V, Ypsilantis P, Giatromanolaki A, Sivridis E, Lialiaris T, Mikroulis D, Simopoulos C, Bougioukas G (2007) Intramyocardial injection of low-dose basic fibroblast growth factor or vascular endothelial growth factor induces angiogenesis in the infarcted rabbit myocardium. Cardiovasc Pathol 16(2):63–68
46. Betsholtz C (2004) Insight into the physiological functions of PDGF through genetic studies in mice. Cytokine Growth Factor Rev 15(4):215–228
47. Lu H, Xu X, Zhang M, Cao R, Brakenhielm E, Li C, Lin H, Yao G, Sun H, Qi L, Tang M, Dai H, Zhang Y, Su R, Bi Y, Zhang Y, Cao Y (2007) Combinatorial protein therapy of angiogenic and arteriogenic factors remarkably improves collaterogenesis and cardiac function in pigs. Proc Natl Acad Sci USA 104(29):12140–12145
48. Zhao YY, Feron O, Dessy C, Han X, Marchionni MA, Kelly RA (1999) Neuregulin signaling in the heart. Dynamic targeting of erbB4 to caveolar microdomains in cardiac myocytes. Circ Res 84(12):1380–1387
49. Bersell K, Arab S, Haring B, Kuhn B (2009) Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. Cell 138(2):257–270
50. Lavine KJ, White AC, Park C, Smith CS, Choi K, Long F, Hui CC, Ornitz DM (2006) Fibroblast growth factor signals regulate a wave of hedgehog activation that is essential for coronary vascular development. Genes Dev 20(12):1651–1666
51. Pola R, Ling LE, Silver M, Corbley MJ, Kearney M, Blake PR, Shapiro R, Taylor FR, Baker DP, Asahara T, Isner JM (2001) The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. Nat Med 7(6):706–711

52. Ahmed RP, Haider KH, Shujia J, Afzal MR, Ashraf M (2010) Sonic Hedgehog gene delivery to the rodent heart promotes angiogenesis via iNOS/netrin-1/PKC pathway. PLoS ONE 5(1):e8576
53. Geisberg CA, Lenihan DJ (2011) Neuregulin in heart failure: Reverse translation from cancer cardiotoxicity to new heart failure therapy. Herz 36(4):306–310
54. Schumacher B, Pecher P, von Specht BU, Stegmann T (1998) Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease. Circulation 97(7):645–650
55. Unger EF, Goncalves L, Epstein SE, Chew EY, Trapnell CB, Cannon RO, Quyyumi AA (2000) Effects of a single intracoronary injection of basic fibroblast growth factor in stable angina pectoris. Am J Cardiol 85(12):1414–1419
56. Laham RJ, Chronos NA, Pike M, Leimbach ME, Udelson JE, Pearlman JD, Pettigrew RI, Whitehouse MJ, Yoshizawa C, Simons M (2000) Intracoronary basic fibroblast growth factor (FGF-2) in patients with severe ischemic heart disease: results of a phase I open-label dose escalation study. J Am Coll Cardiol 36(7):2132–2139
57. Simons M, Annex BH, Laham RJ, Kleiman N, Henry T, Dauerman H, Udelson JE, Gervino EV, Pike M, Whitehouse MJ, Moon T, Chronos NA (2002) Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial. Circulation 105(7):788–793
58. Hendel RC, Henry TD, Rocha-Singh K, Isner JM, Kereiakes DJ, Giordano FJ, Simons M, Bonow RO (2000) Effect of intracoronary recombinant human vascular endothelial growth factor on myocardial perfusion: evidence for a dose-dependent effect. Circulation 101(2):118–121
59. Henry TD, Annex BH, McKendall GR, Azrin MA, Lopez JJ, Giordano FJ, Shah PK, Willerson JT, Benza RL, Berman DS, Gibson CM, Bajamonde A, Rundle AC, Fine J, McCluskey ER (2003) VIVA Investigators.: the VIVA trial: vascular endothelial growth factor in ischemia for vascular angiogenesis. Circulation 107(10):1359–1365
60. Simons M, Bonow RO, Chronos NA, Cohen DJ, Giordano FJ, Hammond HK, Laham RJ, Li W, Pike M, Sellke FW, Stegmann TJ, Udelson JE, Rosengart TK (2000) Clinical trials in coronary angiogenesis: issues, problems, consensus: an expert panel summary. Circulation 102(11):E73–E86
61. Formiga FR, Tamayo E, Simón-Yarza T, Pelacho B, Prósper F, Blanco-Prieto MJ (2011) Angiogenic therapy for cardiac repair base on protein delivery systems. Heart Fail Rev 17(3):449–473
62. Oh KS, Song JY, Yoon SJ, Park Y, Kim D, Yuk SH (2010) Temperature-induced gel formation of core/shell nanoparticles for the regeneration of ischemic heart. J Control Release 146(2):207–211
63. Formiga FR, Pelacho B, Garbayo E, Abizanda G, Gavira JJ, Simon-Yarza T, Mazo M, Tamayo E, Jauquicoa C, Ortiz-de-Solorzano C, Prosper F, Blanco-Prieto MJ (2010) Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia-reperfusion model. J Control Release 147(1):30–37
64. Kobayashi H, Minatoguchi S, Yasuda S, Bao N, Kawamura I, Iwasa M, Yamaki T, Sumi S, Misao Y, Ushikoshi H, Nishigaki K, Takemura G, Fujiwara T, Tabata Y, Fujiwara H (2008) Post-infarct treatment with an erythropoietin-gelatin hydrogel drug delivery system for cardiac repair. Cardiovasc Res 79(4):611–620
65. Iwakura A, Fujita M, Kataoka K, Tambara K, Sakakibara Y, Komeda M, Tabata Y (2003) Intramyocardial sustained delivery of basic fibroblast growth factor improves angiogenesis and ventricular function in a rat infarct model. Heart Vessels 18(2):93–99
66. Sakakibara Y, Tambara K, Sakaguchi G, Lu F, Yamamoto M, Nishimura K, Tabata Y, Komeda M (2003) Toward surgical angiogenesis using slow-released basic fibroblast growth factor. Eur J Cardiothorac Surg 24(1):105–111 discussion 112

67. Rask F, Dallabrida SM, Ismail NS, Amoozgar Z, Yeo Y, Rupnick MA, Radisic M (2010) Photocrosslinkable chitosan modified with angiopoietin-1 peptide, QHREDGS, promotes survival of neonatal rat heart cells. J Biomed Mater Res A 95(1):105–117
68. Silva EA, Mooney DJ (2007) Spatiotemporal control of vascular endothelial growth factor delivery from injectable hydrogels enhances angiogenesis. J Thromb Haemost 5(3):590–598
69. Wang H, Zhang X, Li Y, Ma Y, Zhang Y, Liu Z, Zhou J, Lin Q, Wang Y, Duan C, Wang C (2010) Improved myocardial performance in infarcted rat heart by co-injection of basic fibroblast growth factor with temperature-responsive chitosan hydrogel. J Heart Lung Transplant 29(8):881–887
70. Fujita M, Ishihara M, Morimoto Y, Simizu M, Saito Y, Yura H, Matsui T, Takase B, Hattori H, Kanatani Y, Kikuchi M, Maehara T (2005) Efficacy of photocrosslinkable chitosan hydrogel containing fibroblast growth factor-2 in a rabbit model of chronic myocardial infarction. J Surg Res 126(1):27–33
71. Schumacher B, Pecher P, von Specht BU, Stegmann T (1998) Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease. Circulation 97(7):645–650
72. Achilli F, Malafronte C, Lenatti L, Gentile F, Dadone V, Gibelli G, Maggiolini S, Squadroni L, Di Leo C, Burba I, Pesce M, Mircoli L, Capogrossi MC, Di Lelio A, Camisasca P, Morabito A, Colombo G, Pompilio G (2010) STEM-AMI Investigators.: Granulocyte colony-stimulating factor attenuates left ventricular remodelling after acute anterior STEMI: results of the single-blind, randomized, placebo-controlled multicentre STem cEll Mobilization in Acute Myocardial Infarction (STEM-AMI) Trial. Eur J Heart Fail 12(10):1111–1121
73. Theiss HD, Brenner C, Engelmann MG, Zaruba MM, Huber B, Henschel V, Mansmann U, Wintersperger B, Reiser M, Steinbeck G, Franz WM (2010) Safety and efficacy of SITAgliptin plus GRanulocyte-colony-stimulating factor in patients suffering from Acute Myocardial Infarction (SITAGRAMI-Trial) rationale, design and first interim analysis. Int J Cardiol 145(2):282–284
74. Gao R, Zhang J, Cheng L, Wu X, Dong W, Yang X, Li T, Liu X, Xu Y, Li X, Zhou M (2010) Phase II, randomized, double-blind, multicenter, based on standard therapy, placebo-controlled study of the efficacy and safety of recombinant human neuregulin-1 in patients with chronic heart failure. J Am Coll Cardiol 55(18):1907–14
75. Voors AA, Belonje AM, Zijlstra F, Hillege HL, Anker SD, Slart RH, Tio RA, van t Hof A, Jukema JW, Peels HO, Henriques JP, Ten Berg JM, Vos J, van Gilst WH, van Veldhuisen DJ, HEBE III Investigators (2010) A single dose of erythropoietin in STelevation myocardial infarction. Eur Heart J 31(21):2593-2600
76. Scott RC, Rosano JM, Ivanov Z, Wang B, Chong PL, Issekutz AC, Crabbe DL, Kiani MF (2009) Targeting VEGF-encapsulated immunoliposomes to MI heart improves vascularity and cardiac function. FASEB J 23(10):3361–3367
77. Cleland JL, Duenas ET, Park A, Daugherty A, Kahn J, Kowalski J, Cuthbertson A (2001) Development of poly-(D, L-lactide–coglycolide) microsphere formulations containing recombinant human vascular endothelial growth factor to promote local angiogenesis. J Control Release 72(1–3):13–24
78. Rocha FG, Sundback CA, Krebs NJ, Leach JK, Mooney DJ, Ashley SW, Vacanti JP, Whang EE (2008) The effect of sustained delivery of vascular endothelial growth factor on angiogenesis in tissue-engineered intestine. Biomaterials 29(19):2884–2890
79. Cohen-Sela E, Chorny M, Koroukhov N, Danenberg HD, Golomb G (2009) A new double emulsion solvent diffusion technique for encapsulating hydrophilic molecules in PLGA nanoparticles. J Control Release 133(2):90–95
80. Kumar PS, Ramakrishna S, Saini TR, Diwan PV (2006) Influence of microencapsulation method and peptide loading on formulation of poly(lactide-co-glycolide) insulin nanoparticles. Pharmazie 61(7):613–617
81. Bilati U, Allemann E, Doelker E (2005) Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles. Eur J Pharm Biopharm 59(3):375–388

82. Vila A, Sanchez A, Tobio M, Calvo P, Alonso MJ (2002) Design of biodegradable particles for protein delivery. J Control Release 78(1–3):15–24
83. Garbayo E, Ansorena E, Lanciego JL, Aymerich MS, Blanco-Prieto MJ (2008) Sustained release of bioactive glycosylated glial cell-line derived neurotrophic factor from biodegradable polymeric microspheres. Eur J Pharm Biopharm 69(3):844–851
84. Lee J, Bhang SH, Park H, Kim BS, Lee KY (2010) Active blood vessel formation in the ischemic hindlimb mouse model using a microsphere/hydrogel combination system. Pharm Res 27(5):767–774
85. Sun Q, Silva EA, Wang A, Fritton JC, Mooney DJ, Schaffler MB, Grossman PM, Rajagopalan S (2010) Sustained release of multiple growth factors from injectable polymeric system as a novel therapeutic approach towards angiogenesis. Pharm Res 27(2):264–271
86. Golub JS, Kim YT, Duvall CL, Bellamkonda RV, Gupta D, Lin AS, Weiss D, Guldberg TW, Robert RE (2010) Sustained VEGF delivery via PLGA nanoparticles promotes vascular growth. Am J Physiol Heart Circ Physiol 298(6):H1959–H1965
87. Brar BK, Stephanou A, Wagstaff MJ, Coffin RS, Marber MS, Engelmann G, Latchman DS (1999) Heat shock proteins delivered with a virus vector can protect cardiac cells against apoptosis as well as against thermal or hypoxic stress. J Mol Cell Cardiol 31(1):135–146
88. Laham RJ, Sellke FW, Edelman ER, Pearlman JD, Ware JA, Brown DL, Gold JP, Simons M (1999) Local perivascular delivery of basic fibroblast growth factor in patients undergoing coronary bypass surgery: results of a phase I randomized, double-blind, placebo-controlled trial. Circulation 100(18):1865–1871
89. Laflamme MA, Murry CE (2011) Heart regeneration. Nature 473(7347):326–335
90. Murry CE, Reinecke H, Pabon LM (2006) Regeneration gaps: observations on stem cells and cardiac repair. J Am Coll Cardiol 47(9):1777–1785
91. Whelan RS, Kaplinskiy V, Kitsis RN (2010) Cell death in the pathogenesis of heart disease: mechanisms and significance. Annu Rev Physiol 72:19–44
92. Jezierska-Wozniak K, Mystkowska D, Tutas A, Jurkowski MK (2011) Stem cells as therapy for cardiac disease:a review. Folia Histochem Cytobiol 49(1):13–25
93. Taylor DA, Atkins BZ, Hungspreugs P, Jones TR, Reedy MC, Hutcheson KA, Glower DD, Kraus WE (1998) Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. Nat Med 4(8):929–933
94. Menasche P, Alfieri O, Janssens S, McKenna W, Reichenspurner H, Trinquart L, Vilquin JT, Marolleau JP, Seymour B, Larghero J, Lake S, Chatellier G, Solomon S, Desnos M, Hagege AA (2008) The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. Circulation 117(9):1189–1200
95. Winitsky SO, Gopal TV, Hassanzadeh S, Takahashi H, Gryder D, Rogawski MA, Takeda K, Yu ZX, Xu YH, Epstein ND (2005) Adult murine skeletal muscle contains cells that can differentiate into beating cardiomyocytes in vitro. PLoS Biol 3(4):e87
96. Menasche P (2007) Skeletal myoblasts as a therapeutic agent. Prog Cardiovasc Dis 50(1):7–17
97. Dib N, Michler RE, Pagani FD, Wright S, Kereiakes DJ, Lengerich R, Binkley P, Buchele D, Anand I, Swingen C, Di Carli MF, Thomas JD, Jaber WA, Opie SR, Campbell A, McCarthy P, Yeager M, Dilsizian V, Griffith BP, Korn R, Kreuger SK, Ghazoul M, MacLellan WR, Fonarow G, Eisen HJ, Dinsmore J, Diethrich E (2005) Safety and feasibility of autologous myoblast transplantation in patients with ischemic cardiomyopathy: four-year follow-up. Circulation 112(12):1748–1755
98. Mozid AM, Arnous S, Sammut EC, Mathur A (2011) Stem cell therapy for heart diseases. Br Med Bull 98:143–159
99. He KL, Yi GH, Sherman W, Zhou H, Zhang GP, Gu A, Kao R, Haimes HB, Harvey J, Roos E, White D, Taylor DA, Wang J, Burkhoff D (2005) Autologous skeletal myoblast transplantation improved hemodynamics and left ventricular function in chronic heart failure dogs. J Heart Lung Transplant 24(11):1940–1949

100. van den Bos EJ, Thompson RB, Wagner A, Mahrholdt H, Morimoto Y, Thomson LE, Wang LH, Duncker DJ, Judd RM, Taylor DA (2005) Functional assessment of myoblast transplantation for cardiac repair with magnetic resonance imaging. Eur J Heart Fail 7(4):435–443
101. McConnell PI, del Rio CL, Jacoby DB, Pavlicova M, Kwiatkowski P, Zawadzka A, Dinsmore JH, Astra L, Wisel S, Michler RE (2005) Correlation of autologous skeletal myoblast survival with changes in left ventricular remodeling in dilated ischemic heart failure. J Thorac Cardiovasc Surg 130(4):1001
102. Emani SM, Ellis MJ, Dibernardo LR, Colgrove S, Glower DD, Taylor DA (2006) Systolic contraction within aneurysmal rabbit myocardium following transplantation of autologous skeletal myoblasts. J Surg Res 135(1):202–208
103. Koh GY, Klug MG, Soonpaa MH, Field LJ (1993) Differentiation and long-term survival of C2C12 myoblast grafts in heart. J Clin Invest 92(3):1548–1554
104. Perez-Ilzarbe M, Agbulut O, Pelacho B, Ciorba C, San J-EE, Desnos M, Hagege AA, Aranda P, Andreu EJ, Menasche P, Prosper F (2008) Characterization of the paracrine effects of human skeletal myoblasts transplanted in infarcted myocardium. Eur J Heart Fail 10(11):1065–1072
105. Shintani Y, Fukushima S, Varela-Carver A, Lee J, Coppen SR, Takahashi K, Brouilette SW, Yashiro K, Terracciano CM, Yacoub MH, Suzuki K (2009) Donor cell-type specific paracrine effects of cell transplantation for post-infarction heart failure. J Mol Cell Cardiol 47(2):288–295
106. Bonaros N, Rauf R, Wolf D, Margreiter E, Tzankov A, Schlechta B, Kocher A, Ott H, Schachner T, Hering S, Bonatti J, Laufer G (2006) Combined transplantation of skeletal myoblasts and angiopoietic progenitor cells reduces infarct size and apoptosis and improves cardiac function in chronic ischemic heart failure. J Thorac Cardiovasc Surg 132(6):1321–1328
107. Farahmand P, Lai TY, Weisel RD, Fazel S, Yau T, Menasche P, Li RK (2008) Skeletal myoblasts preserve remote matrix architecture and global function when implanted early or late after coronary ligation into infarcted or remote myocardium. Circulation 118(14 Suppl):S130–S137
108. Fukushima S, Coppen SR, Lee J, Yamahara K, Felkin LE, Terracciano CM, Barton PJ, Yacoub MH, Suzuki K (2008) Choice of cell-delivery route for skeletal myoblast transplantation for treating post-infarction chronic heart failure in rat. PLoS ONE 3(8):e3071
109. Gavira JJ, Nasarre E, Abizanda G, Perez-Ilzarbe M, de Martino-Rodriguez A, de Jalon JAG, Mazo M, Macias A, Garcia-Bolao I, Pelacho B, Martinez-Caro D, Prosper F (2010) Repeated implantation of skeletal myoblast in a swine model of chronic myocardial infarction. Eur Heart J 31(8):1013–1021
110. Gavira JJ, Perez-Ilzarbe M, Abizanda G, Garcia-Rodriguez A, Orbe J, Paramo JA, Belzunce M, Rabago G, Barba J, Herreros J, Panizo A, de Jalon JA, Martinez-Caro D, Prosper F (2006) A comparison between percutaneous and surgical transplantation of autologous skeletal myoblasts in a swine model of chronic myocardial infarction. Cardiovasc Res 71(4):744–753
111. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P (2001) Bone marrow cells regenerate infarcted myocardium. Nature 410(6829):701–705
112. Liu JF, Wang BW, Hung HF, Chang H, Shyu KG (2008) Human mesenchymal stem cells improve myocardial performance in a splenectomized rat model of chronic myocardial infarction. J Formos Med Assoc 107(2):165–174
113. Waksman R, Fournadjiev J, Baffour R, Pakala R, Hellinga D, Leborgne L, Yazdi H, Cheneau E, Wolfram R, Seabron R, Horton K, Kolodgie F, Virmani R, Rivera E (2004) Transepicardial autologous bone marrow-derived mononuclear cell therapy in a porcine model of chronically infarcted myocardium. Cardiovasc Radiat Med 5(3):125–131
114. Mazo M, Gavira JJ, Abizanda G, Moreno C, Ecay M, Soriano M, Aranda P, Collantes M, Alegria E, Merino J, Penuelas I, Garcia VJM, Pelacho B, Prosper F (2010) Transplantation

of mesenchymal stem cells exerts a greater long-term effect than bone marrow mononuclear cells in a chronic myocardial infarction model in rat. Cell Transplant 19(3):313–328
115. Hatzistergos KE, Quevedo H, Oskouei BN, Hu Q, Feigenbaum GS, Margitich IS, Mazhari R, Boyle AJ, Zambrano JP, Rodriguez JE, Dulce R, Pattany PM, Valdes D, Revilla C, Heldman AW, McNiece I, Hare JM (2010) Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. Circ Res 107(7):913–922
116. Le Blanc K (2006) Mesenchymal stromal cells: tissue repair and immune modulation. Cytotherapy 8(6):559–561
117. Gluckman E (2009) Ten years of cord blood transplantation: from bench to bedside. Br J Haematol 147(2):192–199
118. Henning RJ, Burgos JD, Vasko M, Alvarado F, Sanberg CD, Sanberg PR, Morgan MB (2007) Human cord blood cells and myocardial infarction: effect of dose and route of administration on infarct size. Cell Transplant 16(9):907–917
119. Merx MW, Zernecke A, Liehn EA, Schuh A, Skobel E, Butzbach B, Hanrath P, Weber C (2005) Transplantation of human umbilical vein endothelial cells improves left ventricular function in a rat model of myocardial infarction. Basic Res Cardiol 100(3):208–216
120. Fraser JK, Wulur I, Alfonso Z, Hedrick MH (2006) Fat tissue: an underappreciated source of stem cells for biotechnology. Trends Biotechnol 24(4):150–154
121. Daher SR, Johnstone BH, Phinney DG, March KL (2008) Adipose stromal/stem cells: basic and translational advances: the IFATS collection. Stem Cells 26(10):2664–2665
122. Poglio S, De Toni-Costes F, Arnaud E, Laharrague P, Espinosa E, Casteilla L, Cousin B (2010) Adipose tissue as a dedicated reservoir of functional mast cell progenitors. Stem Cells 28(11):2065–2072
123. Rodbell M (1964) Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. J Biol Chem 239:375–380
124. Rodbell M, Jones AB (1966) Metabolism of isolated fat cells. 3. The similar inhibitory action of phospholipase C (Clostridium perfringens alpha toxin) and of insulin on lipolysis stimulated by lipolytic hormones and theophylline. J Biol Chem 241(1):140–142
125. Rangappa S, Entwistle JW, Wechsler AS, Kresh JY (2003) Cardiomyocyte-mediated contact programs human mesenchymal stem cells to express cardiogenic phenotype. J Thorac Cardiovasc Surg 126(1):124–132
126. Psaltis PJ, Zannettino AC, Worthley SG, Gronthos S (2008) Concise review: mesenchymal stromal cells: potential for cardiovascular repair. Stem Cells 26(9):2201–2210
127. Planat-Benard V, Menard C, Andre M, Puceat M, Perez A, Garcia-Verdugo JM, Penicaud L, Casteilla L (2004) Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. Circ Res 94(2):223–229
128. Leobon B, Roncalli J, Joffre C, Mazo M, Boisson M, Barreau C, Calise D, Arnaud E, Andre M, Puceat M, Penicaud L, Prosper F, Planat-Benard V, Casteilla L (2009) Adipose-derived cardiomyogenic cells: in vitro expansion and functional improvement in a mouse model of myocardial infarction. Cardiovasc Res 83(4):757–767
129. Mazo M, Cemborain A, Gavira JJ, Abizanda G, Araña M, Casado M, Soriano M, Hernández S, Moreno C, Ecay M, Albiasu E, Belzunce M, Orbe J, Páramo JA, Merino J, Peñuelas I, García-Verdugo J.M, Pelacho B, Prosper F (2012) adipose stromal vascular fraction improves cardiac function in chronic myocardial infarction through differentiation and paracrine activity. Cell Transplantation [Epub ahead of print]
130. Mazo M, Planat-Benard V, Abizanda G, Pelacho B, Leobon B, Gavira JJ, Penuelas I, Cemborain A, Penicaud L, Laharrague P, Joffre C, Boisson M, Ecay M, Collantes M, Barba J, Casteilla L, Prosper F (2008) Transplantation of adipose derived stromal cells is associated with functional improvement in a rat model of chronic myocardial infarction. Eur J Heart Fail 10(5):454–462
131. Wang L, Deng J, Tian W, Xiang B, Yang T, Li G, Wang J, Gruwel M, Kashour T, Rendell J, Glogowski M, Tomanek B, Freed D, Deslauriers R, Arora RC, Tian G (2009) Adipose-derived stem cells are an effective cell candidate for treatment of heart failure: an MR imaging study of rat hearts. Am J Physiol Heart Circ Physiol 297(3):H1020–H1031

132. Valina C, Pinkernell K, Song YH, Bai X, Sadat S, Campeau RJ, Le Jemtel TH, Alt E (2007) Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular function, perfusion, and remodelling after acute myocardial infarction. Eur Heart J 28(21):2667–2677
133. Bayes-Genis A, Soler-Botija C, Farre J, Sepulveda P, Raya A, Roura S, Prat-Vidal C, Galvez-Monton C, Montero JA, Buscher D, Belmonte JC (2010) Human progenitor cells derived from cardiac adipose tissue ameliorate myocardial infarction in rodents. J Mol Cell Cardiol 49(5):771–780
134. Zhu XY, Zhang XZ, Xu L, Zhong XY, Ding Q, Chen YX (2009) Transplantation of adipose-derived stem cells overexpressing hHGF into cardiac tissue. Biochem Biophys Res Commun 379(4):1084–1090
135. Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, Ishino K, Ishida H, Shimizu T, Kangawa K, Sano S, Okano T, Kitamura S, Mori H (2006) Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med 12(4):459–465
136. Martin-Puig S, Wang Z, Chien KR (2008) Lives of a heart cell: tracing the origins of cardiac progenitors. Cell Stem Cell 2(4):320–331
137. Urbanek K, Torella D, Sheikh F, De Angelis A, Nurzynska D, Silvestri F, Beltrami CA, Bussani R, Beltrami AP, Quaini F, Bolli R, Leri A, Kajstura J, Anversa P (2005) Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci USA 102(24):8692–8697
138. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114(6):763–776
139. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, Pocius J, Michael LH, Behringer RR, Garry DJ, Entman ML, Schneider MD (2003) Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci USA 100(21):12313–12318
140. Oyama T, Nagai T, Wada H, Naito AT, Matsuura K, Iwanaga K, Takahashi T, Goto M, Mikami Y, Yasuda N, Akazawa H, Uezumi A, Takeda S, Komuro I (2007) Cardiac side population cells have a potential to migrate and differentiate into cardiomyocytes in vitro and in vivo. J Cell Biol 176(3):329–341
141. Matsuura K, Nagai T, Nishigaki N, Oyama T, Nishi J, Wada H, Sano M, Toko H, Akazawa H, Sato T, Nakaya H, Kasanuki H, Komuro I (2004) Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. J Biol Chem 279(12):11384–11391
142. Martin CM, Meeson AP, Robertson SM, Hawke TJ, Richardson JA, Bates S, Goetsch SC, Gallardo TD, Garry DJ (2004) Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. Dev Biol 265(1):262–275
143. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A (2004) Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 95(9):911–921
144. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marban E (2007) Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation 115(7):896–908
145. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin LZ, Cai CL, Lu MM, Reth M, Platoshyn O, Yuan JX, Evans S, Chien KR (2005) Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 433(7026):647–653
146. Scorsin M, Hagege AA, Marotte F, Mirochnik N, Copin H, Barnoux M, Sabri A, Samuel JL, Rappaport L, Menasche P (1997) Does transplantation of cardiomyocytes improve function of infarcted myocardium? Circulation 96(9(Suppl)):II188–II93
147. Li RK, Jia ZQ, Weisel RD, Mickle DA, Zhang J, Mohabeer MK, Rao V, Ivanov J (1996) Cardiomyocyte transplantation improves heart function. Ann Thorac Surg 62(3):654–660 discussion 660–661

148. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292(5819):154–156
149. Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 78(12): 7634–7638
150. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282(5391): 1145–1147
151. Xu C, Police S, Rao N, Carpenter MK (2002) Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res 91(6):501–508
152. Klug MG, Soonpaa MH, Koh GY, Field LJ (1996) Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. J Clin Invest 98(1):216–224
153. Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S, O'Sullivan C, Collins L, Chen Y, Minami E, Gill EA, Ueno S, Yuan C, Gold J, Murry CE (2007) Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25(9):1015–1024
154. Caspi O, Huber I, Kehat I, Habib M, Arbel G, Gepstein A, Yankelson L, Aronson D, Beyar R, Gepstein L (2007) Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. J Am Coll Cardiol 50(19): 1884–1893
155. van Laake LW, Passier R, Monshouwer-Kloots J, Verkleij AJ, Lips DJ, Freund C, den Ouden K, van Oostwaard DW, Korving J, Tertoolen LG, van Echteld CJ, Doevendans PA, Mummery CL (2007) Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction. Stem Cell Res 1(1):9–24
156. Fernandes S, Naumova AV, Zhu WZ, Laflamme MA, Gold J, Murry CE (2010) Human embryonic stem cell-derived cardiomyocytes engraft but do not alter cardiac remodeling after chronic infarction in rats. J Mol Cell Cardiol 49(6):941–949
157. Puymirat E, Geha R, Tomescot A, Bellamy V, Larghero J, Trinquart L, Bruneval P, Desnos M, Hagege A, Puceat M, Menasche P (2009) Can mesenchymal stem cells induce tolerance to cotransplanted human embryonic stem cells? Mol Ther 17(1):176–182
158. van Laake LW, Passier R, Doevendans PA, Mummery CL (2008) Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents. Circ Res 102(9):1008–1010
159. He Q, Trindade PT, Stumm M, Li J, Zammaretti P, Bettiol E, Dubois-Dauphin M, Herrmann F, Kalangos A, Morel D, Jaconi ME (2009) Fate of undifferentiated mouse embryonic stem cells within the rat heart: role of myocardial infarction and immune suppression. J Cell Mol Med 13(1):188–201
160. Zhu WZ, Xie Y, Moyes KW, Gold JD, Askari B, Laflamme MA (2010) Neuregulin/ErbB signaling regulates cardiac subtype specification in differentiating human embryonic stem cells. Circ Res 107(6):776–786
161. Lee AS, Tang C, Cao F, Xie X, van der Bogt K, Hwang A, Connolly AJ, Robbins RC, Wu JC (2009) Effects of cell number on teratoma formation by human embryonic stem cells. Cell Cycle 8(16):2608–2612
162. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
163. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872
164. Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, Vassena R, Bilic J, Pekarik V, Tiscornia G, Edel M, Boue S, Izpisua BJC (2008) Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol 26(11):1276–1284

165. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318(5858):1917–1920
166. Yoshida Y, Yamanaka S (2011) iPS cells: a source of cardiac regeneration. J Mol Cell Cardiol 50(2):327–332
167. Nelson TJ, Martinez-Fernandez A, Yamada S, Perez-Terzic C, Ikeda Y, Terzic A (2009) Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. Circulation 120(5):408–416
168. Mauritz C, Schwanke K, Reppel M, Neef S, Katsirntaki K, Maier LS, Nguemo F, Menke S, Haustein M, Hescheler J, Hasenfuss G, Martin U (2008) Generation of functional murine cardiac myocytes from induced pluripotent stem cells. Circulation 118(5):507–517
169. Martinez-Fernandez A, Nelson TJ, Yamada S, Reyes S, Alekseev AE, Perez-Terzic C, Ikeda Y, Terzic A (2009) iPS programmed without c-MYC yield proficient cardiogenesis for functional heart chimerism. Circ Res 105(7):648–656
170. Liu YH, Karra R, Wu SM (2008) Cardiovascular Stem Cells in Regenerative Medicine: Ready for Prime Time? Drug Discov Today Ther Strateg 5(4):201–207
171. Kang NY, Yun SW, Ha HH, Park SJ, Chang YT (2011) Embryonic and induced pluripotent stem cell staining and sorting with the live-cell fluorescence imaging probe CDy1. Nat Protoc 6(7):1044–1052
172. Hansson EM, Lindsay ME, Chien KR (2009) Regeneration next: toward heart stem cell therapeutics. Cell Stem Cell 5(4):364–377
173. Pelacho B, Mazo M, Gavira JJ, Prosper F (2011) Adult stem cells: from new cell sources to changes in methodology. J Cardiovasc Transl Res 4(2):154–160
174. Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, Epstein SE (2004) Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 94(5):678–685
175. Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt RE, Ingwall JS, Dzau VJ (2006) Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 20(6):661–669
176. Wu KH, Han ZC, Mo XM, Zhou B (2012) Cell delivery in cardiac regenerative therapy. Ageing Res Rev 11(1):32–40
177. Menasche P, Hagege AA, Vilquin JT, Desnos M, Abergel E, Pouzet B, Bel A, Sarateanu S, Scorsin M, Schwartz K, Bruneval P, Benbunan M, Marolleau JP, Duboc D (2003) Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. J Am Coll Cardiol 41(7):1078–1083
178. Hagege AA, Marolleau JP, Vilquin JT, Alheritiere A, Peyrard S, Duboc D, Abergel E, Messas E, Mousseaux E, Schwartz K, Desnos M, Menasche P (2006) Skeletal myoblast transplantation in ischemic heart failure: long-term follow-up of the first phase I cohort of patients. Circulation 114(1 Suppl):108–113
179. Smits PC, van Geuns RJ, Poldermans D, Bountioukos M, Onderwater EE, Lee CH, Maat AP, Serruys PW (2003) Catheter-based intramyocardial injection of autologous skeletal myoblasts as a primary treatment of ischemic heart failure: clinical experience with six-month follow-up. J Am Coll Cardiol 42(12):2063–2069
180. Ince H, Petzsch M, Rehders TC, Chatterjee T, Nienaber CA (2004) Transcatheter transplantation of autologous skeletal myoblasts in postinfarction patients with severe left ventricular dysfunction. J Endovasc Ther 11(6):695–704
181. Siminiak T, Kalawski R, Fiszer D, Jerzykowska O, Rzezniczak J, Rozwadowska N, Kurpisz M (2004) Autologous skeletal myoblast transplantation for the treatment of postinfarction myocardial injury: phase I clinical study with 12 months of follow-up. Am Heart J 148(3):531–537

182. Chachques JC, Acar C, Herreros J, Trainini JC, Prosper F, D'Attellis N, Fabiani JN, Carpentier AF (2004) Cellular cardiomyoplasty: clinical application. Ann Thorac Surg 77(3):1121–1130
183. Siminiak T, Fiszer D, Jerzykowska O, Grygielska B, Rozwadowska N, Kalmucki P, Kurpisz M (2005) Percutaneous trans-coronary-venous transplantation of autologous skeletal myoblasts in the treatment of post-infarction myocardial contractility impairment: the POZNAN trial. Eur Heart J 26(12):1188–1195
184. Biagini E, Valgimigli M, Smits PC, Poldermans D, Schinkel AF, Rizzello V, Onderwater EE, Bountioukos M, Serruys PW (2006) Stress and tissue Doppler echocardiographic evidence of effectiveness of myoblast transplantation in patients with ischaemic heart failure. Eur J Heart Fail 8(6):641–648
185. Dib N, Dinsmore J, Lababidi Z, White B, Moravec S, Campbell A, Rosenbaum A, Seyedmadani K, Jaber WA, Rizenhour CS, Diethrich E (2009) One-year follow-up of feasibility and safety of the first U.S., randomized, controlled study using 3-dimensional guided catheter-based delivery of autologous skeletal myoblasts for ischemic cardiomyopathy (CAuSMIC study). JACC Cardiovasc Interv 2(1):9–16
186. Duckers HJ, Houtgraaf J, Hehrlein C, Schofer J, Waltenberger J, Gershlick A, Bartunek J, Nienaber C, Macaya C, Peters N, Smits P, Siminiak T, van Mieghem W, Legrand V, Serruys PW (2011) Final results of a phase IIa, randomised, open-label trial to evaluate the percutaneous intramyocardial transplantation of autologous skeletal myoblasts in congestive heart failure patients: the SEISMIC trial. EuroIntervention 6(7):805–812
187. Strauer BE, Yousef M, Schannwell CM (2010a) The acute and long-term effects of intracoronary Stem cell Transplantation in 191 patients with chronic heARt failure: the STAR-heart study. Eur J Heart Fail 12(7):721–729
188. Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM (2002) Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation 106(24):3009–3017
189. Britten MB, Abolmaali ND, Assmus B, Lehmann R, Honold J, Schmitt J, Vogl TJ, Martin H, Schachinger V, Dimmeler S, Zeiher AM (2003) Infarct remodeling after intracoronary progenitor cell treatment in patients with acute myocardial infarction (TOPCARE-AMI): mechanistic insights from serial contrast-enhanced magnetic resonance imaging. Circulation 108(18):2212–2218
190. Schächinger V, Assmus B, Britten MB, Honold J, Lehmann R, Teupe C, Abolmaali ND, Vogl TJ, Hofmann WK, Martin H, Dimmeler S, Zeiher AM (2004) Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final oneyear results of the TOPCARE-AMI Trial. J Am Coll Cardiol. 44(8):1690–1699
191. Fernandez-Aviles F, San Roman JA, Garcia-Frade J, Fernandez ME, Penarrubia MJ, de la Fuente L, Gomez-Bueno M, Cantalapiedra A, Fernandez J, Gutierrez O, Sanchez PL, Hernandez C, Sanz R, Garcia-Sancho J, Sanchez A (2004) Experimental and clinical regenerative capability of human bone marrow cells after myocardial infarction. Circ Res 95(7):742–748
192. Perin EC, Dohmann HF, Borojevic R, Silva SA, Sousa AL, Silva GV, Mesquita CT, Belem L, Vaughn WK, Rangel FO, Assad JA, Carvalho AC, Branco RV, Rossi MI, Dohmann HJ, Willerson JT (2004) Improved exercise capacity and ischemia 6 and 12 months after transendocardial injection of autologous bone marrow mononuclear cells for ischemic cardiomyopathy. Circulation 110(11 Suppl 1):II213-218
193. Erbs S, Linke A, Adams V, Lenk K, Thiele H, Diederich KW, Emmrich F, Kluge R, Kendziorra K, Sabri O, Schuler G, Hambrecht R (2005) Transplantation of blood-derived progenitor cells after recanalization of chronic coronary artery occlusion: first randomized and placebo-controlled study. Circ Res 97(8):756–762
194. Patel NR, Lee PS, Kim JH, Weinhouse GL (2005) Koziel H (2005) The influence of diagnostic bronchoscopy on clinical outcomes comparing adult autologous and allogeneic bone marrow transplant patients. Chest 127(4):388–1396

195. Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, Fichtner S, Korte T, Hornig B, Messinger D, Arseniev L, Hertenstein B, Ganser A, Drexler H (2004) Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. Lancet 364(9429):141–148
196. Schaefer A, Meyer GP, Fuchs M, Klein G, Kaplan M, Wollert KC, Drexler H (2006) Impact of intracoronary bone marrow cell transfer on diastolic function in patients after acute myocardial infarction: results from the BOOST trial. Eur Heart J 27(8):929–35
197. Janssens S, Dubois C, Bogaert J, Theunissen K, Deroose C, Desmet W, Kalantzi M, Herbots L, Sinnaeve P, Dens J, Maertens J, Rademakers F, Dymarkowski S, Gheysens O, Van Cleemput J, Bormans G, Nuyts J, Belmans A, Mortelmans L, Boogaerts M, Van de Werf F (2006) Autologous bone marrow-derived stem-cell transfer in patients with STsegment elevation myocardial infarction: double-blind, randomised controlled trial. Lancet 367(9505):113–121
198. Hendrikx M, Hensen K, Clijsters C, Jongen H, Koninckx R, Bijnens E, Ingels M, Jacobs A, Geukens R, Dendale P, Vijgen J, Dilling D, Steels P, Mees U, Rummens JL (2006) Recovery of regional but not global contractile function by the direct intramyocardial autologous bone marrow transplantation: results from a randomized controlled clinical trial. Circulation 114(1 Suppl):I101–107
199. Meyer GP, Wollert KC, Lotz J, Steffens J, Lippolt P, Fichtner S, Hecker H, Schaefer A, Arseniev L, Hertenstein B, Ganser A, Drexler H (2006) Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial. Circulation 113(10):1287–1294
200. Mocini D, Staibano M, Mele L, Giannantoni P, Menichella G, Colivicchi F, Sordini P, Salera P, Tubaro M, Santini M (2006) Autologous bone marrow mononuclear cell transplantation in patients undergoing coronary artery bypass grafting. Am Heart J 151(1):192–197
201. Fuchs S, Kornowski R, Weisz G, Satler LF, Smits PC, Okubagzi P, Baffour R, Aggarwal A, Weissman NJ, Cerqueira M, Waksman R, Serrruys P, Battler A, Moses JW, Leon MB, Epstein SE (2006) Safety and feasibility of transendo- cardial autologous bone marrow cell transplantation in patients with advanced heart disease. Am. J. Cardiol. 97(6):823–829
202. Briguori C, Reimers B, Sarais C, Napodano M, Pascotto P, Azzarello G, Bregni M, Porcellini A, Vinante O, Zanco P, Peschle C, Condorelli G, Colombo A (2006) Direct intramyocardial percutaneous delivery of autologous bone marrow in patients with refractory myocardial angina. Am Heart J 151(3):674–680
203. Lunde K, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Egeland T, Endresen K, Ilebekk A, Mangschau A, Fjeld JG, Smith HJ, Taraldsrud E, Grogaard HK, Bjornerheim R, Brekke M, Muller C, Hopp E, Ragnarsson A, Brinchmann JE, Forfang K (2006) Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. N Engl J Med 355(12):1199–1209
204. Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H, Yu J, Corti R, Mathey DG, Hamm CW, Suselbeck T, Assmus B, Tonn T, Dimmeler S, Zeiher AM (2006) REPAIR-AMI Investigators.: intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 355(12):1210–1221
205. Assmus B, Rolf A, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Tillmanns H, Yu J, Corti R, Mathey DG, Hamm CW, Suselbeck T, Tonn T, Dimmeler S, Dill T, Zeiher AM, Schachinger V (2010) REPAIR-AMI Investigators.: Clinical outcome 2 years after intracoronary administration of bone marrow-derived progenitor cells in acute myocardial infarction. Circ Heart Fail 3(1):89–96
206. Meluzín J, Mayer J, Groch L, Janousek S, Hornácek I, Hlinomaz O, Kala P, Panovský R, Prásek J, Kamínek M, Stanícek J, Klabusay M, Korístek Z, Navrátil M, Dusek L, Vinklárková J (2006) Autologous transplantation of mononuclear bone marrow cells in patients with acute myocardial infarction: the effect of the dose of transplanted cells on myocardial function. Am Heart J. 152(5):975.e9-15.
207. Meluzín J, Janousek S, Mayer J, Groch L, Hornácek I, Hlinomaz O, Kala P, Panovský R, Prásek J, Kamínek M, Stanícek J, Klabusay M, Korístek Z, Navrátil M, Dusek L,

Vinklárková J (2008) Three-, 6-, and 12-month results of autologous transplantation of mononuclear bone marrow cells in patients with acute myocardial infarction. Int J Cardiol. 128(2):185–92
208. de la Fuente LM, Stertzer SH, Argentieri J, Penaloza E, Miano J, Koziner B, Bilos C, Altman PA (2007) Transendocardial autologous bone marrow in chronic myocardial infarction using a helical needle catheter: 1-year follow-up in an open-label, nonrandomized, single-center pilot study (the TABMMI study). Am Heart J 154(1):79.e1-e7
209. Tse HF, Thambar S, Kwong YL, Rowlings P, Bellamy G, McCrohon J, Thomas P, Bastian B, Chan JK, Lo G, Ho CL, Chan WS, Kwong RY, Parker A, Hauser TH, Chan J, Fong DY, Lau CP (2007) Prospective randomized trial of direct endomyocardial implantation of bone marrow cells for treatment of severe coronary artery diseases (PROTECT-CAD trial). Eur Heart J 28(24):2998–3005
210. Stamm C, Kleine HD, Choi YH, Dunkelmann S, Lauffs JA, Lorenzen B, David A, Liebold A, Nienaber C, Zurakowski D, Freund M, Steinhoff G (2007) Intramyocardial delivery of CD133+ bone marrow cells and coronary artery bypass grafting for chronic ischemic heart disease: safety and efficacy studies. J Thorac Cardiovasc Surg 133(3):717–725
211. Zhao Q, Sun Y, Xia L, Chen A, Wang Z (2008) Randomized study of mononuclear bone marrow cell transplantation in patients with coronary surgery. Ann Thorac Surg 86(6):1833–1840
212. Ang KL, Chin D, Leyva F, Foley P, Kubal C, Chalil S, Srinivasan L, Bernhardt L, Stevens S, Shenje LT, Galinanes M (2008) Randomized, controlled trial of intramuscular or intracoronary injection of autologous bone marrow cells into scarred myocardium during CABG versus CABG alone. Nat Clin Pract Cardiovasc Med 5(10):663–670
213. Huikuri HV, Kervinen K, Niemela M, Ylitalo K, Saily M, Koistinen P, Savolainen ER, Ukkonen H, Pietila M, Airaksinen JK, Knuuti J, Makikallio TH (2008) FINCELL Investigators.: effects of intracoronary injection of mononuclear bone marrow cells on left ventricular function, arrhythmia risk profile, and restenosis after thrombolytic therapy of acute myocardial infarction. Eur Heart J 29(22):2723–2732
214. Akar AR, Durdu S, Arat M, Kilickap M, Kucuk NO, Arslan O, Kuzu I, Ozyurda U (2009) Five-year follow-up after transepicardial implantation of autologous bone marrow mononuclear cells to ungraftable coronary territories for patients with ischaemic cardiomyopathy. Eur J Cardiothorac Surg 36(4):633–643
215. van Ramshorst J, Bax JJ, Beeres SL, Dibbets-Schneider P, Roes SD, Stokkel MP, de Roos A, Fibbe WE, Zwaginga JJ, Boersma E, Schalij MJ, Atsma DE (2009) Intramyocardial bone marrow cell injection for chronic myocardial ischemia: a randomized controlled trial. JAMA. 301(19):1997–2004
216. Herbots L, D'hooge J, Eroglu E, Thijs D, Ganame J, Claus P, Dubois C, Theunissen K, Bogaert J, Dens J, Kalantzi M, Dymarkowski S, Bijnens B, Belmans A, Boogaerts M, Sutherland G, Van de Werf F, Rademakers F, Janssens S (2009) Improved regional function after autologous bone marrow-derived stem cell transfer in patients with acute myocardial infarction: a randomized, doubleblind strain rate imaging study. Eur. Heart J. 30 (6):582 662–670.
217. Beitnes JO, Hopp E, Lunde K, Solheim S, Arnesen H, Brinchmann JE, Forfang K, Aakhus S (2009) Long-term results after intracoronary injection of autologous mononuclear bone marrow cells in acute myocardial infarction: the ASTAMI randomised, controlled study. Heart 95(24):1983–1989
218. Plewka M, Krzemińska-Pakuła M, Lipiec P, Peruga JZ, Jezewski T, Kidawa M, Wierzbowska-Drabik K, Korycka A, Robak T, Kasprzak JD (2009) Effect of intracoronary injection of mononuclear bone marrow stem cells on left ventricular function in patients with acute myocardial infarction. Am J Cardiol 104(10):1336–1342
219. Tendera M (2009) Familial idiopathic dilated cardiomyopathy - search for identity. Kardiol Pol 67(3):242

220. Strauer BE, Yousef M, Schannwell CM (2010b) The acute and long-term effects of intracoronary Stem cell Transplantation in 191 patients with chronic heARt failure: the STAR-heart study. Eur J Heart Fail. 12(7):721–9
221. Traverse JH, Henry TD, Ellis SG, Pepine CJ, Willerson JT, Zhao DX, Forder JR, Byrne BJ, Hatzopoulos AK, Penn MS, Perin EC, Baran KW, Chambers J, Lambert C, Raveendran G, Simon DI, Vaughan DE, Simpson LM, Gee AP, Taylor DA, Cogle CR, Thomas JD, Silva GV, Jorgenson BC, Olson RE, Bowman S, Francescon J, Geither C, Handberg E, Smith DX, Baraniuk S, Piller LB, Loghin C, Aguilar D, Richman S, Zierold C, Bettencourt J, Sayre SL, Vojvodic RW, Skarlatos SI, Gordon DJ, Ebert RF, Kwak M, Moyé LA, Simari RD, ResearchNetwork Cardiovascular Cell Therapy (2011) Intracoronary infusion of mononuclear cells from bone marrow or peripheral blood compared with standard therapy in patients after acute myocardial infarction treated by primary percutaneous coronary intervention: results of the randomized controlled HEBE trial. Eur Heart J 32(14):1736–1747
222. Hirsch A, Nijveldt R, van der Vleuten PA, Tijssen JG, van der Giessen WJ, Tio RA, Waltenberger J, Ten Berg JM, Doevendans PA, Aengevaeren WR, Zwaginga JJ, Biemond BJ, van Rossum AC, Piek JJ, Zijlstra F (2011) On behalf of the HEBE investigators.: intracoronary infusion of mononuclear cells from bone marrow or peripheral blood compared with standard therapy in patients after acute myocardial infarction treated by primary percutaneous coronary intervention: results of the randomized controlled HEBE trial. Eur Heart J 32(14):1736–1747
223. Chen SL, Fang WW, Qian J, Ye F, Liu YH, Shan SJ, Zhang JJ, Lin S, Liao LM, Zhao RC (2004) Improvement of cardiac function after transplantation of autologous bone marrow mesenchymal stem cells in patients with acute myocardial infarction. Chin Med J 117(10):1443–1448
224. Hare JM, Traverse JH, Henry TD, Dib N, Strumpf RK, Schulman SP, Gerstenblith G, DeMaria AN, Denktas AE, Gammon RS, Hermiller JB, Reisman MA, Schaer GL, Sherman W (2009) A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J Am Coll Cardiol 54(24):2277–2286
225. Dib N, Henry T, DeMaria A, Itescu S, McCarthy MM, Jaggar SC, Taylor N, Campbell A, Krum H, Bartels KA, Skerrett D, Perin E (2009) The first US study to assess the feasibility and safety of endocardial delivery of allogenic mesenchymal precursor cells in patient with heart failure: Three-month interim analysis. Circulation 120:S810
226. Viswanathan C, Davidson Y, Cooper K, Tipnis S, Pujari G, Kurian VM (2010) Tansplantation of autologous bone marrow derived mesenchymal stem cells transepicardially in patients undergoing coronary bypass surgery. Indian Heart J 62(1):43–48
227. Leistner DM, Fischer-Rasokat U, Honold J, Seeger FH, Schachinger V, Lehmann R, Martin H, Burck I, Urbich C, Dimmeler S, Zeiher AM, Assmus B (2011) Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI): final 5 year results suggest long-term safety and efficacy. Clin Res Cardiol 100(10):925–934
228. Martin-Rendon E, Brunskill SJ, Hyde CJ, Stanworth SJ, Mathur A, Watt SM (2008) Autologous bone marrow stem cells to treat acute myocardial infarction: a systematic review. Eur Heart J 29(15):1807–1818
229. Zhang C, Sun A, Zhang S, Yao K, Wu C, Fu M, Wang K, Zou Y, Ge J (2010) Efficacy and safety of intracoronary autologous bone marrow-derived cell transplantation in patients with acute myocardial infarction: insights from randomized controlled trials with 12 or more months follow-up. Clin Cardiol 33(6):353–360
230. Haider HK, Ashraf M (2008) Strategies to promote donor cell survival: combining preconditioning approach with stem cell transplantation. J Mol Cell Cardiol 45(4):554–566
231. Suuronen EJ, Kuraitis D, Ruel M (2008) Improving cell engraftment with tissue engineering. Semin Thorac Cardiovasc Surg 20(2):110–114
232. Chiarugi P, Giannoni E (2008) Anoikis: a necessary death program for anchorage-dependent cells. Biochem Pharmacol 76(11):1352–1364

233. Mazo M, Pelacho B, Prosper F (2010) Stem cell therapy for chronic myocardial infarction. J Cardiovasc Transl Res 3(2):79–88
234. Menasche P (2009) Stem cell therapy for heart failure: are arrhythmias a real safety concern? Circulation 119(20):2735–2740
235. Huang NF, Li S (2011) Regulation of the matrix microenvironment for stem cell engineering and regenerative medicine. Ann Biomed Eng 39(4):1201–1214
236. Bouten CV, Dankers PY, Driessen-Mol A, Pedron S, Brizard AM, Baaijens FP (2011) Substrates for cardiovascular tissue engineering. Adv Drug Deliv Rev 63(4–5):221–241
237. O'Shaughnessy L (1937) Surgical treatment of cardiac ischemia. The Lancet 229(5917):185–194
238. Wall ST, Walker JC, Healy KE, Ratcliffe MB, Guccione JM (2006) Theoretical impact of the injection of material into the myocardium: a finite element model simulation. Circulation 114(24):2627–2635
239. Cortes-Morichetti M, Frati G, Schussler O, Van Duong JPH, Lauret E, Genovese JA, Carpentier AF, Chachques JC (2007) Association between a cell-seeded collagen matrix and cellular cardiomyoplasty for myocardial support and regeneration. Tissue Eng 13(11):2681–2687
240. Ryu JH, Kim IK, Cho SW, Cho MC, Hwang KK, Piao H, Piao S, Lim SH, Hong YS, Choi CY, Yoo KJ, Kim BS (2005) Implantation of bone marrow mononuclear cells using injectable fibrin matrix enhances neovascularization in infarcted myocardium. Biomaterials 26(3):319–326
241. Landa N, Miller L, Feinberg MS, Holbova R, Shachar M, Freeman I, Cohen S, Leor J (2008) Effect of injectable alginate implant on cardiac remodeling and function after recent and old infarcts in rat. Circulation 117(11):1388–1396
242. Leor J, Tuvia S, Guetta V, Manczur F, Castel D, Willenz U, Petnehazy O, Landa N, Feinberg MS, Konen E, Goitein O, Tsur-Gang O, Shaul M, Klapper L, Cohen S (2009) Intracoronary injection of in situ forming alginate hydrogel reverses left ventricular remodeling after myocardial infarction in swine. J Am Coll Cardiol 54(11):1014–1023
243. Ruvinov E, Leor J, Cohen S (2011) The promotion of myocardial repair by the sequential delivery of IGF-1 and HGF from an injectable alginate biomaterial in a model of acute myocardial infarction. Biomaterials 32(2):565–578
244. Yu J, Gu Y, Du KT, Mihardja S, Sievers RE, Lee RJ (2009) The effect of injected RGD modified alginate on angiogenesis and left ventricular function in a chronic rat infarct model. Biomaterials 30(5):751–756
245. Davis ME, Motion JP, Narmoneva DA, Takahashi T, Hakuno D, Kamm RD, Zhang S, Lee RT (2005) Injectable self-assembling peptide nanofibers create intramyocardial microenvironments for endothelial cells. Circulation 111(4):442–450
246. Davis ME, Hsieh PC, Grodzinsky AJ, Lee RT (2005) Custom design of the cardiac microenvironment with biomaterials. Circ Res 97(1):8–15
247. Hsieh PC, Davis ME, Gannon J, MacGillivray C, Lee RT (2006) Controlled delivery of PDGF-BB for myocardial protection using injectable self-assembling peptide nanofibers. J Clin Invest 116(1):237–248
248. Padin-Iruegas ME, Misao Y, Davis ME, Segers VF, Esposito G, Tokunou T, Urbanek K, Hosoda T, Rota M, Anversa P, Leri A, Lee RT, Kajstura J (2009) Cardiac progenitor cells and biotinylated insulin-like growth factor-1 nanofibers improve endogenous and exogenous myocardial regeneration after infarction. Circulation 120(10):876–887
249. Lin YD, Yeh ML, Yang YJ, Tsai DC, Chu TY, Shih YY, Chang MY, Liu YW, Tang AC, Chen TY, Luo CY, Chang KC, Chen JH, Wu HL, Hung TK, Hsieh PC (2010) Intramyocardial peptide nanofiber injection improves postinfarction ventricular remodeling and efficacy of bone marrow cell therapy in pigs. Circulation 122(11 Suppl):S132–S141
250. Hernandez RM, Orive G, Murua A, Pedraz JL (2010) Microcapsules and microcarriers for in situ cell delivery. Adv Drug Deliv Rev 62(7–8):711–730

251. Yu J, Du KT, Fang Q, Gu Y, Mihardja SS, Sievers RE, Wu JC, Lee RJ (2010) The use of human mesenchymal stem cells encapsulated in RGD modified alginate microspheres in the repair of myocardial infarction in the rat. Biomaterials 31(27):7012–7020
252. Lim GJ, Zare S, Van Dyke M, Atala A (2010) Cell microencapsulation. Adv Exp Med Biol 670:126–136
253. Liu ZC, Chang TM (2010) Artificial cell microencapsulated stem cells in regenerative medicine, tissue engineering and cell therapy. Adv Exp Med Biol 670:68–79
254. Murua A, Portero A, Orive G, Hernandez RM, de Castro M, Pedraz JL (2008) Cell microencapsulation technology: towards clinical application. J Control Release 132(2):76–83
255. Santos E, Zarate J, Orive G, Hernandez RM, Pedraz JL (2010) Biomaterials in cell microencapsulation. Adv Exp Med Biol 670:5–21
256. Christman KL, Lee RJ (2006) Biomaterials for the treatment of myocardial infarction. J Am Coll Cardiol 48(5):907–913
257. Leoni L, Desai TA (2004) Micromachined biocapsules for cell-based sensing and delivery. Adv Drug Deliv Rev 56(2):211–229
258. Leor J, Aboulafia-Etzion S, Dar A, Shapiro L, Barbash IM, Battler A, Granot Y, Cohen S (2000) Bioengineered cardiac grafts: a new approach to repair the infarcted myocardium? Circulation 102(19(Suppl 3)):III56–III61
259. Piao H, Kwon JS, Piao S, Sohn JH, Lee YS, Bae JW, Hwang KK, Kim DW, Jeon O, Kim BS, Park YB, Cho MC (2007) Effects of cardiac patches engineered with bone marrow-derived mononuclear cells and PGCL scaffolds in a rat myocardial infarction model. Biomaterials 28(4):641–649
260. Jin J, Jeong SI, Shin YM, Lim KS, Shin H, Lee YM, Koh HC, Kim KS (2009) Transplantation of mesenchymal stem cells within a poly(lactide-co-epsilon-caprolactone) scaffold improves cardiac function in a rat myocardial infarction model. Eur J Heart Fail 11(2):147–153
261. Fitzpatrick JR, Frederick JR, McCormick RC, Harris DA, Kim AY, Muenzer JR, Gambogi AJ, Liu JP, Paulson EC, Woo YJ (2010) Tissue-engineered pro-angiogenic fibroblast scaffold improves myocardial perfusion and function and limits ventricular remodeling after infarction. J Thorac Cardiovasc Surg 140(3):667–676
262. Caspi O, Lesman A, Basevitch Y, Gepstein A, Arbel G, Habib IH, Gepstein L, Levenberg S (2007) Tissue engineering of vascularized cardiac muscle from human embryonic stem cells. Circ Res 100(2):263–272
263. Xiang Z, Liao R, Kelly MS, Spector M (2006) Collagen-GAG scaffolds grafted onto myocardial infarcts in a rat model: a delivery vehicle for mesenchymal stem cells. Tissue Eng 12(9):2467–2478
264. Frederick JR, Fitzpatrick JR, McCormick RC, Harris DA, Kim AY, Muenzer JR, Marotta N, Smith MJ, Cohen JE, Hiesinger W, Atluri P, Woo YJ (2010) Stromal cell-derived factor-1alpha activation of tissue-engineered endothelial progenitor cell matrix enhances ventricular function after myocardial infarction by inducing neovasculogenesis. Circulation 122(11 Suppl):S107–S117
265. Zimmermann WH, Fink C, Kralisch D, Remmers U, Weil J, Eschenhagen T (2000) Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes. Biotechnol Bioeng 68(1):106–114
266. Zimmermann WH, Didie M, Wasmeier GH, Nixdorff U, Hess A, Melnychenko I, Boy O, Neuhuber WL, Weyand M, Eschenhagen T (2002) Cardiac grafting of engineered heart tissue in syngenic rats. Circulation 106(12 Suppl 1):I151–I157
267. Naito H, Melnychenko I, Didie M, Schneiderbanger K, Schubert P, Rosenkranz S, Eschenhagen T, Zimmermann WH (2006) Optimizing engineered heart tissue for therapeutic applications as surrogate heart muscle. Circulation 114(1 Suppl):I72–I78
268. Zimmermann WH, Melnychenko I, Wasmeier G, Didie M, Naito H, Nixdorff U, Hess A, Budinsky L, Brune K, Michaelis B, Dhein S, Schwoerer A, Ehmke H, Eschenhagen T

(2006) Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. Nat Med 12(4):452–458
269. Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M, Okano T (2002) Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ Res 90(3):e40
270. Tsuda Y, Shimizu T, Yamato M, Kikuchi A, Sasagawa T, Sekiya S, Kobayashi J, Chen G, Okano T (2007) Cellular control of tissue architectures using a three-dimensional tissue fabrication technique. Biomaterials 28(33):4939–4946
271. Furuta A, Miyoshi S, Itabashi Y, Shimizu T, Kira S, Hayakawa K, Nishiyama N, Tanimoto K, Hagiwara Y, Satoh T, Fukuda K, Okano T, Ogawa S (2006) Pulsatile cardiac tissue grafts using a novel three-dimensional cell sheet manipulation technique functionally integrates with the host heart, in vivo. Circ Res 98(5):705–712
272. Sekine H, Shimizu T, Hobo K, Sekiya S, Yang J, Yamato M, Kurosawa H, Kobayashi E, Okano T (2008) Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts. Circulation 118(14 Suppl):S145–S152
273. Bel A, Planat-Bernard V, Saito A, Bonnevie L, Bellamy V, Sabbah L, Bellabas L, Brinon B, Vanneaux V, Pradeau P, Peyrard S, Larghero J, Pouly J, Binder P, Garcia S, Shimizu T, Sawa Y, Okano T, Bruneval P, Desnos M, Hagege AA, Casteilla L, Puceat M, Menasche P (2010) Composite cell sheets: a further step toward safe and effective myocardial regeneration by cardiac progenitors derived from embryonic stem cells. Circulation 122(11 Suppl):S118–S123
274. Miyagawa S, Sawa Y, Sakakida S, Taketani S, Kondoh H, Memon IA, Imanishi Y, Shimizu T, Okano T, Matsuda H (2005) Tissue cardiomyoplasty using bioengineered contractile cardiomyocyte sheets to repair damaged myocardium: their integration with recipient myocardium. Transplantation 80(11):1586–1595
275. Gilbert TW, Sellaro TL, Badylak SF (2006) Decellularization of tissues and organs. Biomaterials 27(19):3675–3683
276. Tan MY, Zhi W, Wei RQ, Huang YC, Zhou KP, Tan B, Deng L, Luo JC, Li XQ, Xie HQ, Yang ZM (2009) Repair of infarcted myocardium using mesenchymal stem cell seeded small intestinal submucosa in rabbits. Biomaterials 30(19):3234–3240
277. Valentin JE, Turner NJ, Gilbert TW, Badylak SF (2010) Functional skeletal muscle formation with a biologic scaffold. Biomaterials 31(29):7475–7484
278. Tottey S, Johnson SA, Crapo PM, Reing JE, Zhang L, Jiang H, Medberry CJ, Reines B, Badylak SF (2011) The effect of source animal age upon extracellular matrix scaffold properties. Biomaterials 32(1):128–136
279. Brown BN, Freund JM, Han L, Rubin JP, Reing JE, Jeffries EM, Wolf MT, Tottey S, Barnes CA, Ratner BD, Badylak SF (2011) Comparison of three methods for the derivation of a biologic scaffold composed of adipose tissue extracellular matrix. Tissue Eng Part C Methods 17(4):411–421
280. Chen CH, Wei HJ, Lin WW, Chiu I, Hwang SM, Wang CC, Lee WY, Chang Y, Sung HW (2008) Porous tissue grafts sandwiched with multilayered mesenchymal stromal cell sheets induce tissue regeneration for cardiac repair. Cardiovasc Res 80(1):88–95
281. Wei HJ, Chen CH, Lee WY, Chiu I, Hwang SM, Lin WW, Huang CC, Yeh YC, Chang Y, Sung HW (2008) Bioengineered cardiac patch constructed from multilayered mesenchymal stem cells for myocardial repair. Biomaterials 29(26):3547–3556
282. Singelyn JM, DeQuach JA, Seif-Naraghi SB, Littlefield RB, Schup-Magoffin PJ, Christman KL (2009) Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering. Biomaterials 30(29):5409–5416
283. Ott HC, Matthiesen TS, Goh SK, Black LD, Kren SM, Netoff TI, Taylor DA (2008) Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med 14(2):213–221

284. Ott HC, Clippinger B, Conrad C, Schuetz C, Pomerantseva I, Ikonomou L, Kotton D, Vacanti JP (2010) Regeneration and orthotopic transplantation of a bioartificial lung. Nat Med 16(8):927–933
285. Uygun BE, Soto-Gutierrez A, Yagi H, Izamis ML, Guzzardi MA, Shulman C, Milwid J, Kobayashi N, Tilles A, Berthiaume F, Hertl M, Nahmias Y, Yarmush ML, Uygun K (2010) Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nat Med 16(7):814–820

Chapter 6
Development of Biological Approaches to Improve Muscle Healing After Injury and Disease

Jonathan D. Proto and Johnny Huard

Abstract Unique among adult tissues, skeletal muscle possesses a robust regeneration capacity dependent upon satellite cells (SC), the professional muscle stem cell. SCs are normally quiescent in a niche located within the basal lamina but outside the muscle membrane, or sarcolemma. Activated SCs proliferate and migrate to the site of injury. Through asymmetric division, a portion of SCs return to quiescence while daughter cells committed to the myogenic lineage, termed myoblasts, fuse and eventually form new muscle fibers. Yet, despite this potential, severely damaged muscle requires medical intervention. In addition to injuries resulting from blunt trauma, work, or sports-related accidents, there are a number of muscle diseases, both inherited, and acquired. At this time, there are no pharmaceutical drugs that can be used to effectively accelerate muscle healing following injury. Here, we discuss new biological approaches to improve muscle healing such as growth factor delivery, myoblast transfer therapy, stem cell-based therapy, gene therapy, and combining biological therapeutics.

6.1 Introduction

6.1.1 Musculoskeletal Injury and Disease

In the United States alone, approximately 30 % of the population suffers from some form of musculoskeletal condition. This percentage increases to 69 % when focusing on those 75 years old or older [1]. Conditions may range from injuries,

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such as bone fractures, to congenital diseases, such as muscular dystrophy and osteogenesis imperfecta, to age-related conditions, such as arthritis, osteoporosis, or sarcopenia. Within the body are three types of muscle tissue: skeletal, cardiac, and smooth. In this chapter, for our discussion of regenerative medicine for musculoskeletal disorders, we will focus specifically on skeletal muscle injury and disease. Compared to other tissues, evolution has endowed skeletal muscle with a high capacity for repair. Post-natal skeletal muscle regeneration occurs through the activation of quiescent satellite cells (SC), the professional muscle stem cell [2]. Therefore, muscle healing serves as an excellent example of stem cell-mediated tissue regeneration.

Upon activation by injury, SC re-enter the cell cycle, giving rise to a population of myoblasts, committed muscle precursors. A portion of SCs, or "reserve cells," return to a quiescent state, maintaining the SC pool [3]. Yet, despite this robust regeneration potential, severely damaged muscle requires medical intervention. In addition to injuries resulting from blunt trauma, work, or sports-related accidents, there are a number of muscle diseases, both inherited, and acquired. While modern medicine offers many therapeutic options for treating muscle disorders, many of these are inefficient, ineffectively repairing damaged tissue. Such injuries may result in atrophy and degeneration, the long-term consequences of which can greatly affect an individual's quality of life [1].

Muscle injuries can be induced by strain during sports or work activities, aging, or exposure to toxic substances. Among athletes, about 35–45 % of all sports-related injuries involve muscle damage [4]. Often overlooked, work-related musculoskeletal disorders (WMSDs) are a type of recurrent muscle strain that often leads to fibrotic scar formation, thereby predisposing muscle to future injury [5]. These types of injuries have been studied in groups as diverse as house cleaners to forest loggers, highlighting the broad range of those affected by WMSDs [6]. Another type of induced injury, muscle atrophy, or muscle wasting, can result from denervation, immobilization, or even malnutrition. Typically, in cases where atrophy is due to immobilization, adult muscle is able to recover to its former size. It is interesting to note, however, that if atrophy occurs during adolescence, satellite cell number appears to be irreversibly reduced, decreasing future regenerative capacity [7]. Additionally, stem cell functional decline is observed during aging, where injury results in extensive fibrosis rather than functional regeneration [8]. Such an observation underlines the importance of stem and progenitor cells in tissue repair.

There are also a number of inherited skeletal muscle diseases, or myopathies. The rarest types of congenital myopathies may be caused by mutation in genes regulating ion channels, metabolism, and mitochondrial function. The largest group of congenital myopathies, muscular dystrophy, contains about 40 different genetic disorders characterized by ongoing fiber degeneration resulting in functional decline and wasting [7]. Two important X-linked dystrophies, Duchenne, and Becker muscular dystrophy (DMD and BMD, respectively) result from mutations in the dystrophin gene, whose product is an important cytoskeletal element for muscle membrane integrity. The dystrophin gene is quite large,

accounting for approximately 1 % of the X chromosome. Due to its large size (2.4 Mb), it is particularly prone to deletions and point mutations. Dystrophin serves as a structural connection between the actin cytoskeleton and the extracellular matrix, allowing for the transmission of external forces into the cell. Dystrophin deficiency results in loss of membrane integrity, and subsequent degeneration and necrosis [9].

DMD, the most severe dystrophinopathy, is fatal, affecting 1 in 3,500 male births. At the time of birth, there are no overt signs of pathology, but once the infant reaches the walking stage, he may be perceived as clumsy. Excessive degeneration results in kyphosis and scoliosis of the spine, and by early adolescence, approximately 95 % of patients are confined to a wheelchair. By the age of 20, about 90 % die due to respiratory or cardiovascular failure. Whereas there is no functional dystrophin expressed in DMD patients, with the exception of few revertant fibers, expression of a truncated function form of dystrophin results in BMD. Many BMD patients enjoy a normal lifespan, but in some of the more severe forms, patients may die as young as 40 [7].

There are also autosomal muscular dystrophies, such as limb girdle muscular dystrophy (LGMD). This condition may be caused by mutations in various components of the sarcoglycan complex, which again, maintains membrane integrity. LGMD principally affects proximal muscles of the trunk and limbs, whereby it derives its name. Although there is a lot of variability, many of the LGMD subtypes are not severe in phenotype, and may not arise until later in life [10].

6.1.2 Current Treatments

At this time, there are no pharmaceutical drugs that can be used to effectively accelerate muscle healing following injury. Nonsteroidal anti-inflammatory drugs (NSAIDs) are often prescribed to ease the pain and inflammation associated with injury. NSAIDs relieve these symptoms temporarily through the inhibition of cyclooxygenase 1 and 2 (COX-1,2), reducing the synthesis of prostaglandins, principal participants in inflammation. However, studies show that blocking inflammation does in fact delay wound healing [11]. We have previously also reported that treatment with NS-398, a specific inhibitor of COX-2, immediately following injury delays regeneration and increases fibrotic scarring [12]. Inflammation plays a critical role not only in the clearance of cellular debris, but also in SC activation. Thus, blocking inflammation early in wound repair could in turn delay SC activation, and therefore delay recovery from injury. Indeed, we found that NS-398 treatment decreased myogenic progenitor cell proliferation and differentiation. In agreement with this hypothesis, a study by Mackey and colleagues found that indomethacin, an often prescribed NSAID, decreases exercise-induced SC proliferation [13]. Therefore, while NSAIDs relieve the pain associated with injury, they are likely detrimental to the overall regeneration process.

Similarly, there is no FDA approved treatment for chronic muscle wasting diseases, such as muscular dystrophy. Rather, current clinical therapies focus on disease management to improve patient quality of life. Off label prescription of corticosteroids, such as prednisone and deflazacort, is the current gold standard for DMD and BMD [9]. Corticosteroids have had a tremendous impact on patient quality of life, delaying wheelchair use by up to 5 years. At the same time, use of corticosteroids for more than 2 years, the length of time necessary for clinical benefit, results in numerous side effects, including growth retardation and eye cataract development [14]. Some LGMD patients benefit from a similar treatment, but the majority of these patients receive benefit only from physical therapy [10].

6.2 The Biology of Muscle Regeneration

The muscle repair process may be divided into four phases: degeneration, inflammation, regeneration, and remodeling/fibrosis. Although the early events of muscle injury vary, typically the injury is initiated by damage to cytoskeletal elements, leading to loss of membrane integrity. Once the membrane has been compromised, calcium (Ca^{2+}) homeostasis is disrupted, leading to its intracellular accumulation. Subsequently, Ca^{2+} influx results in protease activation, eventually leading to necrosis of the myofiber [7].

This leads into the second stage of repair, inflammation, in which mononuclear cells, first neutrophils, and followed by macrophages, invade the tissue to clear necrotic debris, and activate SCs. Neutrophils accumulate rapidly, often within an hour of injury. Although the exact identity of the chemoattractant molecule responsible for neutrophil accumulation is unknown, both muscle and endothelial cells are capable of producing inflammatory cytokines such as interleukin-1 beta (IL-1), IL-6, monocyte chemoattractant protein 1 (MCP-1), and tumor necrosis factor alpha (TNFα) [15]. These factors also act as vasodilators, stimulating edema, and monocyte accumulation. Macrophages, derived from monocytes, have the primary roles of phagocytosis of cellular debris, and the release of stimulatory cytokines, which activate SC, and support myogenic differentiation [16].

SCs are normally quiescent in a niche located within the basal lamina but outside the muscle membrane, or sarcolemma. Activated SCs proliferate and migrate to the site of injury (Fig. 6.1). Through asymmetric division, thought to be mediated via Notch-1 signaling [17], a portion of SC return to quiescence, while daughter cells committed to the myogenic lineage, termed myoblasts, fuse, and eventually form new muscle fibers [17]. Maintenance of the SC pool is largely dependent on the transcription factor paired box 7 (Pax7), which is expressed solely in muscle SCs. In the mouse, there is no single membrane marker to identify SCs, so a combination of two or more can be used for isolation by fluorescence activated cell sorting (FACS), including CD34, integrin-α7, and m-cadherin, among others [18]. In humans, there is not a reliable set of markers to isolate SCs, although in practice, CD56 is used to isolate muscle progenitor cells [19].

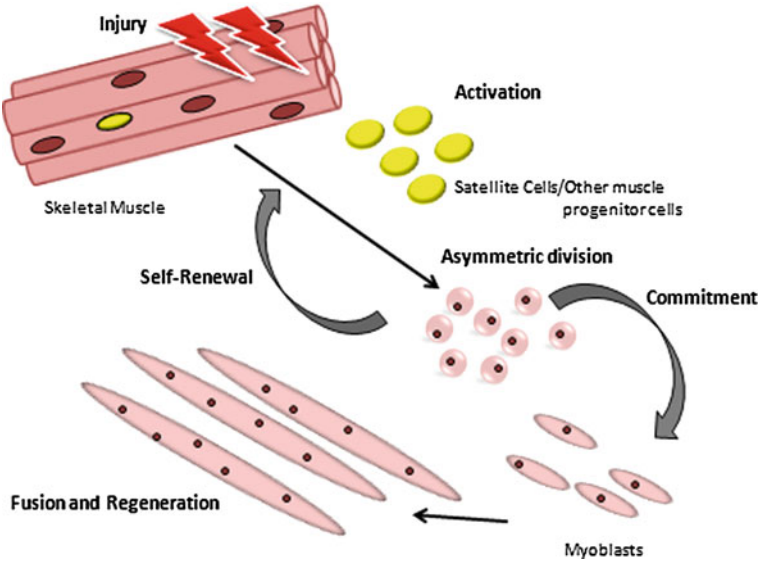

Fig.6.1 Skeletal muscle is regenerated through the activation of muscle stem cells. Disruption of skeletal muscle homeostasis by injury results in satellite cell activation. During population expansion, muscle stem cells undergo asymmetric division to produce myoblasts, committed muscle precursors, while a portion retains "stemness" in order to repopulate the niche. Following a proliferative phase, myoblasts eventually fuse to produce new muscle fibers

The regenerative phase begins approximately 1 week post-injury. Downstream of Pax7, myogenic lineage progression is controlled by members of the basic helix-loop-helix transcription factor family, including MyoD and MyF5. During this time, Pax7+MyoD- SC return to the niche, while Pax7+MyoD+ myoblasts persist (Fig. 6.1) [20]. Eventually myoblasts fuse to form new myofibers, which can be identified histologically by central nucleation. Finally, the tissue is remodeled, the fibers begin to grow in size and the nuclei are repositioned at the edge of the fiber. Fibrotic scar tissue can develop during this time, which decreases the function of muscle and predisposes the tissue to future injury. Work from our group has found transforming growth factor beta-1 (TGFβ1) to be a key mediator of skeletal muscle fibrosis [12, 21]. TGFβ1 induces its further expression in myogenic cells, exacerbating matrix deposition, and the transition of myogenic cells to myofibroblasts [21]. During youth, muscle is likely to repair efficiently, but with aging comes a decrease in regeneration and an increase in fibrosis. Excessive fibrosis during aging has been found to be related to canonical Wnt signaling, and can be reversed in mice by treatment with Wnt inhibitors [22].

The identification of the molecular mechanisms regulating the stages of muscle repair has opened the door to specific targeting of key processes for therapeutic intervention. As we discussed earlier, there has yet to be an FDA approved drug for the acceleration of skeletal muscle regeneration. In the past two decades, there has been a large development of biological, rather than pharmaceutical,

approaches to muscle repair. In the next section, we will discuss growth factor, gene, and cell-based experimental approaches for the healing of muscle injuries.

6.3 Biological Approaches to Muscle Healing After Injury and Disease

6.3.1 Growth Factor Therapy

The timing and advancement of tissue regeneration depends upon communication among myogenic, endothelial, and inflammatory cells. Numerous studies over the years have identified a number of critical growth factors responsible for this. Some may be held in an inactive form in the extracellular matrix, and are not released until injury, such as hepatocyte growth factor (HGF), while others may be released from cells up stimulation or activation, such as TNFα [23]. Growth factors may function by stimulating cell migration, proliferation, differentiation, or death, and unlike hormones, which can act systemically, growth factors usually act in a local, paracrine manner [24]. Due to these facts, a growth factor delivery approach to tissue regeneration has been under investigation for several years. The basic hypothesis of growth factor therapy is that targeted release of specific therapeutic proteins will initiate or augment the natural healing process, accelerating regeneration and reducing fibrotic scaring.

Some of the first evidence of the therapeutic potential of growth factor delivery came from preclinical canine studies in which the angiogenic protein basic fibroblast growth factor (bFGF) was delivered to infarcted myocardium via intracoronary injection. Treated dogs showed improved systolic function associated with an increase in vessel formation, compared to controls [25]. Positive results from such studies led to clinical trials for the treatment of coronary artery disease. Trials proceeded through phase one, but halted at phase two due to a lack of efficacy, as measured by exercise tolerance [26]. Many believe the fatal flaw of these trials was the delivery method, as a protein's half life is very short. For example, in a phase 2 clinical trial investigating the efficacy of vascular endothelial growth factor (VEGF) delivery for coronary artery disease, investigators found that a VEGF bolus delivered intravenously has a half life of only approximately 30 min [27]. Due to such a short half life, even a supraphysiological dose is still likely to be depleted rapidly, and there may be potential side effects.

Researchers have had to develop innovative approaches to overcome this limitation, resulting in biomaterials-based delivery. Such strategies offer many advantages, most notably controlled release. In the most simple example, release from biodegradable polymers can easily be controlled by altering material properties such as pore size, cross-linking, and therefore degradation rate [24]. Additionally, the delivery of multiple factors is now being developed. As each factor involved with wound healing has its own temporospacial distribution, this

approach presents two important problems: (1) selection of the proper cocktail and (2) controlling the timing of individual factors within the same material(s).

Rather than selecting specific cocktails of growth factors, a recent approach has been the isolation and use of autologous platelet-rich plasma (PRP). PRP is easily collected by centrifugation, concentrating platelets from blood about 4–8x. Within platelets, α-granules contain a variety of factors, including platelet-derived growth factor (PDGF), VEGF, TGFβ1, epidermal growth factor (EGF), bFGF, and insulin-like growth factor 1 (IGF-1) [28]. Just prior to injection, the platelets are activated by supplementation with Ca^{2+} and thrombin [29]. Within 1 hour, approximately 95 % of α-granule derived proteins are released; however, the platelets themselves have been found to persist for up to a week, continuously releasing growth factors [30]. Clinical studies of the use of PRP for muscle injury are sparse. However, in one unpublished human study, Sanchez and colleagues treated 20 athletes for muscle tears by PRP injection. Reportedly, the patients recovered their full muscle function in about half the time as expected [31]. Much needs to be done to standardize the use of PRP, and to verify that the presence of certain growth factors, such as TGFβ1, does not have deleterious effects, such as the promotion of fibrosis.

When a specific combination of factors is chosen, the timing of the release of individual factors remains an important issue. Using an injectable and biodegradable alginate scaffold, Borselli and colleagues demonstrated that both IGF-1 and VEGF could be delivered in a controlled manner to regenerate skeletal muscle in a rat hind limb ischemia model. They chose VEGF for its angiogenic properties and IGF-1 for its multiple roles in muscle cell differentiation, including myoblast proliferation, survival, and differentiation [32]. Not only did the investigators rely on the physical properties of the scaffold to control release, but they took into account the biophysical properties of IGF-1 and VEGF. Due to its low molecular weight, 80 % of the IGF-1 was released within the first 24 h. VEGF, however, was released much more slowly, and in a sustained manner. Finally, they found that either VEGF or IGF alone increased muscle diameter by 10 % compared to control, but dual treatment increased it by 25 % [33].

6.3.2 Cell Therapy: Myoblast Transplant Therapy

As an alternative to growth factor delivery, cell therapy for muscle injury is a popular area of research for the treatment of chronic muscle diseases such as muscular dystrophy [34]. Due to the ease of myoblast identification and isolation, several attempts at allogeneic myoblast transplants, also known as myoblast transfer therapy (MTT), have been made in the last two decades. Unfortunately, limited success has been recorded. Several studies have reported modest restoration of dystrophin expression and approximately 15 % of the patients have experienced an increase in muscle strength [3]. Despite high hopes, it has become apparent that MTT targeted to one muscle cannot restore “enough” dystrophin to

improve the overall DMD pathology at this point in time. Due to their inability to extravasate from circulation, myoblasts cannot be delivered systemically [35]. One additional obstacle standing even in the way of local cellular therapies for skeletal muscle is the inefficiency of cell transfer, resulting from poor donor cell survival [36]. Despite a regimen of cyclosporine A, which is necessary to control rejection, several reports have established that approximately 90–95 % of transplanted cells die within 3 days of transplant [37, 38]. Many researchers have suggested that inflammatory stress mediated by leukocytes of the innate immune system could account for the high percentage of myoblast death upon transplantation [35].

Several different techniques have been used to enhance the efficiency of myoblast engraftment. One group has attempted to utilize an innate cellular protective mechanism: heat shock protein (HSP) induction. HSPs can intervene directly in the apoptotic pathway by down regulating caspase-3 activity. It has been reported that maintaining cells at 42 °C for 60 min is enough to increase myoblast survival following exposure to TNF-α or glucose oxidase-induced oxidative stress by approximately 10 %. This modest increase in vitro translated to approximately a 4-fold increase in engraftment at 28 days post-injection [39]. Other groups have tried to use growth factors to improve donor cell survival. In one report, researchers transplanted myoblasts along with extended release HGF into infarcted rat myocardium. Co-administration of HGF and myoblasts was able to decrease donor cell apoptosis at 24 h, although longer time points were not examined [40]. This study was aimed at improving survival in cardiac muscle, but one could hypothesize that a similar effect would be produced in skeletal muscle. In yet another study, bFGF was co-administered with myoblasts transduced to express Factor IX, the protein deficient in hemophilia B. Muscle injected with both transduced myoblasts and bFGF demonstrated increased factor IX expression compared to myoblast injections alone, implying that survival or proliferation had been improved [41]. Further examples of growth factor utilization in MTT include myostatin inhibition, IL-1 receptor antagonist expression, and leukemia inhibitory factor co-administration [35]. While combination therapy of growth factor and cell transplants seems promising, such an approach may make it more difficult to separate cell-mediated from factor-mediated therapeutic effects. However, the previously mentioned studies indicate combination therapy was superior to cell or growth factor delivery alone.

6.3.3 Cell Therapy: Alternate Sources

Another way to improve the efficacy of cell therapies is to optimize selection of the donor cell source. For example, there is immense evidence suggesting that mesenchymal stem cells (MSCs) possess immunomodulatory properties. Using a hamster model of limb-girdle muscular dystrophy, one group demonstrated that intramuscular injection of human bone marrow MSCs significantly reduced IL-6 and CD45 expression, suggesting a reduction in inflammation, and leukocyte

infiltration. Furthermore, the MSCs were able to significantly improve muscle regeneration, compared to saline injected controls, by activating host progenitor cells as well as by fusing with preexisting fibers [42]. In vitro evidence suggests that MSCs may exert particularly useful effects on macrophage phenotypes. So-called "wound healing," or alternatively activated macrophages, typically classified as an M2 phenotype, exert many anti-inflammatory, and pro-wound healing effects. M2a macrophages, which are activated via IL-4 and IL-10, stimulate myoblast proliferation and protect myotubes from M1 mediated membrane lysis [43]. MSCs have been demonstrated to bias macrophages towards an M2a phenotype in vitro [44]. Therefore, use of MSCs may have the potential to decrease inflammation in the host and improve muscle regeneration through multiple mechanisms.

Some may argue that a more myogenic cell source than MSCs is required to regenerate significant amounts of normal (donor-derived) rather than dystrophic (host-derived) muscle, which is still susceptible to continued degeneration. Within skeletal muscle, multiple stem/progenitor cells have been isolated, in addition to SCs [3]. Our group has identified a highly myogenic cell population in murine muscle, termed muscle-derived stem cells (MDSCs), which exhibit a higher intramuscular engraftment capacity compared to myoblasts [45]. Isolated by a modified preplate technique, which separates cell populations based on their adhesion characteristics, MDSCs are slow to adhere and appear to represent a progenitor population less committed to the myogenic lineage, as they are able to undergo multi-lineage differentiation and long-term proliferation in vivo [46]. When donor cells are transplanted into an inflammatory environment, such as that found in DMD, free radicals released from activated leukocytes can induce oxidative stress in donor cells. Evidence from murine studies suggests that the relatively high level of MDSC engraftment may be due to a strong resistance to stress-induced apoptosis resulting from high levels of the cellular antioxidant glutathione and the enzyme superoxide dismutase [47]. MDSCs have also been found to aid in the repair of normal injured muscle. Using a skeletal muscle contusion injury model, MDSC transplantation accelerated healing by enhancing angiogenesis [48]. In addition to skeletal muscle, murine MDSCs have been found to aid in the repair of bone and cartilage defects, and improve heart function in murine models of acute myocardial infarction [49–51]. Slowly adhering cells isolated from human muscle have also been found to display similar skeletal and cardiac muscle regeneration properties [52, 53]. Furthermore, clinical trials of human slowly adhering cell-based therapies are currently underway for the treatment of urinary incontinence [54]. Additionally, Rouger and colleagues recently reported that systemic delivery of allogeneic wild type canine slowly adhering muscle-derived cells to dystrophic dogs significantly improves disease phenotype. Donor cells were found to not only contribute to muscle regeneration, but they replenished the SC niche, resulting in long-term dystrophin expression [55]. If such results are able to translate to humans, slowly adhering muscle-derived cells may be able to be delivered systemically, an option not possible for myoblasts, which cannot extravasate from circulation.

Our group has also identified myogenic endothelial cells within human skeletal muscle. Using FACS, this population is able to be identified by the coexpression of myogenic and endothelial cell surface markers (CD56, CD34, CD144). This unique endothelial population, so-called myoendothelial cells, may represent another human muscle-derived progenitor cell. Compared to myoblasts (CD56), they demonstrate a significantly higher survival rate under hydrogen peroxide-induced oxidative stress and display superior muscle regeneration in immunodeficient mice [56].

In addition to tissue-resident adult stem cells, two pluripotent cell types have been considered for cell therapies: embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells. However, due to the relatively new nature of iPS cell technology, autologous therapies may be far off. Much more likely in the near future is the use of iPS cells for patient-specific drug screening. In this scenario, reprogramming a somatic cell from a patient with a particular disease would provide disease-specific, and patient-specific, pluripotent cells. Such a pool of cells could then be derived into any tissue of interest and used as a tool for drug screening [57]. However, reprogramming is a highly inefficient process. Comparing the reprogramming efficiency of skeletal muscle progenitors and committed myoblasts, Tan and colleagues demonstrated the case of myogenic cells, the efficiency of reprogramming negatively correlates with the level of somatic cell differentiation [58]. Thus, in the future, the selection of stem and progenitor cells for iPS cell generation may greatly improve upon this process. If used for regenerative medicine, both iPS cells and ESCs would obviously need to be differentiated down the skeletal muscle lineage prior to transplant to prevent the formation of teratomas. Human ESCs have been differentiated into myoblasts able to regenerate muscle in vivo. However, the process is quite inefficient [59]. In addition to the barriers that exist for all cell types, such as immunogenicity, survival, and differentiation, pluripotent cells also have the additional risk of tumor formation upon transplant [19]. Therefore, specific and well developed protocols remain to be developed in order to produce fully committed cells from iPS cells and ESCs, and verify a lack of tumorogenicity.

6.3.4 Gene Therapy

When the dystrophin gene, whose product is missing in DMD, was identified in 1987, scientists thought that a genetic approach to treatment would provide a cure for DMD/BMD [60]. The delivery of a therapeutic gene to diseased tissue, the basis of gene therapy, has had a modest level of success in treating multiple musculoskeletal diseases. However, significant hurdles remain for gene therapy: off target delivery, immune rejection, and mutation [61]. The most efficient method of gene delivery is virus-mediated transfer. Essentially, the gene of interest is packaged into a viral vector stripped of the endogenous genes required for replication. In early proof-of-concept studies for gene transfer to muscle, viral

vectors were delivered by intramuscular injection. While intramuscular injection resulted in highly efficient gene transfer, this only occurred locally, in the injection area [62]. For a disease like DMD, the number of injections required to treat a patient would be staggering. This is one of the many hurdles facing gene therapy approaches for treating global muscle diseases. Therefore, a muscle-targeted systemic delivery approach is desirable.

Many viruses, including retroviruses, lentiviruses, adenoviruses, adeno-associated viruses (AAV) have all been tested in preclinical models of muscular dystrophy with limited success [61]. The largest hurdle to overcome has been the large size of the dystrophin gene, preventing packaging in most viral vectors. This has been partially overcome through the use of so-called mini-dystrophin, which lacks a large portion of the rod domain, yet is still functional [63]. However, just as the current treatments for muscular dystrophy are aimed at improving quality of life, gene therapy could take a similar approach. For example, AAV vector-mediated delivery of a dominant negative mutant of IkB Kinase β, a critical kinase for inflammatory macrophage activation, has been shown to increase muscle regeneration and decrease necrosis in locally treated skeletal muscle of aged *mdx* mice, a murine DMD model [64].

Recombinant AAV (rAAV) vector mediated gene transfer has shown promise in recent years. AAV can infect both dividing and nondividing cells, an attractive characteristic for gene delivery to post-mitotic muscle fibers. Additionally, unlike lentiviruses, AAV does not insert into the genome, and unlike adenoviruses, AAV has a low immunogenicity [62]. In the case of DMD the viruses should be targeted to the appropriate tissue, skeletal, and cardiac muscle. There are many different serotypes of AAV, each with a unique tropism, some with a natural tropism towards muscle [62]. Despite these positive attributes, problems remain for rAAV gene delivery. For example, AAV serotype 2 (AAV2) has been extensively investigated and is the only serotype approved for clinical trials in humans. However, a large percentage of the population has pre-existing, neutralizing antibodies to this serotype [65]. In order to overcome a humoral response, innovation has led to the creation of chimeric viral capsids, which have the dual benefit of targeting specific tissues and reducing immunogenicity [66].

6.3.5 Combining Biological Therapeutics

Given the pros and cons of the numerous biological strategies discussed thus far, it is reasonable to hypothesize that a combination of two or more approaches might give the most successful therapeutic results. By combining cell or gene therapy with biomaterial science, many groups have developed novel combinatorial regenerative therapies.

Recently, by altering the elasticity of growth surfaces, conditions designed to more closely resemble a stem cell niche have been used to expand muscle stem cells in vitro without loss of “potency” [67]. Extending this concept in vivo,

Carnio and colleagues used a 3-D collagen sponge to deliver wild type myogenic cells to *mdx* mouse muscle, with the idea that the scaffold would serve as a type of cellular reservoir. Compared to direct injection of cells, implantation of the seeded scaffold resulted in the generation of approximately three times more dystrophin positive (donor derived) fibers. Not only did they find donor cells more proliferative when implanted in a scaffold, but they found a significant increase in cell survival [68]. Whether or not the scaffold truly acted as a stem cell niche, the support it provided for cell delivery warrants further investigation for local cell transplants.

In addition to use as a cell delivery tool, scaffolds can be used to deliver therapeutic genes in a controlled release manner. Synthetic polymers or modified natural polymers can be used to deliver either virus particles or naked plasmid DNA. Generally, this can be done in one of two ways. First, virus particles can be encapsulated within the material, so that particles are released as the material degrades. Secondly, virus particles can be bound to the scaffold surface, a method termed solid-phase delivery [69]. For example, cyclic acetal monomers containing diacrylate end groups have been used to form a porous biomaterial scaffold containing IGF-1 plasmid DNA [70]. While the use of plasmid DNA could help to overcome an immune response, it also compromises the high efficiency of viral gene transfer. However, biomaterials could also be used to increase transfection efficiency. Osada and colleagues were able to significantly enhance skeletal muscle transfection efficiency by packaging plasmid DNA into complex, engineered micelles delivered by IV injection [71]. Alternatively, adsorbing viruses to a biomaterial surface offer a few unique options. For example, viral capsids can be modified by chemical conjugation to polymer antibody or polymer-protein complex, increasing target specificity. Using this method, one group conjugated adenovirus to the biodegradable polymer 2-hydroxypropyl meth-acrylamide (HPMA), which in turn was bound to FGF-2 [72]. In this way, the virus was targeted to cells with the FGF-2 receptor.

Rather than using a biomaterial to deliver a viral vector, or delivering a virus directly to host tissue, stem cells can be used as a gene delivery vehicle. For example, rather than delivering IGF-1 to tissue to enhance regeneration, Subramanian and colleagues transduced canine myoblasts with AAV2-IGF. They hypothesized that IGF would not only increase cell survival, but also promote angiogenesis in vivo. In murine studies, AAV2-IGF myoblasts were subcutaneously injected in the form of a matrigel plug. Three weeks post injection, the plug was found to be vascularized far more than the cell-free control, and associated with an increase in donor cell survival and proliferation [73]. Additionally, rather than through the transplantation of transduced cells, growth factors can also be used to pretreat cells prior to injection. Mesangioblasts, a type of vessel associated progenitor, can differentiate into mesenchymal lineages and have the added benefit of systemic delivery. Pretreatment with stromal-derived factor 1 (SDF-1) and TNFα significantly increased donor cell migration to injured murine muscle. Furthermore, transient transfection with α4 integrin combined with pretreatment enhanced donor cell migration 5–6 fold [74].

Another interesting twist on gene delivery to stem cells is the use of vectors to expand the differentiation potential of a particular stem cell type. An attractive stem cell source, MSCs are capable of myogenic differentiation, but with a low efficiency. Lentiviral-mediated expression of MyoD, a critical muscle transcription factor, was shown to significantly increase the myogenic capacity of human adipose stem cells, while decreasing adipogenesis. Furthermore, MyoD-expressing adipose stem cells were able to differentiate and regenerate skeletal muscle in a murine muscle injury model [75].

6.4 Conclusion

6.4.1 Translation to Clinic: Recent Clinical Trials

Recently, Bowles and colleagues completed a randomized double-blind placebo-controlled phase 1 clinical trial of gene therapy for DMD using a recombinant AAV vector containing the mini-dystrophin transgene [76]. As AAV 2 has been approved previously for clinical trials, the investigators made rationally designed mutations to the AAV2 capsid in order to increase muscle tropism and decrease immune recognition. Results demonstrated that the recombinant vector, AAV2.5, elicited no cellular immune response, although mini-dystrophin expression was low. This may be attributed to preexisting humoral immunity to AAV2 that is able to cross react with AAV2.5. However, these results established the safety of recombinant AAV vectors for gene delivery and identified the next issues that need to be addressed for future development [76].

Clinical trials of various stem cell therapies are also under way. A phase 2 clinical trial of bone marrow derived stem cell-based therapy for the treatment of severe leg ischemia has recently completed in 2010. With 35 patients enrolled, this study evaluated the safety and efficacy of intramuscular stem cell injection to promote blood vessel formation in patients who were not eligible for surgical intervention [77]. A similar study is currently recruiting patients with peripheral artery disease, although they will specifically be performing intramuscular injection of autologous CD133+ stem cells, as opposed to a mixed population of bone marrow progenitors [78].

6.4.2 Conclusion

Biomedical research has led to the development of numerous biological regenerative therapies for the treatment of muscle injuries and disease. Beginning with growth factor delivery and myoblast transplants, regenerative medicine for muscle healing has expanded to stem cell, gene, and biomaterials based approaches.

Once each pursued as separate methods, innovation and collaboration between disparate disciplines has led to combinatorial approaches and improved success.

References

1. Andersson G (2008) American academy of orthopaedic surgeons: the burden of musculoskeletal diseases in the United States: prevalence societal and economic cost. American Academy of Orthopaedic Surgeons, Rosemont
2. Brooks SV (2003) Current topics for teaching skeletal muscle physiology. Adv Physiol Educ 27(1–4):171–182
3. Tedesco FS, Dellavalle A, Diaz-Manera J, Messina G, Cossu G (2010) Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. J Clin Invest 120(1):11–19
4. Andia I, Sanchez M, Maffulli N (2011) Platelet rich plasma therapies for sports muscle injuries: any evidence behind clinical practice? Expert Opin Biol Ther 11(4):509–518
5. Stauber WT, Knack KK, Miller GR, Grimmett JG (1996) Fibrosis and intercellular collagen connections from four weeks of muscle strains. Muscle Nerve 19(4):423–430
6. Hagg GM (2000) Human muscle fibre abnormalities related to occupational load. Eur J Appl Physiol 83(2–3):159–165
7. Tiidus PM (2008) Skeletal muscle damage and repair. Human Kinetics, Champaign
8. Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA (2005) Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 433(7027):760–764
9. McNeil DE, Davis C, Jillapalli D, Targum S, Durmowicz A, Cote TR (2010) Duchenne muscular dystrophy: drug development and regulatory considerations. Muscle Nerve 41(6):740–745
10. Rocha CT, Hoffman EP (2010) Limb-girdle and congenital muscular dystrophies: current diagnostics, management, and emerging technologies. Curr Neurol Neurosci Rep 10(4): 267–276
11. Mishra DK, Friden J, Schmitz MC, Lieber RL (1995) Anti-inflammatory medication after muscle injury. A treatment resulting in short-term improvement but subsequent loss of muscle function. J Bone Joint Surg Am 77(10):1510–1519
12. Shen W, Li Y, Tang Y, Cummins J, Huard J (2005) NS-398, a cyclooxygenase-2-specific inhibitor, delays skeletal muscle healing by decreasing regeneration and promoting fibrosis. Am J Pathol 167(4):1105–1117
13. Mackey AL, Kjaer M, Dandanell S, Mikkelsen KH, Holm L, Dossing S, Kadi F, Koskinen SO, Jensen CH, Schroder HD, Langberg H (2007) The influence of anti-inflammatory medication on exercise-induced myogenic precursor cell responses in humans. J Appl Physiol 103(2):425–431
14. Biggar WD, Harris VA, Eliasoph L, Alman B (2006) Long-term benefits of deflazacort treatment for boys with duchenne muscular dystrophy in their second decade. Neuromuscul Disord 16(4):249–255
15. Nagaraju K (2001) Immunological capabilities of skeletal muscle cells. Acta Physiol Scand 171(3):215–223
16. Chazaud B, Sonnet C, Lafuste P, Bassez G, Rimaniol AC, Poron F, Authier FJ, Dreyfus PA, Gherardi RK (2003) Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth. J Cell Biol 163(5):1133–1143
17. Conboy IM, Rando TA (2002) The regulation of notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell 3(3):397–409
18. Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM (2008) Self-renewal and expansion of single transplanted muscle stem cells. Nature 456(7221):502–506

19. Peault B, Rudnicki M, Torrente Y, Cossu G, Tremblay JP, Partridge T, Gussoni E, Kunkel LM, Huard J (2007) Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. Mol Ther 15(5):867–877
20. Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA, Beauchamp JR (2004) Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? J Cell Biol 166(3): 347–357
21. Li Y, Foster W, Deasy BM, Chan Y, Prisk V, Tang Y, Cummins J, Huard J (2004) Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: a key event in muscle fibrogenesis. Am J Pathol 164(3):1007–1019
22. Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C, Rando TA (2007) Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. Science 317(5839):807–810
23. Miller KJ, Thaloor D, Matteson S, Pavlath GK (2000) Hepatocyte growth factor affects satellite cell activation and differentiation in regenerating skeletal muscle. Am J Physiol Cell Physiol 278(1):C174–C181
24. Chen FM, Zhang M, Wu ZF (2010) Toward delivery of multiple growth factors in tissue engineering. Biomaterials 31(24):6279–6308
25. Yanagisawa-Miwa A, Uchida Y, Nakamura F, Tomaru T, Kido H, Kamijo T, Sugimoto T, Kaji K, Utsuyama M, Kurashima C et al (1992) Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. Science 257(5075):1401–1403
26. Simons M, Annex BH, Laham RJ, Kleiman N, Henry T, Dauerman H, Udelson JE, Gervino EV, Pike M, Whitehouse MJ, Moon T, Chronos NA (2002) Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial. Circulation 105(7):788–793
27. Eppler SM, Combs DL, Henry TD, Lopez JJ, Ellis SG, Yi JH, Annex BH, McCluskey ER, Zioncheck TF (2002) A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. Clin Pharmacol Ther 72(1):20–32
28. Creaney L, Hamilton B (2008) Growth factor delivery methods in the management of sports injuries: the state of play. Br J Sports Med 42(5):314–320
29. Mishra A, Pavelko T (2006) Treatment of chronic elbow tendinosis with buffered platelet-rich plasma. Am J Sports Med 34(11):1774–1778
30. Marx RE (2004) Platelet-rich plasma: evidence to support its use. J Oral Maxillofac Surg 62(4):489–496
31. Sanchez M, Anitua E, Andia I (2005) Application of autologous growth factors on skeletal muscle healing. In: 2nd international conference on regenerative medicine
32. Mourkioti F, Rosenthal N (2005) IGF-1, inflammation and stem cells: interactions during muscle regeneration. Trends Immunol 26(10):535–542
33. Borselli C, Storrie H, Benesch-Lee F, Shvartsman D, Cezar C, Lichtman JW, Vandenburgh HH, Mooney DJ (2010) Functional muscle regeneration with combined delivery of angiogenesis and myogenesis factors. Proc Natl Acad Sci USA 107(8):3287–3292
34. Bogdanovich S, Perkins KJ, Krag TO, Khurana TS (2004) Therapeutics for Duchenne muscular dystrophy: current approaches and future directions. J Mol Med (Berl) 82(2): 102–115
35. Liu Z, Wu Y, Chen BG (2006) Myoblast therapy: from bench to bedside. Cell Transplant 15(6):455–462
36. Fan Y, Maley M, Beilharz M, Grounds M (1996) Rapid death of injected myoblasts in myoblast transfer therapy. Muscle Nerve 19(7):853–860
37. Beauchamp JR, Morgan JE, Pagel CN, Partridge TA (1999) Dynamics of myoblast transplantation reveal a discrete minority of precursors with stem cell-like properties as the myogenic source. J Cell Biol 144(6):1113–1122
38. Huard J, Acsadi G, Jani A, Massie B, Karpati G (1994) Gene transfer into skeletal muscles by isogenic myoblasts. Hum Gene Ther 5(8):949–958

39. Bouchentouf M, Benabdallah BF, Tremblay JP (2004) Myoblast survival enhancement and transplantation success improvement by heat-shock treatment in mdx mice. Transplantation 77(9):1349–1356
40. Tambara K, Premaratne GU, Sakaguchi G, Kanemitsu N, Lin X, Nakajima H, Sakakibara Y, Kimura Y, Yamamoto M, Tabata Y, Ikeda T, Komeda M (2005) Administration of control-released hepatocyte growth factor enhances the efficacy of skeletal myoblast transplantation in rat infarcted hearts by greatly increasing both quantity and quality of the graft. Circulation 112(9 Suppl):I129–134
41. Yao SN, Smith KJ, Kurachi K (1994) Primary myoblast-mediated gene transfer: persistent expression of human factor IX in mice. Gene Ther 1(2):99–107
42. Shabbir A, Zisa D, Leiker M, Johnston C, Lin H, Lee T (2009) Muscular dystrophy therapy by nonautologous mesenchymal stem cells: muscle regeneration without immunosuppression and inflammation. Transplantation 87(9):1275–1282
43. Villalta SA, Nguyen HX, Deng B, Gotoh T, Tidball JG (2009) Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. Hum Mol Genet 18(3):482–496
44. Kim J, Hematti P (2009) Mesenchymal stem cell-educated macrophages: a novel type of alternatively activated macrophages. Exp Hematol 37(12):1445–1453
45. Qu-Petersen Z, Deasy B, Jankowski R, Ikezawa M, Cummins J, Pruchnic R, Mytinger J, Cao B, Gates C, Wernig A, Huard J (2002) Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J Cell Biol 157(5):851–864
46. Deasy BM, Gharaibeh BM, Pollett JB, Jones MM, Lucas MA, Kanda Y, Huard J (2005) Long-term self-renewal of postnatal muscle-derived stem cells. Mol Biol Cell 16(7): 3323–3333
47. Urish KL, Vella JB, Okada M, Deasy BM, Tobita K, Keller BB, Cao B, Piganelli JD, Huard J (2009) Antioxidant levels represent a major determinant in the regenerative capacity of muscle stem cells. Mol Biol Cell 20(1):509–520
48. Ota S, Uehara K, Nozaki M, Kobayashi T, Terada S, Tobita K, Fu FH, Huard J (2011) Intramuscular transplantation of muscle-derived stem cells accelerates skeletal muscle healing after contusion injury via enhancement of angiogenesis. Am J Sports Med 39(9):1912–1922
49. Deasy BM, Li Y, Huard J (2004) Tissue engineering with muscle-derived stem cells. Curr Opin Biotechnol 15(5):419–423
50. Payne TR, Oshima H, Okada M, Momoi N, Tobita K, Keller BB, Peng H, Huard J (2007) A relationship between vascular endothelial growth factor, angiogenesis, and cardiac repair after muscle stem cell transplantation into ischemic hearts. J Am Coll Cardiol 50(17): 1677–1684
51. Rose T, Peng H, Shen HC, Usas A, Kuroda R, Lill H, Fu FH, Huard J (2003) The role of cell type in bone healing mediated by ex vivo gene therapy. Langenbecks Arch Surg 388(5): 347–355
52. Chirieleison SM, Feduska JM, Schugar RC, Askew Y, Deasy BM (2011) Human muscle-derived cell populations isolated by differential adhesion rates: phenotype and contribution to skeletal muscle regeneration in Mdx/SCID mice. Tissue Eng Part A 18(3–4):232–241
53. Okada M, Payne TR, Drowley L, Jankowski RJ, Momoi N, Beckman S, Chen WC, Keller BB, Tobita K, Huard J (2011) Human skeletal muscle cells with a slow adhesion rate after isolation and an enhanced stress resistance improve function of ischemic hearts. Mol Ther 20(1):138–145
54. Carr LK, Steele D, Steele S, Wagner D, Pruchnic R, Jankowski R, Erickson J, Huard J, Chancellor MB (2008) 1-year follow-up of autologous muscle-derived stem cell injection pilot study to treat stress urinary incontinence. Int Urogynecol J Pelvic Floor Dysfunct 19(6):881–883
55. Rouger K, Larcher T, Dubreil L, Deschamps JY, Le Guiner C, Jouvion G, Delorme B, Lieubeau B, Carlus M, Fornasari B, Theret M, Orlando P, Ledevin M, Zuber C, Leroux I, Deleau S, Guigand L, Testault I, Le Rumeur E, Fiszman M, Cherel Y (2011) Systemic

delivery of allogenic muscle stem cells induces long-term muscle repair and clinical efficacy in duchenne muscular dystrophy dogs. Am J Pathol 179(5):2501–2518
56. Zheng B, Cao B, Crisan M, Sun B, Li G, Logar A, Yap S, Pollett JB, Drowley L, Cassino T, Gharaibeh B, Deasy BM, Huard J, Peault B (2007) Prospective identification of myogenic endothelial cells in human skeletal muscle. Nat Biotechnol 25(9):1025–1034
57. Inoue H, Yamanaka S (2011) The use of induced pluripotent stem cells in drug development. Clin Pharmacol Ther 89(5):655–661
58. Tan KY, Eminli S, Hettmer S, Hochedlinger K, Wagers AJ (2011) Efficient generation of iPS cells from skeletal muscle stem cells. PLoS One 6(10):e26406
59. Darabi R, Baik J, Clee M, Kyba M, Tupler R, Perlingeiro RC (2009) Engraftment of embryonic stem cell-derived myogenic progenitors in a dominant model of muscular dystrophy. Exp Neurol 220(1):212–216
60. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM (1987) Complete cloning of the duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell 50(3):509–517
61. Miyagoe-Suzuki Y, Takeda S (2010) Gene therapy for muscle disease. Exp Cell Res 316(18):3087–3092
62. Tang Y, Cummins J, Huard J, Wang B (2010) AAV-directed muscular dystrophy gene therapy. Expert Opin Biol Ther 10(3):395–408
63. Wang B, Li J, Xiao X (2000) Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model. Proc Natl Acad Sci USA 97(25):13714–13719
64. Tang Y, Reay DP, Salay MN, Mi MY, Clemens PR, Guttridge DC, Robbins PD, Huard J, Wang B (2010) Inhibition of the IKK/NF-kappaB pathway by AAV gene transfer improves muscle regeneration in older mdx mice. Gene Ther 17(12):1476–1483
65. Sun JY, Anand-Jawa V, Chatterjee S, Wong KK (2003) Immune responses to adeno-associated virus and its recombinant vectors. Gene Ther 10(11):964–976
66. Mendell JR, Clark KR (2006) Challenges for gene therapy for muscular dystrophy. Curr Neurol Neurosci Rep 6(1):47–56
67. Gilbert PM, Havenstrite KL, Magnusson KE, Sacco A, Leonardi NA, Kraft P, Nguyen NK, Thrun S, Lutolf MP, Blau HM (2010) Substrate elasticity regulates skeletal muscle stem cell self-renewal in culture. Science 329(5995):1078–1081
68. Carnio S, Serena E, Rossi CA, De Coppi P, Elvassore N, Vitiello L (2011) Three-dimensional porous scaffold allows long-term wild-type cell delivery in dystrophic muscle. J Tissue Eng Regen Med 5(1):1–10
69. Jang JH, Schaffer DV, Shea LD (2011) Engineering biomaterial systems to enhance viral vector gene delivery. Mol Ther 19(8):1407–1415
70. Falco EE, Wang MO, Thompson JA, Chetta JM, Yoon DM, Li EZ, Kulkami MM, Shah S, Pandit A, Roth JS, Fisher JP (2011) Porous EH and EH-PEG scaffolds as gene delivery vehicles to skeletal muscle. Pharm Res 28(6):1306–1316
71. Osada K, Shiotani T, Tockary TA, Kobayashi D, Oshima H, Ikeda S, Christie RJ, Itaka K, Kataoka K (2012) Enhanced gene expression promoted by the quantized folding of pDNA within polyplex micelles. Biomaterials 33(1):325–332
72. Fisher KD, Stallwood Y, Green NK, Ulbrich K, Mautner V, Seymour LW (2001) Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. Gene Ther 8(5):341–348
73. Subramanian IV, Fernandes BC, Robinson T, Koening J, Lapara KS, Ramakrishnan S (2009) AAV-2-mediated expression of IGF-1 in skeletal myoblasts stimulates angiogenesis and cell survival. J Cardiovasc Transl Res 2(1):81–92
74. Galvez BG, Sampaolesi M, Brunelli S, Covarello D, Gavina M, Rossi B, Constantin G, Torrente Y, Cossu G (2006) Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability. J Cell Biol 174(2):231–243

75. Goudenege S, Pisani DF, Wdziekonski B, Di Santo JP, Bagnis C, Dani C, Dechesne CA (2009) Enhancement of myogenic and muscle repair capacities of human adipose-derived stem cells with forced expression of MyoD. Mol Ther 17(6):1064–1072
76. Bowles DE, McPhee SW, Li C, Gray SJ, Samulski JJ, Camp AS, Li J, Wang B, Monahan PE, Rabinowitz JE, Grieger JC, Govindasamy L, Agbandje-McKenna M, Xiao X, Samulski RJ (2011) Phase 1 gene therapy for duchenne muscular dystrophy using a translational optimized AAV vector. Mol Ther 20(2):443–455
77. TCA Cellular Therapy (2000) In. Phase II safety/efficacy study of a combination stem cell therapycell therapy that develops mature stable vessel formation in ischemic limbs. In: ClinicalTrials.gov [Internet], (Bethesda (MD): National library of medicine (US). [cited 2011 Sept 23])
78. University of Wisconsin, Madison (2000) In. Stem cell revascularization in patients with critical limb ischemia. In: ClinicalTrials.gov [Internet], (Bethesda (MD): National library of medicine (US). [cited 2011 Sept 23])

Chapter 7
Mesenchymal Stem Cells in Bone and Cartilage Regeneration

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Abstract Most damage to bone and cartilage, as well as bone and cartilage diseases, is curable by routine medical procedures. However, some are either incurable or difficult to cure. Often these damages or diseases result in large-scale tissue loss. Treatment of these conditions is the subject of regenerative medicine. One valuable regenerative medicine tool is stem cell-based therapy. The use of mesenchymal stem cells (MSCs) in bone and cartilage regeneration is the subject of the present chapter, which is organized into three main parts. First, bone and cartilage structure, their repair capacity, and current therapy will be briefly described. In the second section, the main features of MSCs that render them appropriate cell candidates for bone and cartilage regeneration will be discussed, followed by a brief description of scaffolds and growth factors that may accompany MSCs in order to enhance their regenerative effects. Finally, in the third section, some examples of the application of MSCs in bone and cartilage regeneration will be explained.

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7.1 Part I: Bone and Cartilage Repair

7.2 Bone

7.2.1 Structure

Bone is a specialized form of connective tissue responsible for several important tasks in the body: it protects vital organs, facilitates movement, stores minerals, and plays a role in calcium and phosphate homeostasis. In addition, bone houses precursor cells that include hematopoietic as well as mesenchymal stem cells (MSCs). This variety of tasks enables the bone to have a complex dynamic structure with continual destruction and formation throughout life [1, 2].

Bone tissue is structurally composed of an extracellular matrix (ECM) and cells. The ECM includes minerals, organic materials, and water. The mineral part of the bone matrix is mainly composed of calcium and phosphate in the form of hydroxyapatite (HA) crystals. Other minerals include nonorganic salts of magnesium, potassium, fluoride, and citrate in trace amounts [3, 4].

Organic materials constitute about 30 % of dried bone weight of which the main component is collagen I. Other collagen isoforms including types III, V, and XII are also present in trace amounts [5, 6]. Bone matrix is slightly metachromatic, indicating the presence of glycosaminoglycan (GAG) which includes chondroitin sulfate and keratin sulfate. GAGs are responsible for the bone's compressive resistance. These macromolecules also play a role in matrix mineralization.

Noncollagenous proteins constitute about 5 % of bone matrix and are involved in organization, turnover, and matrix mineralization. The most abundant noncollagenous ECM protein in the bone is osteonectin, which binds to collagen through its calcium-binding domain. Osteonectin expression occurs in the early stage of osteoblastic differentiation [5–8]. Osteopontin (OPN) is a phosphorylated sialoprotein involved in cell movement and regulation of matrix mineralization [9]. In the absence of calcium, this protein has a random coil formation, but in the presence of calcium, its beta sheet structure is seen. It has been shown that OPN is involved in bone remodeling induced by mechanical stress [10]. Osteocalcin constitutes about 15 % of noncollagenous protein in the bones. It is vitamin K dependent and has an affinity for the Ca^{2+}/HA complex. This protein is a late marker of the osteogenic phenotype and plays a role in bone remodeling [11]. Fibronectin and vitronectin are other noncollagenous proteins present in bone ECM that have crucial roles in cell signaling.

There are three main cells in bone tissue: osteoblasts, osteocytes, and osteoclasts. Osteoblasts are morphologically cuboidal and reside on bone surfaces. These cells originate from bone progenitors in the bone marrow and periosteum in response to inducers. The main function of osteoblasts is to produce organic ECM, also known as osteoids. Osteoblasts also regulate matrix mineralization as well as osteoclast activity. Osteoblasts have a high alkaline phosphatase activity [12–14].

Osteocytes are star-shaped cells with less organelles than osteoblasts. Osteocytes are osteoblasts embedded in bone ECM. The main role of osteocytes is mechanotransduction, osteoid calcification, and regulation of calcium blood levels [12–14].

Osteoclasts are multinuclear cells formed by the fusion of several mononuclear hematopoietic precursors. These cells and osteoblasts have crucial roles in bone remodeling, which is a complex process where old bone is continuously and gradually digested by osteoclasts and replaced by new bone secreted by osteoblasts [12–14].

7.2.2 Repair

Bone is a tissue capable of repairing its defects. Most bone lesions can heal spontaneously or with minimal intervention. At the fracture site, a series of events happens to promote the repair process. Upon damage, inflammation occurs followed by migration, proliferation, and differentiation of mesenchymal cells to the damaged site [15, 16]. In some disorders, including osteogenesis imperfecta (OI), Paget disease, hyperparathyroidism, osteomyelitis, and osteoporosis, the repair process does not work properly. These diseases, along with large bone defects resulting from trauma and tumor resection, are problematic conditions in the orthopedic field.

7.2.3 Current Therapy

Among the different methods of bone repair, autograft is the gold standard for bone defect reconstruction [17]. In this treatment, bone is obtained from another site within the patient's body, such as the iliac crest and then transplanted into the defective site. An autograft does not trigger the host immune response. Nevertheless, due to the limited availability of autografts, in addition to the necessity for extensive surgery, pain, and possibility of infection and paresthesia, a better treatment is needed [18–20].

Although the use of the allogenic graft for repairs does not have some of the limitations of the autologous graft, such as pain and quantity; however, this method triggers the host immune response. Furthermore, the irradiation and freeze-drying processes used during allograft preparation may remove the cells and damage allograft osteoconductivity, hence decreasing their regenerative capacity [21].

Xenografts, on the other hand, may be considered as an alternative. However, some disadvantages limit their application, including the possibility of viral transmission, toxicity, and immunogenicity associated with these types of transplantable materials [22, 23]. Another option is to use metal implants such as titanium, stainless steel, and cobalt chromium, all of which are biocompatible, processable, strong, and relatively inexpensive. In contrast to biodegradable materials, metal implants are not as easily degradable. However, they may release toxic ions which lead to an inflammatory response or infection at the transplant site. Furthermore, to remove these implants an additional operation is required [24, 25]. Therefore, finding an appropriate substitute for lost bone tissue is of utmost importance in orthopedic and maxillofacial surgeries.

7.3 Articular Cartilage

7.3.1 Structure

Articular cartilage is a hyaline cartilage covering the ends of bones in joints. It is histologically composed of a few chondrocytes, an abundant ECM, and a moderate amount of interstitial fluid. Chondrocytes constitute less than 5 % of cartilage structure. They do not directly interact; rather each single cell acts as a functional unit that is responsible for production and organization of its surrounding ECM. Each chondrocyte possesses high metabolic activity; however, cartilage tissue exhibits low metabolic activity due to the scarcity of its cell content [26–28].

Cartilage matrix includes collagen, proteoglycan, and noncollagenous proteins. Collagen constitutes about two-thirds of cartilage's dry weight in an adult and it forms an interwoven stroma responsible for the tissue's tensile strength. Collagen type II is the dominant collagen in cartilage. Other collagens include collagen IX, XI, and VI. Collagen IX and XI are interwoven with collagen II to make a complex network that physically entraps other macromolecules. Collagen type VI binds chondrocytes to the matrix through an integrin receptor that is involved in mechanical signal transduction [29].

Proteoglycans such as hyaluronan, keratin sulfate, and chondroitin sulfate are responsible for cartilage's compressive strength. Keratin sulfate and chondroitin sulfate are joined to a core protein to form a proteoglycan monomer, the most important of which is aggrecan. More than 300 aggrecans are linked to a linear molecule of hyaluronan to form the proteoglycan aggregates. The negative charge seen in proteoglycans holds a considerable amount of water. Noncollagenous proteins include anchorin and cartilage oligomeric protein that mediate chondrocyte binding to the matrix. Interstitial fluid plays a crucial role in exchanges of nutrients and oxygen in the cartilage, which has no blood vessels [30].

There are four structural zones: superficial, middle, deep, and calcified distinguishable in articular cartilage. The superficial zone is composed of flattened chondrocytes that secrete a superficial zone protein (SZP) which is also known as lubricin, thus providing a lubricating surface for joint movement. The middle zone mostly contains collagen II and proteoglycan aggregates. The deep zone is characterized by small round chondrocytes that are arranged in short columns perpendicular to the free surfaces and the calcified zone is characterized by a calcified matrix with the presence of small chondrocytes [31, 32].

7.3.2 Repair

The process of tissue repair involves the stages of necrosis, inflammation, and repair. The existence of blood vessels is necessary for triggering an inflammatory response, which brings repair cells including monocytes and macrophages to the

injury site. Cartilage undergoes necrosis in response to an injury but there is no inflammatory stage due to the avascular nature of cartilage tissue. If the injury occurs on cartilage surfaces and subchondral bone is not affected, there will be no repair since chondrocytes in mature cartilage have lost their ability to undergo proliferation and cannot participate in the repair process. If the injury affects the articular cartilage subchondral bone, blood will fill the injured site, initiating an inflammatory response followed by the formation of repair tissue, which is fibrocartilage in nature [33, 34].

7.3.3 Current Therapy

Treatment of hyaline cartilage injury and degeneration is a challenge for orthopedic surgeons. Current therapies in use in the clinic include marrow stimulation techniques such as micro fracture, osteochondral mosaicplasty, and cell-based treatments [34, 35].

Marrow stimulating techniques attempt to solve articular cartilage damage through an arthroscopic procedure. Firstly, damaged cartilage is opened by drilling or punching until the underlying bone is exposed. Then, the subchondral bone is perforated to generate a blood clot within the defect. Repair material produced by this method is often fibrocartilage, which is not as good mechanically as hyaline cartilage [36].

Autologous osteochondral mosaicplasty, known also as osteoarticular transfer system (OATS) is technically challenging in the clinic. Osteochondral tissue is usually obtained from a nonweight bearing area of the patient's own articular cartilage. This method has disadvantages that include morbidity of the donor site, tissue supply limitation and unsuitable mechanical properties, as well as thickness of the obtained tissue [37, 38]. The use of autologous tissue could be considered an alternative but it is associated with high cost, risk of immunologic rejection, and transmission of pathogens [39].

There are two types of cell-based treatments for cartilage defects: autologous chondrocyte implantation (ACI) [40] and MSC-based cell therapy. ACI involves the preparation of chondrocytes from an intact region of cartilage, followed by their culture expansion and surgical transplantation. This technique involves a two-step surgical procedure: one for collecting tissue and the other for transplantation. Obtaining sufficient chondrocytes from biopsies is challenging, therefore expansion of chondrocytes is inevitable. It has been reported that chondrocytes expanded in culture gradually undergo dedifferentiation and lose morphological features as well as specialized functions [41]. Limitations associated with current therapies for cartilage injuries have motivated investigators to search for an alternative reliable strategy. In this context, MSC-based cartilage regeneration has gained considerable attention.

7.4 Part II: Mesenchymal Stem Cells

Treatment of large bone and cartilage lesions is of primary concern in the field of regenerative medicine. As previously mentioned, the current therapies are often not appropriate. Advances in the stem cell field hold great promise in regenerative medicine. In the field of bone and cartilage regenerative medicine, MSCs have gained considerable attention owing to their unique characteristics. In this section, the key features of MSCs which render them appropriate cellular candidates for cell-based treatment of bone and cartilage defects will be discussed, followed by a brief description of scaffolds and growth factors that may be used along with MSCs to enhance their regenerative effects.

7.5 Key Characteristics of MSCs

MSCs are adult stem cells found in varying somatic tissues. These cells are capable of differentiating into mesenchymal cells including bone, cartilage, and adipose lineages. The key characteristics of MSCs are their ability to form colonies that result from the activity of the cells, referred to as colony-forming unit fibroblasts (CFU-F). The frequency of CFU-F in bone marrow samples is reported to be about 1 cell/10^4–10^5 mononuclear cells [42]. Currently, MSCs are characterized by their ability for plastic adherence and capability of multi-lineage differentiation. Although no distinct single surface antigen has been identified for MSCs, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has suggested markers that should be verified on isolated cells before they can be given the designation of MSC. These include positive expressions of CD73, CD105, CD90, and no expression of CD34 and CD45, and other hematopoietic markers [43]. The following features render MSC appropriate cellular candidates for cell-based treatment of bone and cartilage lesions.

7.5.1 MSCs Lack Ethical Concerns

Several stem cell types, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and MSCs have the capability to differentiate into bone and cartilage, hence they can be used in bone and cartilage regeneration. ESCs are derived from the blastocyst inner cell mass and can be directed toward differentiation into varying cell lineages, including osteoblastic as well as chondrocytic cell lineages under suitable culture conditions [44–46]. Despite increasing interest in ESCs, their clinical application is highly limited due to concerns associated with these cells. The primary concern is ethical; to derive the cells, at least one human embryo must be used. Additionally, some reports have indicated that transplantation of ESCs has led

to teratoma formation in an animal model [47, 48]. Because of the above-mentioned concerns with ESCs, scientists have tried to establish ESC-like stem cells, known as iPSCs from somatic cells by plasmid or adenovirus-based transduction [49]. Actually, iPSCs are patient-specific ESCs that lack ethical concerns and do not trigger an immune response. Despite this prominent capability, there are important issues that need to be considered before their clinical applications. The method by which iPSCs are generated, i.e., through plasmid or adenovirus-based transduction is a main concern. MSCs, as postnatal adult stem cells, have none of these concerns.

7.5.2 MSCs Reside in Multiple Tissues

MSCs are readily available and can be derived from a variety of tissues including trabecular bone [50], synovium [51], adipose tissue [52], skeletal muscle [53], periosteum [54], dermis [55], blood [56], deciduous teeth [57], amniotic fluid [58], and umbilical cord blood [59]. In this regard, bone marrow, adipose tissue, and birth-associated tissues, including umbilical cord, and dental pulp, have gained considerable attention. Two of the most common sources for MSCs are bone marrow and adipose tissue.

7.5.3 MSCs can be Differentiated Along an Osteocytic and Chondrocytic Lineage

MSCs possess multilineage differentiation potentials. Their osteogenic and chondrogenic properties were the first differentiation capacities reported when initially discovered by Friedenstein et al. [42]. These investigators plated marrow cells in plastic dishes and removed nonadherent cells 4 h after culture initiation. According to their observations, adherent cells produced small aggregates of 2–4 cells with fibroblastic morphologies. These cells remained quiescent for a few days and then underwent proliferation. The most important feature of these cells was their capacity to produce small deposits of bone and cartilage-like tissue [42, 60]. The original observations of Fridenstein et al. have been confirmed by the other studies. Today bone and cartilage differentiation have been considered one of the criteria for cells to be designated as MSCs.

7.5.4 Non-Immunogenic Properties of MSCs

MSCs possess immunologically specific characteristics that enable them to be general donors for therapeutic applications. Immunologic phenotypes of MSCs are: MHC I+, MHC II−, CD 40−, CD80−, and CD 86− [61]. Graft rejection

by the immune system occurs when T cells are fully activated. T cells require two signals to become fully activated, of which the first signal is provided through the T cell receptor that interacts with the peptide-MHC molecule I on the membrane of antigen presenting cells (APC). A second signal, the co-stimulatory signal, is provided by the interaction among co-stimulatory molecules, including CD80 and CD86, that are expressed on the membranes of APC and T cells [62]. MSCs do not trigger T cell activation owing to the absence of CD80 and CD86 in their membranes [63]. Most likely, MSCs inhibit CD4+ and CD8+ T cell activation as well as proliferation by secreting indoleamine 2,3-dioxygenase (IDO), nitric oxide, transforming growth factor beta (TGF-beta), and prostaglandin E2 [64, 65]. It has been demonstrated that MSCs inhibit B cell differentiation into plasma cells [66, 67]. MSCs are also able to inhibit dendritic cell (DC) proliferation and differentiation [68]. For these reasons, there is hope that allogeneic MSCs could replace autologous and allogenic bone and osteochondral grafts, which have known limitations in terms of availability and risk of pathogen transmission, respectively. At the allogenic setting, it could be possible to develop a cell bank for the storage of donor MSCs for cell therapy.

7.5.5 Injury-Seeking Capability of MSCs

One of the most important capabilities of MSCs is their migration capability in response to signals produced by an injured bone [69, 70]. This is of considerable importance when it comes to the systematic injection of these cells when used for diseases such as osteoarthritis (OA). The precise mechanisms of cell trafficking in the blood, transmigration through endothelial cells, and homing to the injured site are not thoroughly understood; it has been speculated that chemokines and their receptors regulate this process [61]. MSCs express a series of chemokine receptors that play a role in their migration in response to a chemokine gradient produced at the damaged site. These include CCR1, CCR3, CCR4, CCR5, CCR7, CCR9, and CX3CR1 [71]. The CXCR4 receptor and its specific chemokine (stromal cell-derived factor 1 or SDF1) plays an important role in stem cell trafficking, particularly hematopoietic stem cells (HSCs). It has been proposed that SDF1/CXCR4 could be a homing signal for MSCs in bone repair [72].

7.6 Bone and Cartilage Scaffolds

In most regeneration strategies, MSCs are used in combination with scaffolds. Scaffolds are spongy 3-dimensional biocompatible materials that house MSCs and allow them to undergo proliferation as well as matrix deposition. Meanwhile, they withstand the dynamic mechanical environment of the tissue intended to be regenerated. An ideal scaffold must be biodegradable and undergo degradation at

the time the cells deposit their ECM. In this way, the implant integrates into the host tissue and repair is accomplished. Scaffold materials as well as their degradation byproducts must not be immunogenic.

7.6.1 Bone Scaffolds

Materials used to fabricate bone scaffolds include bioceramics, polymers, and composites of polymers plus bioceramics. There are two categories of bioceramics: naturally occurring (i.e., coral hydroxyapatite) and synthesized (i.e., synthetic hydroxyapatite and tricalcium phosphate). Bioceramics are widely used in the fields of orthopedics [73] and maxillofacial surgeries [74] due to their high mechanical strength. There are, however, several disadvantages that limit the application of bioceramics. These biomaterials possess low biodegradability, low tensile strength, and are very brittle [73].

Polymers can be divided into two categories: natural and synthetic. The most widely used natural polymers in bone tissue engineering include collagen, alginate, and chitosan. Immunogenecity and the possibility of pathogen transmission are the most prominent disadvantages of natural polymers [75]. Some common synthetic bone polymers include polyglycolic acid (PGA), polylactic acid (PLA), polylactic-co-glycolic acid (PLGA), polycaprolacton (PCL), polypropylene fumarate (PPF), polycaprolacton fumarate, and polycaprolacton diacrylate. Synthetic polymers are appropriate options as bone engineering scaffolds since they can be fabricated in large quantities and the risk of infection, toxicity, and immunogenicity is low compared to natural polymers. In addition to their mechanical, physical, and chemical properties, the elastic module, acidity, and hydrophilicity of the polymers are adjustable. The disadvantages of synthetic polymers include their very low strength that worsens with the introduction of pores to form tissue engineering scaffolds. Most of these materials are non-osteoconductive [76].

Composite scaffolds are fabricated using bioceramics and polymers. Many polymer-based scaffolds that include bioactive bioceramics, for example hydroxyapatite/PLLA, have been produced [77, 78]. In composite scaffolds, bioceramics serve to make the scaffold osteoconductive and provide reinforcement, whereas the polymer serves to solve the problem of poor degradability, low tensile strength, and brittleness of the ceramics [79].

7.6.2 Cartilage Scaffolds

There are two main categories of cartilage scaffolds: natural and synthetic polymers. Natural polymer scaffolds are fabricated using proteins and polysaccharides. Protein-based scaffolds include collagen gel/membrane, fibrin glue, and platelet-rich plasma (PRP). Carbohydrate-based scaffolds include hyaluronic acid (HA),

alginate, agarose, and chitosan. Collagen and hyaluronan-based scaffolds are the most currently used materials in the clinic [80]. The drawback of collagen is its immunogenicity [81]. HA is highly biocompatible. It completely degrades and absorbs after 3 months. The byproducts of HA degradation are, however, believed to cause chondrolysis [82].

Among the synthetic scaffolds for cartilage engineering are PLA, PGA, carbon fibers, dacron, and Teflon [83, 84]. The mechanical property of synthetic scaffolds can be controlled during the fabrication process. These scaffolds are not expensive and do not possess immunogenicity. Release of growth factors from scaffolds can be improved by regulating their degradation rate and kinetics of growth factor delivery [85]. PLA and PGA are the most commonly used synthetic scaffolds in the field of cartilage engineering [86].

One important category of cartilage scaffolds is hydrogels, which are designed to be applied as injectable materials. This is of utmost importance since surgery can be avoided by their use. Hydrogels are fabricated using either of the above-mentioned natural or synthetic polymers [87].

7.7 Bone and Cartilage Growth Factors

Sometimes MSCs are used in combination with either osteogenic or chondrogenic growth factors to promote bone and cartilage regeneration, respectively. Growth factors are signaling molecules that bind to a specific receptor on the cell and influence special cell functions.

7.7.1 Bone Growth Factors

Growth factors that play a role in bone development and growth have the potential to be used in bone engineering. These growth factors include bone morphogenetic protein (BMP), particularly BMP-2, 4, and 7 which effect the induction of bone formation [88]; insulin-like growth factor (IGF) that plays a role in bone metabolism [89] especially at the time of fracture healing [90]; fibroblast growth factor-2 (FGF-2) which has a bone healing effect [91]; and vascular endothelial growth factor (VEGF) whose osteogenic effects are mediated indirectly through induction of vascularization [75].

7.7.2 Cartilage Growth Factors

Many differentiation and growth factors are involved in cartilage development and control tissue homeostasis. These well-known factors include TGF-beta which promotes MSC chondrogenic differentiation [92]; BMPs increase MSC chondrogenic

differentiation [93]; FGF-2 promotes cell proliferation as well as chondrogenic differentiation [94]; and IGF-I which has an anabolic effect on cartilage matrix synthesis [95].

7.8 Part III: MSC-Based Treatment of Bone and Cartilage Defects

To accomplish regeneration, on some occasions MSCs are applied without any biomaterial, for example, in OI or in cartilage resurfacing of the osteoarthritic joint. In most regeneration strategies, i.e., in the reconstruction of large bone and cartilage defects, MSCs are usually transplanted in combination with scaffolds or growth factors. For the purpose of regeneration, MSCs can be present either in an undifferentiated or differentiated state. Following, some examples of the application of MSCs in the regeneration of bone and cartilage defects will be discussed.

7.9 Bone Regeneration

7.9.1 Large Bone Defects

Large bone defects, also known as critical-sized bone defects, are one of the problems in the field of regenerative medicine. Many attempts have, thus far, been made to efficiently improve regeneration of tissue defects using MSCs. In this context, the groundbreaking investigation by Bruder et al. has involved the reconstruction of segmental bone defects (21 mm) in a canine femur [96]. In clinical trials, the pioneers could be regarded as Quatro et al. who have reported successful repairs of massive 4–8 cm defects in four human cases, aged 16–41 years. These authors have seeded MSCs on porous HA ceramic scaffolds and transplanted them into the defects. Good integration of the implant with host bone has led to recovery of limb function in all cases. A limitation associated with human studies has been the inability of the researchers to perform histological studies of the repair tissue [97]. Morishita et al. have reported results from three patients with benign bone tumors who were treated with MSC transplantation. According to their study, MSCs were first seeded on porous HA ceramics and differentiated into bone cells prior to transplantation into the bone cavities that were left after removal of the tumors [98].

In the MSC-based bone reconstruction field, the study by Gronthose et al. is interesting. These researchers have attempted to reconstruct the mandible of a 56-year-old man who underwent an ablative tumor surgery 8 years prior, by removing a large portion of his mandible (a length of 7 cm from the left paramedian region to the right retromolar region). In this study, the scaffold was

fabricated to conform to the defect shape and consisted of a titanium mesh loaded with HA blocks coated with rhBMP-7. Bone marrow-derived MSCs were loaded into a scaffold and differentiated along bone cell lineages. Prior to the main surgery, the construct was implanted in the patient's own latisimus dorsi muscle to encourage growth of heterotopic bone as well as ingrowth of vessels from the thoracodorsal artery. Seven weeks later, the construct was removed from the muscle and transplanted into the patient's mandible. During the first 6 months, the patient reported continuous improvement both in the quality of life and in self-confidence. Between months 7 and 13, a fracture occurred in the external titanium mesh scaffold and the regenerated bone became partially infected, probably due to oral flora. Unfortunately, the patient died of cardiac arrest 15 months after implantation of the replacement mandible [99].

7.9.2 Distraction Osteogenesis

Distraction osteogenesis is a surgical process used to reconstruct skeletal deformities and lengthen the long bones of the body. For this purpose, a corticotomy is used to fracture the bone into two segments; the two ends of the bone are gradually moved apart during the distraction phase, allowing new bone to form in the gap [100]. When the desired or possible length is reached, a consolidation phase follows in which the bone is allowed to continue healing. To shorten the consolidation period as well as to enhance the quality of repair tissue, MSCs can be applied. One study that has used MSCs' bone-regenerating capacity in distraction osteogenesis is the work conducted by Kitoh et al. These authors have conducted a clinical study that treated three femora and two tibia in two patients who suffered from achondroplasia and pseudoarthrosis, using differentiated autologous MSCs (osteoblast-like cells) along with autologous PRP injected into the distracted callus [101].

7.9.3 Osteomyelitis

Osteomyelitis is a heterogeneous disorder in terms of clinical signs, pathophysiology, and treatment. This difficult to cure disorder occurs after a fracture in which the involved bones are infected. *Staphylococcus aureus* is the most common organism seen in osteomyelitis. Diagnosis of osteomyelitis and determination of the extent of infection is performed by a bone biopsy, its culture, and radiological examination. The treatment of these diseases needs the cooperation of multiple disciplines, including orthopedic surgeons, neurosurgeons, rheumatologists, vascular surgeons, and specialists in infectious diseases. Treatment of osteomyelitis often requires the removal of infectious tissue and use of antibiotics [102].

Until now, there has been no in vivo study regarding the application of MSCs in the treatment of osteomyelitis. In an in vitro study design, Hou et al. have

presented a construct with potential benefit for osteomyelitis treatment. This construct was an antibiotic delivery system made of vancomycin alginate beads embedded in a fibrin gel seeded with bone marrow-derived MSCs. The researchers have proposed that local delivery of antibiotics by this system would be more effective than routine systemic delivery in treating bone infections. Furthermore, the presence of MSCs in the system would assist with regeneration of the bone defects [103].

7.9.4 Osteonecrosis

Osteonecrosis or avascular necrosis of the femoral head is a painful condition that may result in the collapse of bone end, necessitating total replacement of the hip joint. Core decompression along with bone graft is often used to treat osteonecrosis. There are several studies in which whole bone marrow or marrow stromal cells have been used to treat osteonecrosis.

In a study by Hernigou et al. in 2002, concentrated bone marrow was injected into the femoral head by a small trocar. The patient was then followed for 5–10 years by Harris hip scoring, radiography, and the need for hip replacement. According to the results, the individuals who received more CFU-F cells have more satisfactory outcomes. In this study there was no control group [104]. In a blinded study that treated osteonecrotic bone by bone marrow cell implantation, Gangji et al. included a control group. In their study, 13 patients (18 hips at stages I and II of osteonecrosis of the femur head) were included. Two years after treatment, significant reductions in pain were observed in the marrow cell implanted patients. Additionally, in this group the numbers of necrotic lesions reduced by 35 %. According to the findings, osteonecrosis worsened in five out of eight hips from the control group compared to one hip from the treatment group [105]. Wang et al. have transplanted 30–50 ml of concentrated marrow into 45 patients with avascular necrosis and observed satisfactory results. In their study, hip replacement was performed for 7 out of 59 femurs [106].

In contrast to the three above-mentioned studies that have used marrow cells, Ji et al. have investigated the transplantation of bone marrow stromal cells along with decalcified bone matrix in osteonecrosis and reported satisfactory outcomes [107].

7.9.5 Nonunion Fractures

Most bone fractures heal spontaneously; the remainder (approximately 15–20 %) do not heal appropriately, resulting in delayed union or nonunion of the bone [108]. In the past, attempts have been made to apply marrow progenitor cells to treat nonunion of the bone [109, 110]. In this context, Hernigou et al. have injected concentrated marrow into 60 noninfected atrophic nonunions of the tibia. Each

nonunion received a relatively constant volume of concentrated bone marrow. The number of progenitor cells transplanted was estimated by counting the CFU-F. The volume of mineralized bone formation was determined by comparing preoperative computerized tomography scans with scans performed 4 months following the injection. According to their findings, there was a positive correlation between the volume of mineralized callus at 4 months and the number and concentration of CFU-F in the graft. There was a negative correlation between the time needed to obtain union and the concentration of FCF-U in the graft [111].

7.9.6 Osteogenesis Imperfecta

OI is a group of inherited heterogeneous disorders of the connective tissue in which production of collagen I is defective. The clinical outcome of the diseases ranges from no clinical signs to short stature, mobility impairments, severe skeletal deformities, and death. In OI, fracture may occur in any bone, however, it is more common in the extremities [112].

A routine treatment for OI is bisphosphonate, which results in increased bone mass and reduction of fracture frequency. MSC therapy would be another option. In this context, Horwitz et al. were the first to use marrow cell potential for treating three children with OI. They transplanted allogeneic bone marrow. Three months after cell engraftment, representative specimens of trabecular bone showed histologic changes indicative of new dense bone formation. In addition, all patients had increased total body bone mineral content compared with predicted values for healthy children with similar changes in weight [113]. Another clinical trial by the same group confirmed the effectiveness of MSC transplantation [114]. As further evidence for the usefulness of stem cell transplantation in treating OI, a report by Panaroni et al. was interesting. They have shown that intrauterine transplantation of adult stem cells resulted in 20 % more synthesis of collagen I in a murine OI model [115].

7.9.7 Hypophosphatasia

Hypophosphatasia, sometimes considered to be a fatal metabolic bone disorder, is an inherited disease characterized by defective bone and teeth mineralization, and deficiency of serum and bone alkaline phosphatase activity. This disorder is due to a mutation in the gene encoding tissue-nonspecific alkaline phosphatase (TNAP) [116]. Some researchers have attempted to use MSCs in treating hypophosphatasia.

Tadokoro et al. have used MSCs to cure an 8-month-old infant. MSCs were obtained by culture expansion of fresh marrow from the patient's father. Some of the MSCs were further cultured under osteogenic conditions on a culture dish or porous hydroxyapatite ceramics, resulting in cultured osteoblasts, and osteogenic

constructs, respectively. The MSCs and osteoblasts were injected into the patient, and the constructs locally implanted. The infant's respiratory condition improved, and the retrieved construct showed de novo bone derived from both donor and patient cells. These researchers demonstrated the importance of allogeneic MSC transplantation for hypophosphatasia and the constructs as an alternative to bone fragments that provided further osteogenic capability in the patient [117].

In a recently conducted research, MSCs obtained from a hypophosphatasia patient were transfected with a vector that carried the TNSALP gene, loaded onto HA scaffolds, and transplanted into nude mice for a period of 6 months. According to the findings, none of the control samples and MSCs/HA composites showed osteogenesis, but osteogenesis was noted in two out of the four transfected cells/HA groups. This approach could provide a promising treatment for hypophosphatasia [118].

7.10 Regeneration of Articular Cartilage

7.10.1 Cartilage Defects Following Trauma

Articular cartilage of the knee joint is often damaged in an athlete after a fall; its repair is considered a challenging surgery for orthopedists. In this context, some authors have attempted to regenerate articular cartilage defects using MSC-based treatments. For example, in a study by Kuroda et al. the researchers have reported successful reconstruction of a 20 × 30 mm full-thickness cartilage defect (international cartilage repair society classification (ICRS) grade IV) in the weight-bearing area of the medial femoral condyle of the right knee in a 31-year-old male who performed judo. They transplanted MSC/collagen gel into the cartilage defects and observed the formation of hyaline cartilage in the histological sections. The patient returned to a normal life 7 months post-implantation [119]. Wakitani et al. have transplanted autologous MSCs combined with collagen gel into two patients with full thickness articular cartilage defects in their patella and reported significant improvements in patient pain and walking ability 6 months post-transplantation [120].

In another clinical trial by Wakitani et al. undifferentiated MSC/collagen membrane was transplanted into articular cartilage defects in the patello-femoral joints of three patients: a 31-year-old female, 44-year-old male, and 45-year-old male. Six months after transplantation, the patients' clinical symptoms improved. The improvements have been maintained over follow-up periods of 17–27 months. Histology of the first patient 1 year after transplantation revealed that the defect had been repaired by fibrocartilaginous tissue. Magnetic resonance imaging of the second patient 1 year after transplantation showed complete coverage of the defect, but they were unable to determine whether the material that covered the defect was hyaline cartilage [121]. In this context, Royan Institute has an ongoing clinical trial.

7.10.2 Osteoarthritis

OA is a group of progressive joint disorders in which the biomechanical characteristics of cartilage changes result in disability, which is referred to as degenerative arthritis associated with joint destruction [122]. This disease progressively involves articular cartilage, subchondral bone, ligaments, and synovial membrane.

Some attempts have been made to treat osteoarthritic joints using MSCs. In this context, a report by Wakitani et al. (2002) on 24 patients with knee OA is remarkable. In this clinical trial, adherent cells from bone marrow aspirates were embedded in collagen gel and transplanted into articular cartilage defects in the medial femoral condyle of 12 patients. The remaining 12 subjects served as cell-free controls [123]. According to their findings, although clinical improvement was not significantly different, the arthroscopic, and histologic grading score was better in the cell-transplanted group.

In the above-mentioned study, MSCs were introduced through an invasive approach (surgery) into the defect. Some authors have attempted to introduce the cells by injection. Using this approach, Centeno et al. have culture-expanded autologous MSCs and transplanted the cells through an intra-articular injection into the knee of a 46-year-old OA patient. They reported that 90 % of the patient's pain was reduced 2 years post-injection [124]. Davachi et al. have used this strategy to introduce cells into the knee joints of four OA patients and reported the strategy as encouraging, but not excellent [125]. Using this strategy we have injected autologous MSCs in six female volunteer patients with knee OA and observed more satisfactory outcomes (unpublished data).

7.10.3 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder that potentially affects numerous tissues and organs, but primarily attacks synovial joints. This systemic autoimmune disease is associated with progressive reduction of the ECM and joint destruction. Proinflammatory cytokines including TNF-alpha and IL-6 are believed to be responsible for the creation of RA symptoms [126, 127]. Current therapy is based mainly on suppressing the symptoms by using analgesia and anti-inflammatory drugs, including steroids. Although such therapy is effective in relieving pain and inflammation, it does not regenerate damaged cartilage. It has been reported that cartilage-regenerating methods including cell-based treatment strategies using autologous chondrocytes do not work in RA since the inflammatory condition in the joint prevents cartilage formation or causes destruction of the newly formed cartilage.

In contrast to chondrocyte-based cell therapy, an allogenic MSC injection would result in a considerable reduction in inflammation and formation of new cartilage in RA due to its immunosuppressive and anti-inflammatory qualities

[128]. In support of this idea, injection of MSCs in the mouse animal model of collagen-induced arthritis (CIA) has prevented severe arthritis and lowered the serum level of inflammatory cytokines [129].

7.11 Conclusion

MSCs possess characteristics that render them appropriate cell candidates for regenerating incurable or difficult to cure bone and cartilage diseases. In the past, several attempts have been made to exploit the capability of MSCs to cure disorders such as large bone defects, distraction osteogenesis, osteonecrosis, nonunion fractures, OA, hypophosphatasia, cartilage defects following trauma, OA, and RA. All these applications are in the trial phase and further investigations are needed to confirm the beneficial effects of MSCs that have been reported by authors worldwide, before MSC-based cell therapy becomes a routine therapeutic procedure.

References

1. Rodan GA (1992) Introduction to bone biology. Bone 13(Suppl 1):S3–S6
2. Green D, Walsh D, Mann S, Oreffo RO (2002) The potential of biomimesis in bone tissue engineering: lessons from the design and synthesis of invertebrate skeletons. Bone 30(6):810–815
3. Posner AS (1985) The mineral of bone. Clin Orthop Relat Res 200:87–99
4. Rey C, Kim HM, Gerstenfeld L, Glimcher MJ (1995) Structural and chemical characteristics and maturation of the calcium–phosphate crystals formed during the calcification of the organic matrix synthesized by chicken osteoblasts in cell culture. J Bone Miner Res 10(10):1577–1588
5. Young MF, Kerr JM, Ibaraki K, Heegaard AM, Robey PG (1992) Structure, expression, and regulation of the major noncollagenous matrix proteins of bone. Clin Orthop Relat Res 281:275–294
6. Robey PG (1996) Vertebrate mineralized matrix proteins: structure and function. Connect Tissue Res 35(1–4):131–136
7. Maurer P, Hohenester E, Engel J (1996) Extracellular calcium-binding proteins. Curr Opin Cell Biol 8(5):609–617
8. Sasaki T, Hohenester E, Gohring W, Timpl R (1998) Crystal structure and mapping by site-directed mutagenesis of the collagen-binding epitope of an activated form of BM-40/SPARC/osteonectin. EMBO J 17(6):1625–1634
9. de Oliveira PT, Nanci A (2004) Nanotexturing of titanium-based surfaces upregulates expression of bone sialoprotein and osteopontin by cultured osteogenic cells. Biomaterials 25(3):403–413
10. Terai K, Takano-Yamamoto T, Ohba Y, Hiura K, Sugimoto M, Sato M, Kawahata H, Inaguma N, Kitamura Y, Nomura S (1999) Role of osteopontin in bone remodeling caused by mechanical stress. J Bone Miner Res 14(6):839–849
11. Ducy P, Geoffroy V, Karsenty G (1996) Study of osteoblast-specific expression of one mouse osteocalcin gene: characterization of the factor binding to OSE2. Connect Tissue Res 35(1–4):7–14
12. Moore KL (1992) Clinically oriented anatomy, 3rd edn. Williams & Wilkins, Baltimore
13. Junqueira LCU, Carneiro J, Contopoulos AN (1975) Basic histology. In: A concise medical library for practitioner and student. p. v. Lange Medical Publications, Los Altos

14. Gartner LP, Hiatt JL (2007) Color textbook of histology, 3rd edn. Saunders/Elsevier, Philadelphia
15. Braddock M, Houston P, Campbell C, Ashcroft P (2001) Born again bone: tissue engineering for bone repair. News Physiol Sci 16:208–213
16. Burchardt H, Enneking WF (1978) Transplantation of bone. Surg Clin North Am 58(2):403–427
17. Gazdag AR, Lane JM, Glaser D, Forster RA (1995) Alternatives to autogenous bone graft: efficacy and indications. J Am Acad Orthop Surg 3(1):1–8
18. Suh H (2000) Tissue restoration, tissue engineering and regenerative medicine. Yonsei Med J 41(6):681–684
19. Ikada Y (2006) Challenges in tissue engineering. J R Soc Interface 3(10):589–601
20. Damien CJ, Parsons JR (1991) Bone graft and bone graft substitutes: a review of current technology and applications. J Appl Biomater 2(3):187–208
21. Finkemeier CG (2002) Bone-grafting and bone-graft substitutes. J Bone Joint Surg Am 84-A(3):454–464
22. Lanza RP, Butler DH, Borland KM, Staruk JE, Faustman DL, Solomon BA, Muller TE, Rupp RG, Maki T, Monaco AP et al (1991) Xenotransplantation of canine, bovine, and porcine islets in diabetic rats without immunosuppression. Proc Natl Acad Sci U S A 88(24):11100–11104
23. Butler D (1998) Poll reveals backing for xenotransplants. Nature 391(6665):315
24. Zhang P, Hamamura K, Yokota H (2008) A brief review of bone adaptation to unloading. Genomics Proteomics Bioinformatics 6(1):4–7
25. Jacobs JJ, Sumner DR, Galante JO (1993) Mechanisms of bone loss associated with total hip replacement. Orthop Clin North Am 24(4):583–590
26. Buckwalter JA (1983) Articular cartilage. Instr Course Lect 32:349–370
27. Poole CA (1997) Articular cartilage chondrons: form, function and failure. J Anat 191(Pt 1): 1–13
28. Bhosale AM, Richardson JB (2008) Articular cartilage: structure, injuries and review of management. Br Med Bull 87:77–95
29. Eyre D (2002) Collagen of articular cartilage. Arthritis Res 4(1):30–35
30. Buckwalter JA, Mankin HJ (1998) Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr Course Lect 47:477–486
31. Darling EM, Hu JC, Athanasiou KA (2004) Zonal and topographical differences in articular cartilage gene expression. J Orthop Res 22(6):1182–1187
32. Becerra J, Andrades JA, Guerado E, Zamora-Navas P, Lopez-Puertas JM, Reddi AH (2010) Articular cartilage: structure and regeneration. Tissue Eng Part B Rev 16(6):617–627
33. Dhinsa BS, Adesida AB (2012) Current clinical therapies for cartilage repair, their limitation and the role of stem cells. Curr Stem Cell Res Ther 7(2):143–148
34. Frenkel SR, Di Cesare PE (1999) Degradation and repair of articular cartilage. Front Biosci 4:D671–D685
35. Mithoefer K, McAdams TR, Scopp JM, Mandelbaum BR (2009) Emerging options for treatment of articular cartilage injury in the athlete. Clin Sports Med 28(1):25–40
36. Buckwalter JA, Lohmander S (1994) Operative treatment of osteoarthrosis. Current practice and future development. J Bone Joint Surg Am 76(9):1405–1418
37. Hangody L, Kish G, Karpati Z, Udvarhelyi I, Szigeti I, Bely M (1998) Mosaicplasty for the treatment of articular cartilage defects: application in clinical practice. Orthopedics 21(7):751–756
38. Rose T, Craatz S, Hepp P, Raczynski C, Weiss J, Josten C, Lill H (2005) The autologous osteochondral transplantation of the knee: clinical results, radiographic findings and histological aspects. Arch Orthop Trauma Surg 125(9):628–637
39. Williams RJ 3rd, Ranawat AS, Potter HG, Carter T, Warren RF (2007) Fresh stored allografts for the treatment of osteochondral defects of the knee. J Bone Joint Surg Am 89(4):718–726

40. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 331(14):889–895
41. Schnabel M, Marlovits S, Eckhoff G, Fichtel I, Gotzen L, Vecsei V, Schlegel J (2002) Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthr Cartil 10(1):62–70
42. Friedenstein AJ, Chailakhjan RK, Lalykina KS (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3(4):393–403
43. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. Cytotherapy 8(4):315–317
44. Robertson EJ (1997) Derivation and maintenance of embryonic stem cell cultures. Methods Mol Biol 75:173–184. doi:10.1385/0-89603-441-0:173
45. Woll NL, Heaney JD, Bronson SK (2006) Osteogenic nodule formation from single embryonic stem cell-derived progenitors. Stem Cells Dev 15(6):865–879
46. Nakagawa T, Lee SY, Reddi AH (2009) Induction of chondrogenesis from human embryonic stem cells without embryoid body formation by bone morphogenetic protein 7 and transforming growth factor beta 1. Arthritis Rheum 60(12):3686–3692
47. Daar AS, Bhatt A, Court E, Singer PA (2004) Stem cell research and transplantation: science leading ethics. Transplant Proc 36(8):2504–2506
48. Knoepfler PS (2009) Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. Stem Cells 27(5):1050–1056. doi:10.1002/stem.37
49. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
50. Noth U, Osyczka AM, Tuli R, Hickok NJ, Danielson KG, Tuan RS (2002) Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells. J Orthop Res 20(5):1060–1069
51. De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP (2001) Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum 44(8):1928–1942
52. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7(2):211–228
53. Bosch P, Musgrave DS, Lee JY, Cummins J, Shuler T, Ghivizzani TC, Evans T, Robbins TD (2000) Huard: osteoprogenitor cells within skeletal muscle. J Orthop Res 18(6):933–944
54. Nakahara H, Goldberg VM, Caplan AI (1991) Culture-expanded human periosteal-derived cells exhibit osteochondral potential in vivo. J Orthop Res 9(4):465–476
55. Young HE, Steele TA, Bray RA, Hudson J, Floyd JA, Hawkins K, Thomas K, Austin T, Edwards C, Cuzzourt J, Duenzl M, Lucas PA, Black AC Jr (2001) Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec 264(1):51–62
56. He Q, Wan C, Li G (2007) Concise review: multipotent mesenchymal stromal cells in blood. Stem Cells 25(1):69–77
57. Huang GT, Gronthos S, Shi S (2009) Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 88(9):792–806
58. Baghaban Eslaminejad M, Jahangir S, Aghdami N (2011) Mesenchymal stem cells from murine amniotic fluid as a model for preclinical investigation. Arch Iran Med 14(2):96–103
59. Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DO, Tukun A, Uckan D, Can A (2007) Biology of stem cells in human umbilical cord stroma: in situ and in vitro surveys. Stem Cells 25(2):319–331
60. Friedenstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4(5):267–274

61. Chamberlain G, Fox J, Ashton B, Middleton J (2007) Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells 25(11):2739–2749
62. Javazon EH, Beggs KJ, Flake AW (2004) Mesenchymal stem cells: paradoxes of passaging. Exp Hematol 32(5):414–425
63. DelaRosa O, Lombardo E, Beraza A, Mancheno-Corvo P, Ramirez C, Menta R, Rico L, Camarillo E, Garcia L, Abad JL, Trigueros C, Delgado M, Buscher D (2009) Requirement of IFN-gamma-mediated indoleamine 2,3-dioxygenase expression in the modulation of lymphocyte proliferation by human adipose-derived stem cells. Tissue Eng Part A 15(10):2795–2806
64. Ryan JM, Barry F, Murphy JM, Mahon BP (2007) Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol 149(2):353–363
65. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, Risso M, Gualandi F, Mancardi GL, Pistoia V, Uccelli A (2006) Human mesenchymal stem cells modulate B-cell functions. Blood 107(1):367–372
66. Asari S, Itakura S, Ferreri K, Liu CP, Kuroda Y, Kandeel F, Mullen Y (2009) Mesenchymal stem cells suppress B-cell terminal differentiation. Exp Hematol 37(5):604–615
67. Ramasamy R, Fazekasova H, Lam EW, Soeiro I, Lombardi G, Dazzi F (2007) Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle. Transplantation 83(1):71–76
68. Zhang W, Ge W, Li C, You S, Liao L, Han Q, Deng W, Zhao RC (2004) Effects of mesenchymal stem cells on differentiation, maturation, and function of human monocyte-derived dendritic cells. Stem Cells Dev 13(3):263–271
69. Fox JM, Chamberlain G, Ashton BA, Middleton J (2007) Recent advances into the understanding of mesenchymal stem cell trafficking. Br J Haematol 137(6):491–502
70. Granero-Molto F, Weis JA, Miga MI, Landis B, Myers TJ, O'Rear L, Longobardi L, Jansen ED, Mortlock DP, Spagnoli A (2009) Regenerative effects of transplanted mesenchymal stem cells in fracture healing. Stem Cells 27(8):1887–1898
71. Granero-Molto F, Weis JA, Longobardi L, Spagnoli A (2008) Role of mesenchymal stem cells in regenerative medicine: application to bone and cartilage repair. Expert Opin Biol Ther 8(3):255–268
72. Lapidot T, Dar A, Kollet O (2005) How do stem cells find their way home? Blood 106(6):1901–1910
73. Habraken WJ, Wolke JG, Jansen JA (2007) Ceramic composites as matrices and scaffolds for drug delivery in tissue engineering. Adv Drug Deliv Rev 59(4–5):234–248
74. Gladstone HB, McDermott MW, Cooke DD (1995) Implants for cranioplasty. Otolaryngol Clin North Am 28(2):381–400
75. Lee SH, Shin H (2007) Matrices and scaffolds for delivery of bioactive molecules in bone and cartilage tissue engineering. Adv Drug Deliv Rev 59(4–5):339–359
76. Middleton JC, Tipton AJ (2000) Synthetic biodegradable polymers as orthopedic devices. Biomaterials 21(23):2335–2346
77. Eslaminejad MB, Mirzadeh H, Mohamadi Y, Nickmahzar A (2007) Bone differentiation of marrow-derived mesenchymal stem cells using beta-tricalcium phosphate–alginate–gelatin hybrid scaffolds. J Tissue Eng Regen Med 1(6):417–424
78. Zandi M, Mirzadeh H, Mayer C, Urch H, Eslaminejad MB, Bagheri F, Mivehchi H (2010) Biocompatibility evaluation of nano-rod hydroxyapatite/gelatin coated with nano-HAp as a novel scaffold using mesenchymal stem cells. J Biomed Mater Res A 92(4):1244–1255
79. Wang M (2006) Composite scaffolds for bone tissue engineering. Am J Biochem Biotechnol 2(2):80–84
80. Chajra H, Rousseau CF, Cortial D, Ronziere MC, Herbage D, Mallein-Gerin F, Freyria AM (2008) Collagen-based biomaterials and cartilage engineering. Application to osteochondral defects. Biomed Mater Eng 18(1 Suppl):S33–S45

81. Wakitani S, Goto T, Young RG, Mansour JM, Goldberg VM, Caplan AI (1998) Repair of large full-thickness articular cartilage defects with allograft articular chondrocytes embedded in a collagen gel. Tissue Eng 4(4):429–444
82. Knudson W, Casey B, Nishida Y, Eger W, Kuettner KE, Knudson CB (2000) Hyaluronan oligosaccharides perturb cartilage matrix homeostasis and induce chondrocytic chondrolysis. Arthritis Rheum 43(5):1165–1174
83. Carranza-Bencano A, Armas-Padron JR, Gili-Miner M, Lozano MA (2000) Carbon fiber implants in osteochondral defects of the rabbit patella. Biomaterials 21(21):2171–2176
84. Defrere J, Franckart A (1992) Teflon/polyurethane arthroplasty of the knee: the first 2 years preliminary clinical experience in a new concept of artificial resurfacing of full thickness cartilage lesions of the knee. Acta Chir Belg 92(5):217–227
85. Ferretti M, Marra KG, Kobayashi K, Defail AJ, Chu CR (2006) Controlled in vivo degradation of genipin crosslinked polyethylene glycol hydrogels within osteochondral defects. Tissue Eng 12(9):2657–2663
86. Wang X, Grogan SP, Rieser F, Winkelmann V, Maquet V, Berge ML, Mainil-Varlet P (2004) Tissue engineering of biphasic cartilage constructs using various biodegradable scaffolds: an in vitro study. Biomaterials 25(17):3681–3688
87. Sharma B, Williams CG, Khan M, Manson P, Elisseeff JH (2007) In vivo chondrogenesis of mesenchymal stem cells in a photopolymerized hydrogel. Plast Reconstr Surg 119(1):112–120
88. Yoon BS, Lyons KM (2004) Multiple functions of BMPs in chondrogenesis. J Cell Biochem 93(1):93–103
89. Matsuda N, Lin WL, Kumar NM, Cho MI, Genco RJ (1992) Mitogenic, chemotactic, and synthetic responses of rat periodontal ligament fibroblastic cells to polypeptide growth factors in vitro. J Periodontol 63(6):515–525
90. Chen FM, Zhao YM, Wu H, Deng ZH, Wang QT, Zhou W, Liu Q, Dong GY, Li K, Wu ZF, Jin Y (2006) Enhancement of periodontal tissue regeneration by locally controlled delivery of insulin-like growth factor-I from dextran-co-gelatin microspheres. J Control Release 114(2):209–222
91. Kato T, Kawaguchi H, Hanada K, Aoyama I, Hiyama Y, Nakamura T, Kuzutani K, Tamura M, Kurokawa T, Nakamura K (1998) Single local injection of recombinant fibroblast growth factor-2 stimulates healing of segmental bone defects in rabbits. J Orthop Res 16(6):654–659
92. Grimaud E, Heymann D, Redini F (2002) Recent advances in TGF-beta effects on chondrocyte metabolism. Potential therapeutic roles of TGF-beta in cartilage disorders. Cytokine Growth Factor Rev 13(3):241–257
93. Sekiya I, Larson BL, Vuoristo JT, Reger RL, Prockop DJ (2005) Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma. Cell Tissue Res 320(2):269–276
94. Solchaga LA, Penick K, Porter JD, Goldberg VM, Caplan AI, Welter JF (2005) FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. J Cell Physiol 203(2):398–409
95. Vinatier C, Bouffi C, Merceron C, Gordeladze J, Brondello JM, Jorgensen C, Weiss P, Guicheux J, Noel D (2009) Cartilage tissue engineering: towards a biomaterial-assisted mesenchymal stem cell therapy. Curr Stem Cell Res Ther 4(4):318–329
96. Bruder SP, Kraus KH, Goldberg VM, Kadiyala S (1998) The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am 80(7):985–996
97. Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, Kon E, Marcacci M (2001) Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med 344(5):385–386
98. Morishita T, Honoki K, Ohgushi H, Kotobuki N, Matsushima A, Takakura Y (2006) Tissue engineering approach to the treatment of bone tumors: three cases of cultured bone grafts derived from patients' mesenchymal stem cells. Artif Organs 30(2):115–118
99. Gronthos S (2004) Reconstruction of human mandible by tissue engineering. Lancet 364(9436):735–736

100. Paley D, Herzenberg JE, Paremain G, Bhave A (1997) Femoral lengthening over an intramedullary nail. A matched-case comparison with Ilizarov femoral lengthening. J Bone Joint Surg Am 79(10):1464–1480
101. Kitoh H, Kitakoji T, Tsuchiya H, Mitsuyama H, Nakamura H, Katoh M, Ishiguro N (2004) Transplantation of marrow-derived mesenchymal stem cells and platelet-rich plasma during distraction osteogenesis—a preliminary result of three cases. Bone 35(4):892–898
102. Sia IG, Berbari EF (2006) Infection and musculoskeletal conditions: osteomyelitis. Best Pract Res Clin Rheumatol 20(6):1065–1081
103. Hou T, Xu J, Li Q, Feng J, Zen L (2008) In vitro evaluation of a fibrin gel antibiotic delivery system containing mesenchymal stem cells and vancomycin alginate beads for treating bone infections and facilitating bone formation. Tissue Eng Part A 14(7):1173–1182
104. Hernigou P, Beaujean F (2002) Treatment of osteonecrosis with autologous bone marrow grafting. Clin Orthop Relat Res 405:14–23
105. Gangji V, Hauzeur JP, Matos C, De Maertelaer V, Toungouz M, Lambermont M (2004) Treatment of osteonecrosis of the femoral head with implantation of autologous bone-marrow cells. A pilot study. J Bone Joint Surg Am 86-A(6):1153–1160
106. Wang BL, Sun W, Shi ZC, Zhang NF, Yue DB, Guo WS, Xu SQ, Lou JN, Li ZR (2010) Treatment of nontraumatic osteonecrosis of the femoral head with the implantation of core decompression and concentrated autologous bone marrow containing mononuclear cells. Arch Orthop Trauma Surg 130(7):859–865
107. Ji WF, Ding WH, Ma ZC, Li J, Tong PJ (2008) Three-tunnels core decompression with implantation of bone marrow stromal cells (bMSCs) and decalcified bone matrix (DBM) for the treatment of early femoral head necrosis. Zhongguo Gu Shang 21(10):776–778
108. Marsh D (1998) Concepts of fracture union, delayed union, and nonunion. Clin Orthop Relat Res (355 Suppl):S22–S30
109. Galois L, Bensoussan D, Diligent J, Pinzano A, Henrionnet C, Choufani E, Stoltz JF, Mainard D (2009) Autologous bone marrow graft and treatment of delayed and non-unions of long bones: technical aspects. Biomed Mater Eng 19(4–5):277–281
110. Siwach RC, Sangwan SS, Singh R, Goel A (2001) Role of percutaneous bone marrow grafting in delayed unions, non-unions and poor regenerates. Indian J Med Sci 55(6):326–336
111. Hernigou P, Poignard A, Beaujean F, Rouard H (2005) Percutaneous autologous bone-marrow grafting for nonunions. Influence of the number and concentration of progenitor cells. J Bone Joint Surg Am 87(7):1430–1437
112. Scaramuzzo L, Raffaelli L, Spinelli MS, Damis G, Maccauro G, Manicone PF (2011) Orthopaedic and dental abnormalities in osteogenesis imperfecta: a review of the literature. J Biol Regul Homeost Agents 25(3):313–321
113. Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK (1999) Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 5(3):309–313
114. Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY, Muul L, Hofmann T (2002) Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. Proc Natl Acad Sci U S A 99(13):8932–8937
115. Panaroni C, Gioia R, Lupi A, Besio R, Goldstein SA, Kreider J, Leikin S, Vera JC, Mertz EL, Perilli E, Baruffaldi F, Villa I, Farina A, Casasco M, Cetta G, Rossi A, Frattini A, Marini JC, Vezzoni P, Forlino A (2009) In utero transplantation of adult bone marrow decreases perinatal lethality and rescues the bone phenotype in the knockin murine model for classical, dominant osteogenesis imperfecta. Blood 114(2):459–468
116. Fraser D (1957) Hypophosphatasia. Am J Med 22(5):730–746
117. Tadokoro M, Kanai R, Taketani T, Uchio Y, Yamaguchi S, Ohgushi H (2009) New bone formation by allogeneic mesenchymal stem cell transplantation in a patient with perinatal hypophosphatasia. J Pediatr 154(6):924–930

118. Katsube Y, Kotobuki N, Tadokoro M, Kanai R, Taketani T, Yamaguchi S, Ohgushi H (2010) Restoration of cellular function of mesenchymal stem cells from a hypophosphatasia patient. Gene Ther 17(4):494–502
119. Kuroda R, Ishida K, Matsumoto T, Akisue T, Fujioka H, Mizuno K, Ohgushi H, Wakitani S, Kurosaka M (2007) Treatment of a full-thickness articular cartilage defect in the femoral condyle of an athlete with autologous bone-marrow stromal cells. Osteoarthr Cartil 15(2):226–231
120. Wakitani S, Mitsuoka T, Nakamura N, Toritsuka Y, Nakamura Y, Horibe S (2004) Autologous bone marrow stromal cell transplantation for repair of full-thickness articular cartilage defects in human patellae: two case reports. Cell Transplant 13(5):595–600
121. Wakitani S, Nawata M, Tensho K, Okabe T, Machida H, Ohgushi H (2007) Repair of articular cartilage defects in the patello-femoral joint with autologous bone marrow mesenchymal cell transplantation: three case reports involving nine defects in five knees. J Tissue Eng Regen Med 1(1):74–79
122. Buckwalter JA, Mankin HJ (1998) Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. Instr Course Lect 47:487–504
123. Wakitani S, Imoto K, Yamamoto T, Saito M, Murata N, Yoneda M (2002) Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. Osteoarthr Cartil 10(3):199–206
124. Centeno CJ, Busse D, Kisiday J, Keohan C, Freeman M, Karli D (2008) Increased knee cartilage volume in degenerative joint disease using percutaneously implanted, autologous mesenchymal stem cells. Pain Physician 11(3):343–353
125. Davatchi F, Abdollahi BS, Mohyeddin M, Shahram F, Nikbin B (2011) Mesenchymal stem cell therapy for knee osteoarthritis. Preliminary report of four patients. Int J Rheum Dis 14(2):211–215
126. Taylor PC, Feldmann M (2009) Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis. Nat Rev Rheumatol 5(10):578–582
127. Nishimoto N (2010) Interleukin-6 as a therapeutic target in candidate inflammatory diseases. Clin Pharmacol Ther 87(4):483–487
128. Ringe J, Sittinger M (2009) Tissue engineering in the rheumatic diseases. Arthritis Res Ther 11(1):211
129. Augello A, Tasso R, Negrini SM, Cancedda R, Pennesi G (2007) Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis. Arthritis Rheum 56(4):1175–1186

Chapter 8
Pancreatic Reprogramming

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Abstract Hidden behind the hype of prospective stem cell-based approaches to treat human disease, reprogramming techniques have finally entered the landscape of regenerative medicine and are quickly becoming one of the most exciting and powerful weapons in the field. In the context of pancreatic regeneration, the reprogramming of non-endocrine adult tissues to cells with phenotypes resembling to those of the hormone-producing cells of the islets of Langerhans is a fertile and dynamic area of research. Here we analyze two of the most studied sources of reprogrammable cells, namely the liver and the acinar compartment of the pancreas. Several groups have now established that the ectopic expression of master pancreatic regulators such as Pdx1, MafA, Ngn3, or BETA2/NeuroD can result in variable degrees of reprogramming toward pancreatic endocrine fates, leading to insulin production in vitro, and reversal of hyperglycemia in vivo. The state of the art and clinical prospects of these novel approaches are discussed in the following chapter.

8.1 Introduction

Reprogramming techniques allow for a swift change of phenotype between different cell types. Unlike differentiation, in which there is a progressive education toward terminal specification from more immature stem or progenitor cells, reprogramming (also termed transdifferentiation) does not have a clear directionality or require the starting cell type to be less differentiated than the ending one.

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The potential of this new tool to produce insulin-producing beta-like cells from other non-stem adult cell sources has not gone unnoticed, and is the subject of the present chapter.

The current prominence of reprogramming as a key element in the repertoire of techniques to induce tissue regeneration could arguably be traced back to the cloning of Dolly the sheep in 1996 [1], which marked the development of somatic cell nuclear transfer (SCNT). This novel technique allowed for the resetting of the epigenetic signature of terminally differentiated mammalian cells by means of the microinjection of their nuclei into enucleated oocytes. Embryos reconstituted in this manner would develop in vitro to the blastocyst stage, from which pluripotent embryonic stem (ES) cells could be derived –thus effectively making a cell "go back in time" to its embryonic pluripotent state.

Such biotechnological feat opened the door to the concept of "therapeutic cloning", which was successfully established in mammalian species as high as non-human primates [2, 3], although not in humans despite early reports that had to be later retracted [4–8]. However, because obtaining dedifferentiation by means of oocyte microinjection was expensive and technically challenging, alternatives that made use of progressively simpler reprogramming agents (first ES cells instead of oocytes [9–12] and then cellular extracts [13–16]) were evolved to the point in which the addition of just a few "master genes" was sufficient to override the cell program responsible for its phenotypic identity. That was precisely the breakthrough achievement of Takahashi and colleagues [17], who reprogrammed murine somatic cells into ES cell-like induced pluripotent stem (iPS) cells by stably transfecting them with four components of the core ES cell circuitry, namely Oct3/4, Klf4, c-Myc, and Sox2. The same results were obtained when the approach was used with cells of human origin, be it with the above "reprogramming cocktail" or one in which Klf4 and c-Myc were replaced by Lin28 and Nanog [18, 19]. In a matter of just a few years, we have witnessed the rise of iPS cells as the purported successors of ES cells and their (relative) fall, chiefly on the account of issues regarding the premature senescence of some of their derivatives [20], their residual epigenetic memory [21–24] and the induction of potentially oncogenic mutations during the process of reprogramming [25–28].

Be it as it may, the consensus is that reprogramming techniques are here to stay, and thus it is expected that their use for the generation of other cell types will recapitulate the steps already described for iPS cells. Especially, relevant in this context are the current efforts to replace the original DNA-based reprogramming strategies [18, 19] by safer, more easily controllable choices such as DNA minicircles [29], episomal vectors [30], transducible proteins [31], and synthetic mRNAs [32].

The pancreas has been from very early on a leading candidate for reprogramming from other tissues. Even before the development of the above technology, the interconversion between liver and pancreas had been extensively studied. From an evolutionary perspective, the fact that some invertebrates have a single organ that acts both as liver and pancreas indicates that the separation of both organs is a relatively recent occurrence. Higher in the evolutionary scale, the cardiac

mesoderm is known to signal the ventral endoderm to instruct early liver specification in vertebrates [33–35], and it has been demonstrated that both organs stem out from a common pool of endodermal progenitors in the early foregut [35–43]. A potential mechanism behind this specification was proposed FGF-dependent [37], and the proximity to the FGF-producing cardiac mesoderm would be the main factor dictating pancreatic (distal) or hepatic (proximal) specification from this progenitor pool.

Similarities between liver and pancreas are evidenced not only by their shared ancestry but also by the makeup of key cellular hardware, such as that needed for glucose secretion and sensing. In fact, it is known that virtually 100 % of the mammalian reservoir of the glucose-sensing enzyme glucokinase, which is responsible for the systemic maintenance of glucose homeostasis by regulating the balance between insulin release and glycogen storage, is restricted to hepatocytes and beta cells [44–46]. Because of this close relationship between the two organs, it does not come as a surprise that certain experimental conditions result in the interconversion of liver and pancreas. Pancreas is typically converted to liver in rats subjected to a copper-depleted diet [47–49] and upon treatment with dexamethasone [50]. The opposite has also been observed following exposure to diethylnitrosamine [51] or as a consequence of tumoral processes [52].

8.2 Directed Reprogramming of Liver to Pancreas

Because of the above evidence, the liver stood out fairly soon as one strong candidate for pancreatic cell reprogramming. As expected, the quest for specific factors that could act as post-developmental switches between liver and pancreas started with the study of Pdx1. The pancreatic and duodenal homeobox 1 (Pdx1) gene is preferentially expressed in the beta cells of the islet, where it exerts a tight control over insulin secretion by binding to the insulin promoter [53]. Because of this role, Pdx1 is the main responsible for the maintenance of the beta cell identity. Experiments in which Pdx1 is conditionally knocked out in adulthood [54, 55] revealed that heterozygous mice display an age-dependent decrease in glucose tolerance, impaired glucose-stimulated insulin release, and higher rates of beta cell apoptosis [56]. The full beta cell-specific knockout resulted in total loss of the beta cell phenotype and mature onset of diabetes [54]. The defective glucose-mediated insulin response in *P. obesus*, a type 2 diabetes mouse model, was also linked to Pdx1 deficiency [57, 58].

The influence of Pdx1 in pancreatic development has also been extensively established. Its expression around e8.5 (10—somite stage) in the murine foregut is the true hallmark of pancreatic versus liver specification. Embryos homozygous for Pdx1 inactivating mutations are born without pancreas [59], a phenotype that has also been described in humans with a single nucleotide mutation of the gene [60]. Coincident with a down-regulation of the pro-intestinal factor *sonic hedgehog (Shh)*, Pdx1 expression will delineate the regions that will later become the dorsal

and ventral pancreatic buds up to e10. At this time, Pdx1 expression decreases, only to resume in the cells that will become beta cells from e11.5 onwards [61]. However, because early events of pancreatic organogenesis occur even in the absence of functional Pdx1, it was hypothesized that this gene needed other factors to coordinate the progression of pancreatic development [62]. Ptf1/p48, a factor whose expression marks exocrine cell specification and phenotype maintenance at a later time, might be one such adjuvant of Pdx1 at the beginning of pancreatic specification [63–66].

The importance of Pdx1 as a "master regulator" of all the molecular events leading first to the specification of the pancreas and then to the formation and maintenance of beta cells led to the hypothesis that its ectopic expression might induce a phenotype switch consistent with the acquisition of beta cell characteristics. The results of this approach when using a variety of cellular substrates, including ES cells and enterocytes, were not particularly encouraging [67–69]. However, when Pdx1 was expressed in liver cells, effective conversion was achieved [70]. The strategy consisted in transducing an adenoviral-based Pdx1 into live mice, which led to preferential expression in the liver and resulted in the downstream activation of insulin 1 and 2 as well as prohormone convertase 1/3 (PC 1/3). A significant increase in circulating insulin levels was detected in treated mice versus the control animals treated with the empty vector alone. In another set of experiments, when the mice were rendered diabetic by means of streptozotocin prior to Pdx1 transduction, the treatment was associated with a permanent reduction of hyperglycemia. Immunohystochemical analysis revealed a number of insulin-positive cells near the central veins of the liver tissue of the recipients, which was theorized to favor the systemic release of insulin.

A key aspect of these results was the subsequent observation that the expression of Pdx1 in the liver persisted long after the adenoviral vehicles had been presumptively cleared from the system. Indeed, exogenous Pdx1 was shown to induce the expression of its endogenous counterpart (a previously documented feedback effect [71, 72]), which in turn was responsible for triggering an irreversible transdifferentiation cascade. This was manifested by the persistence of liver insulin expression and maintenance of normal blood glucose levels for at least 8 months in animals that had been previously rendered diabetic [73]. Pdx1-mediated transdifferentiation was later shown to require a prior 'de-differentiation' step based on the down-regulation of LAP and C/EBPbeta, two redundant proteins that belong to a family of transcription factors involved not only in liver embryonic development but also in the phenotypic identity of adult hepatocytes [74, 75]. Indeed, LAP overexpression in cultured hepatocytes prevented the induction of dedifferentiation and subsequent pancreatic conversion mediated by Pdx1. However, the mere down-regulation of C/EBPbeta alone failed to induce the expression of pancreatic genes other than the pro-endocrine factor Ngn3. As predicted by the authors of the study, the simultaneous administration of Pdx1 and knockdown of C/EBPbeta had a synergistic liver-to-pancreas reprogramming effect.

Were these results due to the conversion of fully differentiated hepatocytes, or perhaps a consequence of the unintended targeting of a rarer subpopulation of

potentially more malleable liver progenitors? Unfortunately, in vitro experiments are rather uninformative because, like most other epithelial cell types subjected to regular culture conditions, hepatocyte populations rapidly become fibroblastic when attached to plastic. Hence, the significance of the experiments designed by Horb and collaborators [76]. These investigators generated transgenic frogs in which the gene Xlhbox8 (the amphibian equivalent to the mammalian Pdx1 [77]) was placed under the control of the early liver-specific promoter for transthyretin (TTR). Of note, the Xlhbox8 gene was "reinforced" by the addition of a VP16 fusion domain. VP16 is a powerful transcriptional transactivator from the herpes simplex virus (HSV) [78, 79], and its inclusion in the cassette obeyed to the rationale that non-pancreatic cells may lack developmentally relevant molecular Pdx1 adjuvants that may be needed for it to be fully functional. A green fluorescent protein (GFP) marker was placed under the control of the pancreatic elastase promoter and added to the transgenic construction in order to tag cell derivatives in which the liver-to-pancreas conversion had been successful. Up to 60 % transgenic tadpoles showed not only GFP, but also insulin, glucagon, and amylase expression in their liver. Of note, the approach did not work when using a VP16-less Xlhbox8 cassette, which adds weight to the notion that, while necessary, Pdx1 may not be sufficient by itself to promote pancreatic transdifferentiation. Another important point worth mentioning is that, because TTR is expressed very early during liver specification, it could be argued that the expression of Xlhbox8 at that time merely helped redirect the normal course of development of multipotent progenitors toward a pancreatic fate. Strictly speaking, this would not be a reprogramming phenomenon. To address this concern, the authors proceeded to transfect immortalized human hepatocytes (HepG2) with the same construct. As previously seen in the transgenic setting, up to 65 % of the transfected cells activated elastase expression. Of these, nearly 15 % were insulin-positive. Confirming earlier observations, the hepatic phenotype was found to be lost in a Pdx1-mediated manner, suggesting that a dedifferentiation step precedes reprogramming toward beta cells. The resulting insulin-producing cells were also PC 1/3, C-peptide, and glucagon-like receptor 1 (GLP-1)-positive, responded to glucose stimulation and increased the secretion of insulin following treatment with betacellulin and GLP-1 [80].

The first systematic analysis of Pdx1 alone versus "enhanced" versions of Pdx1 with VP16 was conducted by Tang and colleagues [81], who, in contrast with the results previously reported by the team that developed the transgenic Xlhbox8-VP16 frogs, were successful at reprogramming rat hepatic cells into pancreatic-like beta cells both with Pdx1 and Pdx1-VP16. Insulin-expressing cells generated through both types of constructs were able to correct diabetes in mice. In the short term, however, the VP16 version of the gene proved slightly superior.

Additional studies showed the synergistic effect of combining Pdx1 with other pancreatic transcription factors such as BETA2/NeuroD, Ngn3, or MafA [82–88], once more confirming that Pdx1 may require the help of other molecular partners to accomplish full transdifferentiation. An interesting study by Wang and collaborators [82] added an unexpected twist to the story by describing the inability of

Pdx1 and Ngn3 to induce in vivo liver-to-pancreas reprogramming when using adeno-associated viruses (AAV), but not when using plasmids together with an irrelevant adenoviral (AV) vector. Because the capsid of adenoviruses (but not AAV) is known to elicit an antigen-dependent immune response, these observations present yet another element that may be instrumental in inducing liver-to-pancreas transdifferentiation.

8.2.1 Acinar Tissue Reprogramming

The exploitation of the liver as a potential source of new beta cells by means of reprogramming was a fertile research field in the early 2000s. However, the enthusiasm had started to plateau by the end of the decade after a plethora of studies failed to capitalize on the promise that hepatocytes could be converted into *bona fide* beta cells potentially usable for the clinical treatment of diabetes. In this context, the recent reports on another source of reprogrammable tissue, the acinar compartment of the pancreas, seem to have revitalized the field again.

Acinar exocrine cells are extraordinarily plastic, as evidenced by their ability to turn into ductal cells [89, 90], hepatic cells [91], and even beta-like cells [92–95], the latter confirmed even by lineage-tracing techniques [96]. The combination of simple growth factors such as leukemia inhibitory factor (LIF) and epidermal growth factor (EGF) in vitro has been shown to reactivate Ngn3 expression, which precedes the acquisition of a beta-like phenotype [97]. During embryonic development, the pro-endocrine factor Ngn3 is activated in a Notch-dependent fashion. Cells in which Notch signaling is inactivated down-regulate the Ngn3 repressor HES-1, an event that unleashes Ngn3 expression and therefore endocrine cell specification [98–105]. Based on this, Baeyens and colleagues [106] proposed that down-regulation of Notch in terminally differentiated acinar cells in vitro could also foster acinar to endocrine cell conversion, a hypothesis that was proven correct in the above LIF + EGF in vitro model.

A new wave of gene-based efforts at selectively converting acinar tissue to beta cells was pioneered by Zhou and coworkers [107], who initiated their research by conducting a systematic and combinatorial screening of 20 transcription factors known to have a drastic effect in beta cell development [108, 109]. The combination of Pdx1, Ngn3, and MafA turned out to be the only one conducive to in vivo reprogramming when shuttled in adenoviruses and injected directly in the acinar-rich pancreatic parenchyma. This is not surprising in view of earlier attempts to transdifferentiate liver cells in which the use of Pdx1, MafA, and BETA2/NeuroD (a downstream target of Ngn3 [110]) had proven superior to other combinations of genes. Similarly, adenoviruses expressing the above genes also induced the transdifferentiation of porcine neonatal pancreatic cell clusters [111].

Of note, in this set of experiments the investigators used Rag1-/- mice, a strain typically used to minimize the occurrence of viral-induced immune responses such as those earlier indicated by Wang and collaborators [82]. As early as 3 days after

the delivery of the viral mix, widespread neogenic insulin-producing cells were detected throughout the exocrine compartment of the pancreas in the proximity of the injection site. The mass of these cells expanded for at least 3 months (well beyond the life expectancy of the adenovirus in the system), once again confirming the notion that reprogramming only requires an initial push before a self-sustained endogenous program takes over the maintenance of the process. The resulting insulin-producing cells were virtually identical to those found in islets, as judged by a rigorous examination of molecular and histological markers. When diabetic mice were treated in this manner, long-term improvement of their hyperglycemic state was noted. Diabetes, however, was not completely reversed. To account for this observation, the authors contended that the neogenic beta cells never congregated in islet-like structures, rather remaining interspersed throughout the parenchyma. Since beta-to-beta cell communication is paramount for the appropriate coupling of the glucose sensing–insulin secreting machinery [112, 113], the relative isolation of these cells could indeed be the reason for their failure to correct the diabetic state of the recipient mice. More recently, the above results were partially confirmed in vitro using the AR42 J acinar cell line [114]. The addition of the zinc-finger islet transcription factor INSM1/IA-1 (which, like BETA2/NeuroD, is located just downstream Ngn3 in the differentiation cascade [115]), was reported to enhance the transdifferentiation potential of Pdx1 and other factors.

8.3 Conclusions

This is a rapidly evolving field, but there is still a tremendous amount of basic research to be done before translation to clinical therapies for diabetes and other conditions characterized by beta cell loss can be envisioned. First, be it from the liver or from the acinar tissue, it is difficult to ascertain at this time if an in vivo microenvironment is a *conditio sine qua non* for efficacious reprogramming. This would seem the preliminary conclusion form the liver studies, whose results have failed to unequivocally establish that true beta cells capable of restoring normoglycemia upon transplantation in diabetic animals can be obtained in vitro. Despite some degree of functionality, in vitro transdifferentiated liver cells do not seem to be *bona fide* beta cells. As it is the case in the maturation of beta cells from pluripotent cell types, the physiological niche provided by a live host may contain key, yet undiscovered factors that make all the difference between a beta-like cell [116] and a true beta cell [117]. The potential role of the immune system in aiding this process, as evidenced by the experiments in which a combination of transgenes caused reprogramming when delivered together with AV, but not through AAV (which otherwise induced similar, if not higher levels of expression) cannot be ruled out.

In vivo interventions would obviously require the development of more "therapy-friendly" approaches to induce reprogramming, especially because the

use of viruses is dangerous and faces a substantial amount of regulatory hurdles [87, 88]. In this context, a number of non DNA-based reprogramming strategies currently under development [105, 118, 119] could have a significant impact down the road. However, before we dismiss the in vitro approach altogether, we should consider that reprogrammed cells may not need to be "true" beta cells to be therapeutically effective. Even if these pseudo-beta cells worked just as an insulin pump, continuously secreting a basal amount of insulin in a non-regulated manner, a therapy based on such cells could still make a tremendous difference in the quality of life of diabetic patients. Indeed, because in type 1 diabetes it is the "true" beta cell the one to elicit the autoimmune attack, we cannot reject the possibility that pseudo-beta cells may have a selective advantage over native ones. Although insulin itself has been shown to be an autoantibody in type 1 diabetes [120–122], the unique surface makeup of these hybrid cells may potentially help evade or reduce the autoimmune response.

Whenever there is a pancreatic islet isolation, the acinar tissue is invariably discarded. Considering that the exocrine cells of the pancreas are represented in a 50:1 ratio versus islets, the utilization of this cell source could dramatically expand the usage of each organ for therapeutic purposes.

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References

1. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385(6619):810–813
2. Munsie MJ, Michalska AE, O'Brien CM, Trounson AO, Pera MF, Mountford PS (2000) Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei. Curr Biol 10(16):989–992
3. Byrne JA, Pedersen DA, Clepper LL, Nelson M, Sanger WG, Gokhale S, Wolf DP, Mitalipov SM (2007) Producing primate embryonic stem cells by somatic cell nuclear transfer. Nature 450(7169):497–502
4. Hwang WS, Lee BC, Lee CK, Kang SK (2005) Cloned human embryonic stem cells for tissue repair and transplantation. Stem Cell Rev 1(2):99–109
5. Hwang WS, Roh SI, Lee BC, Kang SK, Kwon DK, Kim S, Kim SJ, Park SW, Kwon HS, Lee CK, Lee JB, Kim JM, Ahn C, Paek SH, Chang SS, Koo JJ, Yoon HS, Hwang JH, Hwang YY, Park YS, Oh SK, Kim HS, Park JH, Moon SY, Schatten G (2005) Patient-specific embryonic stem cells derived from human SCNT blastocysts. Science 308(5729):1777–1783
6. Hwang WS, Ryu YJ, Park JH, Park ES, Lee EG, Koo JM, Jeon HY, Lee BC, Kang SK, Kim SJ, Ahn C, Hwang JH, Park KY, Cibelli JB, Moon SY (2004) Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. Science 303(5664): 1669–1674
7. Kennedy D (2006) Editorial retraction. Science 311(5759):335
8. French AJ, Adams CA, Anderson LS, Kitchen JR, Hughes MR, Wood SH (2008) Development of Human cloned Blastocysts Following Somatic Cell Nuclear Transfer (SCNT) with Adult Fibroblasts. Stem Cells 26(2): 485–493

9. Cowan CA, Atienza J, Melton DA, Eggan K (2005) Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science 309(5739):1369–1373
10. Pells S, Di Domenico AI, Gallagher EJ, McWhir J (2002) Multipotentiality of neuronal cells after spontaneous fusion with embryonic stem cells and nuclear reprogramming in vitro. Cloning Stem Cells 4(4):331–338
11. Pells S, McWhir J (2004) Studying nuclear reprogramming with cell hybrids. Methods Mol Biol 254:301–312
12. Sullivan S, Pells S, Hooper M, Gallagher E, McWhir J (2006) Nuclear reprogramming of somatic cells by embryonic stem cells is affected by cell cycle stage. Cloning Stem Cells 8(3):174–188
13. Collas P (2003) Nuclear reprogramming in cell-free extracts. Philos Trans R Soc Lond B Biol Sci 358(1436):1389–1395
14. Collas P, Taranger CK (2006) Epigenetic reprogramming of nuclei using cell extracts. Stem Cell Rev 2(4):309–317
15. Collas P, Taranger CK (2006) Toward reprogramming cells to pluripotency. Ernst Schering Res Found Workshop (60): 47–67
16. Collas P, Taranger CK, Boquest AC, Noer A, Dahl JA (2006) On the way to reprogramming cells to pluripotency using cell-free extracts. Reprod Biomed Online 12(6):762–770
17. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
18. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872
19. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318(5858): 1917–1920
20. Feng Q, Lu SJ, Klimanskaya I, Gomes I, Kim D, Chung Y, Honig GR, Kim KS, Lanza R (2010) Hemangioblastic derivatives from human induced pluripotent stem cells exhibit limited expansion and early senescence. Stem Cells 28(4):704–712
21. Bar-Nur O, Russ HA, Efrat S, Benvenisty N (2011) Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet Beta cells. Cell Stem Cell 9(1):17–23
22. Hu Q, Friedrich AM, Johnson LV, Clegg DO (2011) Memory in induced pluripotent stem cells: reprogrammed human retinal-pigmented epithelial cells show tendency for spontaneous redifferentiation. Stem Cells 28(11):1981–1991
23. Ohi Y, Qin H, Hong C, Blouin L, Polo JM, Guo T, Qi Z, Downey SL, Manos PD, Rossi DJ, Yu J, Hebrok M, Hochedlinger K, Costello JF, Song JS, Ramalho-Santos M (2011) Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human iPS cells. Nat Cell Biol 13(5):541–549
24. Rizzi R, Di Pasquale E, Portararo P, Papait R, Cattaneo P, Latronico MV, Altomare C, Sala L, Zaza A, Hirsch E, Naldini L, Condorelli G, Bearzi C (2012) Post-natal cardiomyocytes can generate iPS cells with an enhanced capacity toward cardiomyogenic re-differentation. Cell Death Differ 19:1162–1174
25. Hussein SM, Batada NN, Vuoristo S, Ching RW, Autio R, Narva E, Ng S, Sourour M, Hamalainen R, Olsson C, Lundin K, Mikkola M, Trokovic R, Peitz M, Brustle O, Bazett-Jones DP, Alitalo K, Lahesmaa R, Nagy A, Otonkoski T (2011) Copy number variation and selection during reprogramming to pluripotency. Nature 471(7336):58–62
26. Pera MF (2011) Stem cells: The dark side of induced pluripotency. Nature 471(7336):46–47
27. Deng J, Shoemaker R, Xie B, Gore A, LeProust EM, Antosiewicz-Bourget J, Egli D, Maherali N, Park IH, Yu J, Daley GQ, Eggan K, Hochedlinger K, Thomson J, Wang W, Gao Y, Zhang K (2009) Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. Nat Biotechnol 27(4):353–360
28. Gore A, Li Z, Fung HL, Young JE, Agarwal S, Antosiewicz-Bourget J, Canto I, Giorgetti A, Israel MA, Kiskinis E, Lee JH, Loh YH, Manos PD, Montserrat N, Panopoulos AD, Ruiz S,

Wilbert ML, Yu J, Kirkness EF, Izpisua Belmonte JC, Rossi DJ, Thomson JA, Eggan K, Daley GQ, Goldstein LS, Zhang K (2011) Somatic coding mutations in human induced pluripotent stem cells. Nature 471(7336): 63–67
29. Jia F, Wilson KD, Sun N, Gupta DM, Huang M, Li Z, Panetta NJ, Chen ZY, Robbins RC, Kay MA, Longaker MT, Wu JC (2010) A nonviral minicircle vector for deriving human iPS cells. Nat Methods 7(3):197–199
30. Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, Thomson JA (2009) Human induced pluripotent stem cells free of vector and transgene sequences. Science 324(5928): 797–801
31. Kim D, Kim CH, Moon JI, Chung YG, Chang MY, Han BS, Ko S, Yang E, Cha KY, Lanza R, Kim KS (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell 4(6):472–476
32. Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, Ebina W, Mandal PK, Smith ZD, Meissner A, Daley GQ, Brack AS, Collins JJ, Cowan C, Schlaeger TM, Rossi DJ (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 7(5):618–630
33. Gualdi R, Bossard P, Zheng M, Hamada Y, Coleman JR, Zaret KS (1996) Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. Genes Dev 10(13):1670–1682
34. Douarin NM (1975) An experimental analysis of liver development. Med Biol 53(6): 427–455
35. Jung J, Zheng M, Goldfarb M, Zaret KS (1999) Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science 284(5422):1998–2003
36. Melton D (1997) Signals for tissue induction and organ formation in vertebrate embryos. Harvey Lect 93:49–64
37. Deutsch G, Jung J, Zheng M, Lora J, Zaret KS (2001) A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development 128(6):871–881
38. Lemaigre F, Zaret KS (2004) Liver development update: new embryo models, cell lineage control, and morphogenesis. Curr Opin Genet Dev 14(5):582–590
39. Tremblay KD, Zaret KS (2005) Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. Dev Biol 280(1):87–99
40. Yoshitomi H, Zaret KS (2004) Endothelial cell interactions initiate dorsal pancreas development by selectively inducing the transcription factor Ptf1a. Development 131(4):807–817
41. Zaret KS (2001) Hepatocyte differentiation: from the endoderm and beyond. Curr Opin Genet Dev 11(5):568–574
42. Zaret KS (2000) Liver specification and early morphogenesis. Mech Dev 92(1):83–88
43. Wells JM, Melton DA (1999) Vertebrate endoderm development. Annu Rev Cell Dev Biol 15:393–410
44. Nordlie RC, Foster JD, Lange AJ (1999) Regulation of glucose production by the liver. Annu Rev Nutr 19:379–406
45. Kim HI, Ahn YH (2004) Role of peroxisome proliferator-activated receptor-gamma in the glucose-sensing apparatus of liver and beta-cells. Diabetes 53(Suppl 1):S60–S65
46. Massa ML, Gagliardino JJ, Francini F (2011) Liver glucokinase: an overview on the regulatory mechanisms of its activity. IUBMB Life 63(1):1–6
47. Rao MS, Dwivedi RS, Subbarao V, Usman MI, Scarpelli DG, Nemali MR, Yeldandi A, Thangada S, Kumar S, Reddy JK (1988) Almost total conversion of pancreas to liver in the adult rat: a reliable model to study transdifferentiation. Biochem Biophys Res Commun 156(1):131–136
48. Rao MS, Reddy JK (1995) Hepatic transdifferentiation in the pancreas. Semin Cell Biol 6(3):151–156
49. Rao MS, Subbarao V, Reddy JK (1986) Induction of hepatocytes in the pancreas of copper-depleted rats following copper repletion. Cell Differ 18(2):109–117

50. Shen CN, Slack JM, Tosh D (2000) Molecular basis of transdifferentiation of pancreas to liver. Nat Cell Biol 2(12):879–887
51. Lee BC, Hendricks JD, Bailey GS (1989) Metaplastic pancreatic cells in liver tumors induced by diethylnitrosamine. Exp Mol Pathol 50(1):104–113
52. Wolf HK, Burchette JL Jr, Garcia JA, Michalopoulos G (1990) Exocrine pancreatic tissue in human liver: a metaplastic process? Am J Surg Pathol 14(6):590–595
53. Ohlsson H, Karlsson K, Edlund T (1993) IPF1, a homeodomain-containing transactivator of the insulin gene. EMBO J 12(11):4251–4259
54. Ahlgren U, Jonsson J, Jonsson L, Simu K, Edlund H (1998) Beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. Genes Dev 12(12):1763–1768
55. Li Y, Cao X, Li LX, Brubaker PL, Edlund H, Drucker DJ (2005) Beta-Cell Pdx1 expression is essential for the glucoregulatory, proliferative, and cytoprotective actions of glucagon-like peptide-1. Diabetes 54(2):482–491
56. Johnson JD, Ahmed NT, Luciani DS, Han Z, Tran H, Fujita J, Misler S, Edlund H, Polonsky KS (2003) Increased islet apoptosis in Pdx1 ± mice. J Clin Invest 111(8):1147–1160
57. Leibowitz G, Ferber S, Apelqvist A, Edlund H, Gross DJ, Cerasi E, Melloul D, Kaiser N (2001) IPF1/PDX1 deficiency and beta-cell dysfunction in Psammomys obesus, an animal With type 2 diabetes. Diabetes 50(8):1799–1806
58. Leibowitz G, Melloul D, Yuli M, Gross DJ, Apelqvist A, Edlund H, Cerasi E, Kaiser N (2001) Defective glucose-regulated insulin gene expression associated with PDX-1 deficiency in the Psammomys obesus model of type 2 diabetes. Diabetes 50(Suppl 1):S138–S139
59. Jonsson J, Carlsson L, Edlund T, Edlund H (1994) Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 371(6498):606–609
60. Stoffers DA, Zinkin NT, Stanojevic V, Clarke WL, Habener JF (1997) Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. Nat Genet 15(1):106–110
61. Jonsson J, Ahlgren U, Edlund T, Edlund H (1995) IPF1, a homeodomain protein with a dual function in pancreas development. Int J Dev Biol 39(5):789–798
62. Ahlgren U, Jonsson J, Edlund H (1996) The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. Development 122(5):1409–1416
63. Afelik S, Chen Y, Pieler T (2006) Combined ectopic expression of Pdx1 and Ptf1a/p48 results in the stable conversion of posterior endoderm into endocrine and exocrine pancreatic tissue. Genes Dev 20(11):1441–1446
64. Kawaguchi Y, Cooper B, Gannon M, Ray M, MacDonald RJ, Wright CV (2002) The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet 32(1):128–134
65. Kim SK, MacDonald RJ (2002) Signaling and transcriptional control of pancreatic organogenesis. Curr Opin Genet Dev 12(5):540–547
66. Krapp A, Knofler M, Ledermann B, Burki K, Berney C, Zoerkler N, Hagenbuchle O, Wellauer PK (1998) The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. Genes Dev 12(23):3752–3763
67. Grapin-Botton A, Majithia AR, Melton DA (2001) Key events of pancreas formation are triggered in gut endoderm by ectopic expression of pancreatic regulatory genes. Genes Dev 15(4):444–454
68. Miyazaki S, Yamato E, Miyazaki J (2004) Regulated expression of pdx-1 promotes in vitro differentiation of insulin-producing cells from embryonic stem cells. Diabetes 53(4): 1030–1037
69. Kojima H, Nakamura T, Fujita Y, Kishi A, Fujimiya M, Yamada S, Kudo M, Nishio Y, Maegawa H, Haneda M, Yasuda H, Kojima I, Seno M, Wong NC, Kikkawa R, Kashiwagi A

(2002) Combined expression of pancreatic duodenal homeobox 1 and islet factor 1 induces immature enterocytes to produce insulin. Diabetes 51(5):1398–1408
70. Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seijffers R, Kopolovic J, Kaiser N, Karasik A (2000) Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. Nat Med 6(5):568–572
71. Ben-Shushan E, Marshak S, Shoshkes M, Cerasi E, Melloul D (2001) A pancreatic beta - cell-specific enhancer in the human PDX-1 gene is regulated by hepatocyte nuclear factor 3beta (HNF-3beta), HNF-1alpha, and SPs transcription factors. J Biol Chem 276(20):17533–17540
72. Marshak S, Ben-Shushan E, Shoshkes M, Havin L, Cerasi E, Melloul D (2001) Regulatory elements involved in human pdx-1 gene expression. Diabetes 50(Suppl 1):S37–S38
73. Ber I, Shternhall K, Perl S, Ohanuna Z, Goldberg I, Barshack I, Benvenisti-Zarum L, Meivar-Levy I, Ferber S (2003) Functional, persistent, and extended liver to pancreas transdifferentiation. J Biol Chem 278(34):31950–31957
74. Westmacott A, Burke ZD, Oliver G, Slack JM, Tosh D (2006) C/EBPalpha and C/EBPbeta are markers of early liver development. Int J Dev Biol 50(7):653–657
75. Begay V, Smink J, Leutz A (2004) Essential requirement of CCAAT/enhancer binding proteins in embryogenesis. Mol Cell Biol 24(22):9744–9751
76. Horb ME, Shen CN, Tosh D, Slack JM (2003) Experimental conversion of liver to pancreas. Curr Biol 13(2):105–115
77. Chalmers AD, Slack JM (1998) Development of the gut in Xenopus laevis. Dev Dyn 212(4):509–521
78. Sadowski I, Ma J, Triezenberg S, Ptashne M (1988) GAL4-VP16 is an unusually potent transcriptional activator. Nature 335(6190):563–564
79. Triezenberg SJ, Kingsbury RC, McKnight SL (1988) Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. Genes Dev 2(6):718–729
80. Li WC, Horb ME, Tosh D, Slack JM (2005) In vitro transdifferentiation of hepatoma cells into functional pancreatic cells. Mech Dev 122(6):835–847
81. Tang DQ, Lu S, Sun YP, Rodrigues E, Chou W, Yang C, Cao LZ, Chang LJ, Yang LJ (2006) Reprogramming liver-stem WB cells into functional insulin-producing cells by persistent expression of Pdx1- and Pdx1-VP16 mediated by lentiviral vectors. Lab Invest 86(1):83–93
82. Wang AY, Ehrhardt A, Xu H, Kay MA (2007) Adenovirus transduction is required for the correction of diabetes using Pdx-1 or Neurogenin-3 in the liver. Mol Ther 15(2):255–263
83. Kaneto H, Matsuoka TA, Nakatani Y, Miyatsuka T, Matsuhisa M, Hori M, Yamasaki Y (2005) A crucial role of MafA as a novel therapeutic target for diabetes. J Biol Chem 280(15):15047–15052
84. Kaneto H, Miyatsuka T, Fujitani Y, Noguchi H, Song KH, Yoon KH, Matsuoka TA (2007) Role of PDX-1 and MafA as a potential therapeutic target for diabetes. Diabetes Res Clin Pract 77(Suppl 1):S127–S137
85. Kaneto H, Miyatsuka T, Shiraiwa T, Yamamoto K, Kato K, Fujitani Y, Matsuoka TA (2007) Crucial role of PDX-1 in pancreas development, beta-cell differentiation, and induction of surrogate beta-cells. Curr Med Chem 14(16):1745–1752
86. Matsuoka TA, Kaneto H, Stein R, Miyatsuka T, Kawamori D, Henderson E, Kojima I, Matsuhisa M, Hori M, Yamasaki Y (2007) MafA regulates expression of genes important to islet beta-cell function. Mol Endocrinol 21(11):2764–2774
87. Miyatsuka T, Kaneto H, Kajimoto Y, Hirota S, Arakawa Y, Fujitani Y, Umayahara Y, Watada H, Yamasaki Y, Magnuson MA, Miyazaki J, Hori M (2003) Ectopically expressed PDX-1 in liver initiates endocrine and exocrine pancreas differentiation but causes dysmorphogenesis. Biochem Biophys Res Commun 310(3):1017–1025

88. Kojima H, Fujimiya M, Matsumura K, Younan P, Imaeda H, Maeda M, Chan L (2003) NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. Nat Med 9(5):596–603
89. Rooman I, Heremans Y, Heimberg H, Bouwens L (2000) Modulation of rat pancreatic acinoductal transdifferentiation and expression of PDX-1 in vitro. Diabetologia 43(7): 907–914
90. Means AL, Meszoely IM, Suzuki K, Miyamoto Y, Rustgi AK, Coffey RJ Jr, Wright CV, Stoffers DA, Leach SD (2005) Pancreatic epithelial plasticity mediated by acinar cell transdifferentiation and generation of nestin-positive intermediates. Development 132(16):3767–3776
91. Lardon J, De Breuck S, Rooman I, Van Lommel L, Kruhoffer M, Orntoft T, Schuit F, Bouwens L (2004) Plasticity in the adult rat pancreas: transdifferentiation of exocrine to hepatocyte-like cells in primary culture. Hepatology 39(6):1499–1507
92. Baeyens L, Bouwens L (2009) Cellular plasticity of the pancreas. Biol Chem 390(10): 995–1001
93. Baeyens L, Bouwens L (2008) Can beta-cells be derived from exocrine pancreas? Diabetes Obes Metab 10(Suppl 4):170–178
94. Baeyens L, De Breuck S, Lardon J, Mfopou JK, Rooman I, Bouwens L (2005) In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells. Diabetologia 48(1):49–57
95. Okuno M, Minami K, Okumachi A, Miyawaki K, Yokoi N, Toyokuni S, Seino S (2007) Generation of insulin-secreting cells from pancreatic acinar cells of animal models of type 1 diabetes. Am J Physiol Endocrinol Metab 292(1):E158–E165
96. Minami K, Okuno M, Miyawaki K, Okumachi A, Ishizaki K, Oyama K, Kawaguchi M, Ishizuka N, Iwanaga T, Seino S (2005) Lineage tracing and characterization of insulin-secreting cells generated from adult pancreatic acinar cells. Proc Natl Acad Sci U S A 102(42):15116–15121
97. Baeyens L, Bonne S, German MS, Ravassard P, Heimberg H, Bouwens L (2006) Ngn3 expression during postnatal in vitro beta cell neogenesis induced by the JAK/STAT pathway. Cell Death Differ 13(11):1892–1899
98. Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae S-K, Kittappa R, McKay RDG (2006) Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 442(7104):823
99. Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T, Hrabe de Angelis M, Lendahl U, Edlund H (1999) Notch signalling controls pancreatic cell differentiation. Nature 400(6747): 877–881
100. Cejudo-Martin P, Johnson RS (2005) A new notch in the HIF belt: how hypoxia impacts differentiation. Dev Cell 9(5):575–576
101. Jensen J, Pedersen EE, Galante P, Hald J, Heller RS, Ishibashi M, Kageyama R, Guillemot F, Serup P, Madsen OD (2000) Control of endodermal endocrine development by Hes-1. Nat Genet 24(1):36–44
102. Kadesch T (2004) Notch signaling: the demise of elegant simplicity. Curr Opin Genet Dev 14(5):506–512
103. Lammert E, Brown J, Melton DA (2000) Notch gene expression during pancreatic organogenesis. Mech Dev 94(1–2):199–203
104. Murtaugh LC, Stanger BZ, Kwan KM, Melton DA (2003) Notch signaling controls multiple steps of pancreatic differentiation. Proc Natl Acad Sci U S A 100(25):14920–14925
105. Domínguez-Bendala J, Klein D, Ribeiro M, Ricordi C, Inverardi L, Pastori R, Edlund H (2005) TAT-mediated neurogenin 3 protein transduction stimulates pancreatic endocrine differentiation in vitro. Diabetes 54(3):720–726
106. Baeyens L, Bonne S, Bos T, Rooman I, Peleman C, Lahoutte T, German M, Heimberg H, Bouwens L (2009) Notch signaling as gatekeeper of rat acinar-to-beta-cell conversion in vitro. Gastroenterology 136(5): 1750–1760 e1713

107. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455(7213): 627–632
108. Zhou Q, Law AC, Rajagopal J, Anderson WJ, Gray PA, Melton DA (2007) A multipotent progenitor domain guides pancreatic organogenesis. Dev Cell 13(1):103–114
109. Murtaugh LC, Melton DA (2003) Genes, signals, and lineages in pancreas development. Annu Rev Cell Dev Biol 19:71–89
110. Huang HP, Liu M, El-Hodiri HM, Chu K, Jamrich M, Tsai MJ (2000) Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. Mol Cell Biol 20(9): 3292–3307
111. You YH, Ham DS, Park HS, Rhee M, Kim JW, Yoon KH (2011) Adenoviruses expressing PDX-1, BETA2/NeuroD and MafA induces the transdifferentiation of porcine neonatal pancreas cell clusters and adult pig pancreatic cells into beta-cells. Diabetes Metab J 35(2):119–129
112. Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggren PO, Caicedo A (2006) The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proc Natl Acad Sci U S A 103(7):2334–2339
113. Konstantinova I, Nikolova G, Ohara-Imaizumi M, Meda P, Kucera T, Zarbalis K, Wurst W, Nagamatsu S, Lammert E (2007) EphA-Ephrin-A-mediated beta cell communication regulates insulin secretion from pancreatic islets. Cell 129(2):359–370
114. Zhang T, Saunee NA, Breslin MB, Song K, Lan MS (2012) Functional role of an islet transcription factor, INSM1/IA-1, on pancreatic acinar cell trans-differentiation. J Cell Physiol 227(6):2470–2479
115. Mellitzer G, Bonne S, Luco RF, Van De Casteele M, Lenne-Samuel N, Collombat P, Mansouri A, Lee J, Lan M, Pipeleers D, Nielsen FC, Ferrer J, Gradwohl G, Heimberg H (2006) IA1 is NGN3-dependent and essential for differentiation of the endocrine pancreas. EMBO J 25(6):1344–1352
116. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE (2006) Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 24(11): 1392–1401
117. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, Young H, Richardson M, Smart NG, Cunningham J, Agulnick AD, D'Amour KA, Carpenter MK, Baetge EE (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol 26(4):443–452
118. Domínguez-Bendala J, Ricordi C, Pastori R (2006) Protein transduction: a novel approach to induce in vitro pancreatic differentiation. Cell Transplant 15(Supp. 15):85–90
119. Vargas N, Alvarez-Cubela S, Giraldo JA, Nieto M, Fort NM, Cechin S, Garcia E, Espino-Grosso P, Fraker CA, Ricordi C, Inverardi L, Pastori RL, Dominguez-Bendala J (2011) TAT-mediated transduction of MafA protein in utero results in enhanced pancreatic insulin expression and changes in islet morphology. PLoS ONE 6(8):e22364
120. von Herrath M (2005) Immunology: insulin trigger for diabetes. Nature 435(7039):151–152
121. Kent SC, Chen Y, Bregoli L, Clemmings SM, Kenyon NS, Ricordi C, Hering BJ, Hafler DA (2005) Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope. Nature 435(7039):224–228
122. Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, Yu L, Wegmann DR, Hutton JC, Elliott JF, Eisenbarth GS (2005) Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. Nature 435(7039):220–223

Chapter 9
Pancreatic Regeneration in the Face of Diabetes

Zeeshan Ahmad

Abstract Diabetes affects millions of people worldwide and the incidence is growing day by day. Hyperglycemia, the main culprit in this disease can be managed through the use of intensive insulin therapy and/or oral hypoglycemic agents. However, the ailment is not cured and leaves the patients dependent on treatment for the rest of their lives. Therefore, maintaining an ideal euglycemic state without external intervention is the much-awaited cure for diabetes. It can be achieved through the replacement of lost β cells with new functional β cells or by inducing beta cell regeneration. This chapter reviews the literature regarding various approaches being used today or those expected to be used for this in future.

Abbreviations

Arx	Aristaless-related homeobox
BCG	Bacillus Calmette-Guérin
BMP	Bone Morphogenetic Protein
CCK	Cholecystokinin
CFA	Complete Freund's Adjuvant
CK	Cytokeratin
DPP	Dipeptidyl Peptidase
DPSCs	Dental Pulp Stem Cells
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescent Protein
ES cells	Embryonic Stem cells
FGF	Fibroblast Growth Factor
GABA	γ-Aminobutyric acid

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GFP	Green Fluorescent Protein
GH	Growth Hormone
GIP	Glucose-Dependent Insulinotropic Polypeptide
GLP-1	Glucagon-Like Peptide-1
GSCs	Germline Stem Cells
GTC-1 cells	high GIP-expressing subpopulation of STC-1 cells
HbA1c	hemoglobin A1c
HDAD	Helper-Dependent Adenovirus
hES cells	human Embryonic Stem cells
HGF	Hepatocyte Growth Factor
hiPS cells	Human induced Pluripotent Stem cells
HSCT	Hematopoeitic Stem Cell Transplantation
HSLs	Hepatic Stem-Like cells
ICA	Islet-like Cell Aggregate
ICM	Inner Cell Mass
IEC-6 cells	Normal rat small intestine-derived immature intestinal stem cells
IPF1	Insulin Promoter Factor 1
iPS cells	Induced Pluripotent Stem cells
LIF	Leukemia Inhibitory Factor
MafA	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (avian)
mTert	Mouse Telomerase reverse transcriptase
NAD	Nicotinamide Adenine Dinucleotide
NeuroD	Neurogenic Differentiation
Ngn3	Neurogenin3
NOD	Non-Obese Diabetic
PAK	Pancreas After Kidney
Pax4	Paired box 4
Pdx1	Pancreatic and duodenal homeobox 1
PL	Placental Lactogen
PMPs	Pancreatic Multipotent Progenitors
PRL	Prolactin
PTA	Pancreas Transplant Alone
RA	Retinoic Acid
SCID	Severe Combined Immuno Deficiency
SHCs	Small Hepatocytes
SPK	Simultaneous Pancreas-Kidney
SSCs	Spermatogonial Stem Cells
STC-1 cells	mouse neuroendocrine tumor-derived cells
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
UCB	Umbilical Cord Blood
YFP	Yellow Fluorescent Protein

9.1 Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia due to reduced insulin production or insulin resistance in the body. Specifically in type 1 diabetes insulin production is markedly reduced due to the destruction of insulin producing β cells as a result of autoimmunity. On the other hand, type 2 diabetes results from a combined effect of insulin resistance and reduced insulin secretion by β cells. While type 2 diabetes can be sometimes managed through the use of oral hypoglycemic agents or lifestyle changes, type 1 patients are exclusively dependent on insulin injections for their whole life span [1, 2]. Intensive insulin therapy combined with continuous glucose monitoring currently remains the best treatment for type 1 diabetes. If carefully followed such therapy can improve the quality of life and prevent or at least delay the development of end organ complications, e.g., nephropathy and retinopathy. However, during long-term treatment some patients develop an impaired hypoglycemia awareness that is dangerous and can prove to be fatal [3]. Therefore, an ideal glucose homeostasis without the fear of hypoglycemia can only be achieved through physiological β cell replacement. This can be achieved through whole pancreas or islet transplantation and this is the only form of β cell replacement therapy that is presently available in certain clinics.

9.2 Pancreas and Islet Transplantation

Kelly et al. [4] reported the first case of pancreas transplantation in 1967. Since then about 30,000 patients have been transplanted worldwide [5]. There are three main categories of pancreas transplantation: simultaneous pancreas–kidney transplantation (SPK) that accounts for the majority of the pancreas transplants performed, pancreas after kidney transplantation (PAK), and pancreas transplant alone (PTA) [3]. Most successful among them is SPK with highest graft survival at 10 years post-transplant [6]. Whole pancreas transplantation is efficient in restoring the normal physiological glycemic control and improving the quality of life. The main disadvantage of pancreas transplantation is the involvement of major surgery and the use of immunosuppressive drugs. That is why an ideal candidate for pancreas transplant is one with end-stage renal disease who will receive a kidney transplant anyway. In the same surgical procedure pancreas can also be included with little extra effort while the patient receives the same immunosuppressive drugs. One major problem with pancreas transplantation is the availability of donor pancreas compared to the huge number of patients on the waiting list.

As compared to pancreas transplantation, the procedure of islet transplantation is less invasive as it requires no major surgery. However, the use of immunosuppressive drugs is common between both procedures. Although islet

transplantation was already being investigated in the late twentieth century [7], the success of the procedure was highly limited with only 8 % individuals remaining insulin independent at one year [8]. The hope in clinical islet transplantation increased after the group of J. Shapiro, at Edmonton University, showed increased islet graft survival and achievement of insulin independence in seven patients using a glucocorticoid-free immunosuppressive regimen [9]. Afterwards, the Edmonton protocol was evaluated at multiple centers around the world with variable success rates. In the most experienced centers about 90 % insulin-independence rate was noticed [10]. However, long-term follow-up showed that graft function decreases over time requiring subsequent islet transplants. Therefore, the major goal of islet transplantation is now limited to avoid severe hypoglycemia and to achieve near-normal glycemic control rather than complete insulin independence [3]. Just like pancreas transplantation, islet transplantation faces a shortage of donors, as in the Edmonton protocol one patient required islets from at least two donor pancreas [9]. This shortage of donor pancreas/islets, therefore, emphasizes the need to understand and promote endogenous regeneration of pancreas in order to combat the disease without or with minimal number of external islets. In parallel to that, efforts must be directed in a way to generate β cells in vitro for transplantation or to generate β cells in vivo from other cell types, e.g., by means of gene therapy. Finally, xenotransplantation of humanized and encapsulated islets can be a therapeutic possibility as well. In the following sections, these issues are discussed individually.

9.3 Endogenous Pancreas Regeneration

Based on numerous studies done in rodents as well as in humans, it is now well known that the pancreas has the ability to regenerate under certain conditions. Pancreatic β cell mass undergoes compensatory changes in response to changes in the metabolic demand. Physiologically, β cell mass can expand in response to increased insulin demand, e.g., during pregnancy or obesity. Such adaptive expansion may involve increase in β cell replication, decrease in β cell death, increase in β cell size (hypertrophy) and insulin secretion, and possibly also β cell neogenesis, that is, differentiation of β cells from some kind of progenitors.

9.3.1 Expansion of β Cell Mass During Pregnancy

In rodents, the β cell mass increases 2–5-fold during pregnancy. This compensatory expansion is achieved through hypertrophy and enhanced β cell proliferation [11–14] and comes back to normal after birth, mainly through increased apoptosis [15]. Compensatory changes in β cell mass during pregnancy are, therefore, reversible and well regulated. In contrast to rodents not much data is available

regarding the changes in β cell mass during human pregnancy. Increase in insulin resistance during human pregnancy is compensated by enhanced insulin secretion to keep the blood glucose level at normal [16, 17]. Analysis of pancreas from pregnant women showed nearly 1.4–2.4-fold increase in β cell mass [18, 19]. Unlike during rodent pregnancy, this increase is not achieved by hypertrophy or replication. Instead, there is a possible neogenesis supported by the appearance of new smaller islets and duct cells positive for insulin [19]. Regardless of the mechanism involved, the regulated change in β cell mass is important to prevent diabetes during pregnancy or other conditions of high metabolic demand, e.g., obesity, and a failure to meet this compensation is what finally leads to diabetes [20].

9.3.2 Regeneration of β Cells Following Pancreatic Injury

Evidence of β cell regeneration also comes from mouse models of pancreatic injury (chemically or surgically induced), and genetically manipulated mice (to specifically destroy β cells) as well as human diabetic patients. It has been shown in many different studies that β cells can proliferate in response to pancreatic injury [21]. However, what is more controversial is the fact that whether or not there is any β cell neogenesis, e.g., generation of insulin producing β cells from pancreatic ductal cells. While pancreatic ductal cells do give rise to endocrine cells during the embryonic phase of development [22], a similar process in adult life is not clear. The initial evidence for such neogenesis comes from morphological observation, showing the presence of insulin-expressing cells in or near to pancreatic duct epithelium, following pancreatic injury in the form of partial pancreatectomy or pancreatic duct ligation [23, 24]. However, these studies were not conclusive, as they did not involve any lineage tracing to specifically demonstrate the origin of new β cells from ductal cells. Dor et al. [25] for the first time used a genetic lineage tracing approach to prove that nearly all β cells in adult life, during normal conditions or after 70 % pancreatectomy, are originated from pre-existing β cells. It was further supported by a study from Teta et al. [26], where they used two types of nucleotide analogs to sequentially label β cells and concluded that no specialized progenitors are involved in β cell regeneration after 50 % pancreatectomy. However, because such lineage tracing is not 100 % efficient, there still remains a possibility that some β cells might arise from sources other than pre-existing β cells.

Another injury model to study pancreatic regeneration in rodents is pancreatic duct ligation. A study by Inada et al. [27] supported the idea that pancreatic ductal cells can give rise to β cells after duct ligation. To directly show the contribution of ductal cells in β cell regeneration, the authors specifically labeled pancreatic ductal cells by using carbonic anhydrase II-cre mouse line and a β-galactosidase reporter mouse line. Following ductal ligation, indeed they found a significant increase in the number of β-gal positive β cells showing the conversion of ductal

cells into β cells. The idea of β cell generation from ductal progenitors is also supported by a study from Xu et al. [28]. By tracking the Ngn3 promoter activity, the authors were able to show the presence of Ngn3 positive progenitors in the duct epithelium following pancreatic ductal ligation. When isolated and cultured, these Ngn3 positive cells were able to differentiate into endocrine cells in vitro. While these studies provide some convincing evidence in favor of β cell neogenesis, other studies show opposing results. In a study by Lee et al. [29], the authors analyzed pancreatic regeneration following 50 % pancreatectomy and were not able to detect any Ngn3 activity in the pancreas. This study also supported the idea of β cell replication as the primary mean of regeneration following pancreatectomy.

Further studies conducted by Solar et al. [30] and Kopp et al. [31], also provided evidence against neogenesis following pancreatic ductal ligation. Solar et al. [30] labeled the Hnf1β expressing mature duct cells and traced their fate after ductal ligation or alloxan-mediated β cell destruction. It was found that in adult pancreas Hnf1β-positive duct cells can only give rise to duct cells even after the surgically or chemically induced injury. This result was also supported by a recent study from Kopp et al. [31], who traced the fate of Sox9-positive progenitors and found no contribution toward endocrine cells in adult pancreas under normal conditions or after ductal ligation. However, again it is important to mention that such labeling strategies are never 100 % efficient. Therefore, the possibility of ductal origin of β cells in such conditions cannot be completely excluded.

While these studies focused on surgically induced pancreatic injury, other studies investigated regeneration following β cell destruction in specific transgenic mouse models. Nir et al. [32], in their study used transgenic mice expressing diphtheria toxin in β cells following doxycycline administration, leading to 70–80 % β cell destruction while leaving the rest of the pancreas intact. They used a lineage tracing strategy along with β cell ablation to conclude that regeneration of β cells in this scenario is mainly through replication of pre-existing β cells. A similar study was performed by Thorel et al. [33] showing very different results. In this study, the authors used transgenic mice expressing diphtheria toxin receptor only in β cells that lead to β cell destruction following diphtheria toxin administration to the mice. In this way a nearly total ablation of β cells (>99 %) was achieved. Interestingly, in this situation the regeneration of β cells occurred via trans-differentiation of α cells to β cells and not via replication of β cells. The prospect of converting α cells into β cells is very interesting from a therapeutic point of view, provided that this process can be activated in diabetic patients for a required time period in a very controlled manner.

Irrespective of the mechanism involved, all of the above-mentioned studies assure that β cell regeneration does occur in adult rodent pancreas under certain physiological and pathological conditions. However, unlike rodents not much is known about β cell regeneration in adult human pancreas, as it is more difficult to analyze this in humans. In humans, it has been shown that the β cells are much more long lived, and there is almost no turnover of β cells beyond 30 years of age [34, 35]. However, a modest increase in β cell mass can be found under

physiological conditions like pregnancy as mentioned before. Islet regeneration in adult human pancreas probably also occurs during pathological conditions like type 1 diabetes, however, it is not that obvious because the increased rate of apoptosis overcomes that of regeneration [36]. The studies to understand endogenous pancreas regeneration in rodents and humans are conducted in a hope to find out a regenerative mechanism that can be finally applied to a human therapeutic setting.

9.4 Promoting Endogenous Pancreas Regeneration

Endogenous pancreas regeneration can prove to be a promising therapeutic strategy, provided that we learn to promote it in vivo in the pathological environment of diabetes in a controlled manner. Considering this possibility, numerous studies have been conducted to investigate the effect of various proliferation/differentiation-promoting factors on endogenous pancreas regeneration.

9.4.1 Role of Gastrointestinal Hormones

In this regard, the role of gastrointestinal hormones has been extensively studied. Gastrointestinal hormones include GLP-1 (glucagon-like peptide-1), GIP (glucose-dependent insulinotropic polypeptide), Cholecystokinin (CCK), and Gastrin. In addition to the regulation of food intake and digestion, these gut peptides also play a role in glucose-dependent insulin secretion and β cell mass expansion [37]. Due to their beneficial effects they are considered as a potential candidate for the treatment of type 2 diabetes.

It has been shown in many different studies that exogenous GLP-1 can increase the functional β cell mass by increasing proliferation and neogenesis of β cells and by preventing β cell death. In a study by Perfetti et al. [38], continuous GLP-1 administration for 5 days led to a 1.5-fold increase in β cell mass in old glucose intolerant Wistar rats. Furthermore, Zhang et al. [39] showed that continuous administration of GLP-1 can delay the onset of diabetes in NOD (non-obese diabetic) mice that serve as a model for type 1 diabetes. However, Plasma GLP-1 is degraded by the enzyme dipeptidyl peptidase-4 (DPP-IV), leading to a very short half-life in vivo that makes it difficult to be used as a therapeutic agent [40]. Therefore, researchers in the past few years have made a lot of efforts to develop GLP-1 receptor agonists with long half-life or DPP-IV inhibitors that can increase the endogenous GLP-1 plasma levels. In this regard, Exendin-4 (a long-acting GLP-1 agonist) treatment was shown to prevent hyperglycemia and improve glucose tolerance in diabetic mice (db/db) by improving insulin release in conjunction with enhanced β cell proliferation and reduced apoptosis [41]. In another study by Green et al. [42], treatment with Val8GLP1, another long-lasting analog

of GLP-1, improved insulin secretion and glucose tolerance, and increased the average islet area by 1.2-fold in obese diabetic (ob/ob) mice. Additionally, in diabetic mice treatment with a DPP-IV inhibitor improved glycemic control and restored the β cell mass [43]. The beneficial effect of GLP-1/GLP-1 analogs is also supported by in vitro studies, where they were shown to convert pancreatic AR42 J or ductal cells into endocrine cells including β cells [44, 45]. These and many other studies show the beneficial effect of GLP-1, GLP-1 analogs, and DPP-IV inhibitors in the prevention of diabetes, by promoting insulin secretion and functional β cell mass. Some of the GLP-1 agonists and DPP-IV inhibitors have already been approved by FDA for use as antidiabetogenic agents mostly in combination with other antidiabetic drugs [46].

Other gastrointestinal hormones (GIP, CCK, and gastrin) may also facilitate the process of β cell regeneration. The role of GIP in the maintenance of β cell mass was shown in a study where the dominant negative form of GIP receptor was overexpressed in transgenic mice that led to the reduction of total islet and β cell volume [47]. As GIP is also degraded in vivo by DPP-IV, making its half-life very short, more stable analogs are required for its therapeutic application, e.g., in combination with GLP-1 therapy. GIP (LysPAL16) a stable analog of GIP was shown to enhance the differentiation of mouse embryonic stem cells (ES) into insulin-producing cells [48]. Another study showed enhanced insulin production and improved β cell volume in obese diabetic (ob/ob) mice following treatment with three different GIP analogs [49].

Cholecystokinin octapeptide (CCK-8) treatment was shown to increase β cell proliferation and reduce hyperglycemia in streptozotocin-induced diabetic rats [50]. In obese (Leptin ob/ob) mice, loss of CCK resulted in reduced islet size and β cell mass due to increased apoptosis, showing that CCK plays a role in β cell survival [51]. CCK also exerted an antidiabetogenic effect when administered to healthy individuals or type 2 diabetic patients, showing its potential as a therapeutic agent [52].

In pancreatic duct ligated rats, gastrin treatment stimulated β cell neogenesis but not proliferation [53]. Overexpression of gastrin or TGF-α (transforming growth factor-α) alone in pancreas did not show any beneficial effect on β cell mass. However, a combined expression of gastrin and TGF-α led to increase in the islet mass [54]. Increased islet mass was also observed in alloxan-treated mice and streptozotocin-treated rats following co-administration of gastrin and EGF (epidermal growth factor) [55, 56]. In these studies, TGF α and EGF treatment promoted metaplastic-ductule formation (through duct proliferation or transdifferentiation from acinar cells), while gastrin stimulated the differentiation of ductules to β cells.

Collectively, these studies clearly demonstrate the beneficial effect of gut peptides, especially in the treatment of type 2 diabetes, where their insulinotropic and β cell protective effect can be utilized.

9.4.2 Role of Growth Factors

TGF-β signaling plays an important role in embryonic development as well as in adult organisms. Members of the TGF-β superfamily have diverse functions ranging from growth and differentiation during embryogenesis to repair and maintenance in adult tissues [57]. Activin, a member of the TGF-β superfamily is involved in the development of pancreas including both endocrine and exocrine portion [58–60]. Zhang et al. [61] detected the expression of activin β A and β B subunits as well as type II and type IIB activin receptors in the pancreatic duct following streptozotocin treatment or partial pancreatectomy in mice, showing the role of activin in promoting endogenous pancreas regeneration after injury. Furthermore, It was shown that *Xenopus* ectoderm when treated with activin and retinoic acid is converted into pancreatic tissue in vitro [62].

Betacellulin, originally identified in the conditioned medium of mouse insulinoma-derived cell lines, is a peptide ligand belonging to the EGF family [63]. Different studies have suggested the possible role of betacellulin in the growth and differentiation of pancreas. Betacellulin together with activin A converted pancreatic AR42 J cells into insulin- secreting cells [64]. Besides, administration of betacellulin promoted β cell proliferation and/or neogenesis following 90 % pancreatectomy in rats [65]. Similarly, in streptozotocin-treated neonatal rats, a combined treatment with activin A and betacellulin, improved β cell mass and insulin content in pancreas [66]. These studies clearly emphasize the role of activin and betacellulin in normal pancreas development as well as in the promotion of endogenous pancreas regeneration.

Growth factors like activin and betacellulin are, however, not very useful for clinical application because of their effect on multiple organ systems [57]. During a screen to identify compounds that can convert pancreatic AR42 J cells into insulin-producing cells, Umezawa et al. [67] came across a compound named conophylline, a vinca alkaloid obtained from the extract of a plant *Ervatamia microphylla*. Conophylline produced a similar action like activin A, however, the viability of cells treated with conophylline was better. Conophylline treatment improved β cell regeneration in streptozotocin-treated neonatal rats by promoting differentiation and also increased the number of insulin positive cells in in vitro organ culture [68]. The feasibility of clinical application is, therefore, higher with conophylline compared to activin because of its in vivo efficiency and reduced undesired effect like apoptosis [69].

Other growth factors that may enhance the endogenous β cell regeneration include hepatocyte growth factor (HGF), growth hormone, prolactin, and placental lactogen [70]. HGF immunoreactivity was shown in human and rat pancreatic islets in primarily α cells, while c-met (HGF receptor) was preferentially found in β cells [71, 72]. In NOD mice, c-met expression was increased in duct-associated β cells showing a possible role in neogenesis [73]. This is supported by another study where an overexpression of HGF in β cells increased the β cell mass by 2–3-fold [74]. In streptozotocin-induced diabetic mice, intravenous injection of naked HGF

gene improved insulin secretion and protected β cell mass by reducing apoptosis and enhancing proliferation [75]. Islets expressing HGF transgene (via adenoviral gene delivery) showed better outcome after transplantation into streptozotocin-induced diabetic rats [76]. HGF is, therefore, a potential therapeutic agent because of its beneficial effect on islet mass and function both in vitro and in vivo.

Placental lactogen (PL), prolactin (PRL), and growth hormone (GH) are related hormones belonging to the same family [70]. Lactogenic hormones are involved in the expansion of β cell mass during pregnancy [77]. In vitro treatment of rat, mouse, and human islets with homologous PRL, PL, and GH enhanced the β cell proliferation and insulin secretion [78, 79]. In accordance with that, an overexpression of mouse PL-1 in β cells increased the β cell proliferation by 2-fold [80]. Furthermore, a knockout of PRL or GH receptor led to reduction in β cell mass and insulin secretion [81, 82]. Lactogens are, therefore, important β cell mitogens that can be utilized at least in vitro to increase β cell mass, e.g., before islet transplantation.

9.4.3 Role of Nicotinamide

Nicotinamide, a vitamin belonging to vitamin B group, has also been shown to have beneficial effects in protecting against diabetes. Administration of a high dose of nicotinamide improved glucose tolerance in NOD mice [83]. Nicotinamide treatment ameliorated the hyperglycemia by expanding the β cell mass following 70 % pancreatectomy in type 2 diabetic rats. However, this effect was sustained only during the treatment and gradually reversed after the cessation of treatment [84]. Moreover, In vitro treatment of human fetal pancreatic cells with nicotinamide increased endocrine differentiation and improved insulin production [85]. Considering the beneficial effect of nicotinamide a lot of clinical trials have been carried out in type 1 diabetic patients; however, the results are variable [86–89]. Recently, Yoshino et al. [90] showed that nicotinamide mononucleotide treatment can ameliorate glucose intolerance and improve hepatic insulin sensitivity in high-fat-diet and age-induced type 2 diabetic mice, by restoring the levels of NAD^+ (nicotinamide adenine dinucleotide). The beneficial antidiabetic effect of nicotinamide is, therefore, mainly through metabolic improvement rather than enhancement of pancreatic regeneration.

9.4.4 Defeating Autoimmunity to Improve Endogenous Regeneration in Type 1 Diabetes

As mentioned above, there are a variety of compounds that are potentially helpful in the treatment of diabetes by improving β cell regeneration and/or function. However, such treatments, even if they come into clinical practice, are mostly

applicable to type 2 diabetics, and only partially applicable to type 1 diabetic patients because of the existence of autoimmunity that will destroy any upcoming β cells. In view of type 1 diabetes, a potential therapy should therefore ameliorate the autoimmunity and at the same time promote β cell regeneration and protect the remaining β cell mass (as in recent-onset diabetes).

Initial efforts to combat type 1 diabetes, therefore, focused on the use of immunosuppressive drugs like prednisone, azathioprine, or cyclosporine A, to preserve the β cell mass in recent-onset diabetes (where a residual β cell mass is still present) [91]. In these studies the treated individuals showed a slower decline in the plasma C-peptide levels and some patients even achieved an insulin-free state for a limited period of time. However, the toxicity associated with the long-term use of immunosuppressive drugs and lack of sustained benefit after discontinuing the treatment makes this approach infeasible.

In 2003, a study by Kodama et al. [92] demonstrated the permanent reversal of autoimmune diabetes in NOD mice following treatment with complete Freund's adjuvant (CFA) and semi-allogeneic splenocytes. In this study splenocytes were also shown to contribute toward β cell generation along with their immunomodulatory effect. The same protocol was repeated by Chong et al. [93] and confirmed the reversal of diabetes. However, in this study the regenerated β cells arose from the host only and splenocytes did not contribute at all. Irrespective of the fact that splenocytes contribute to β cell mass or not, these studies definitely demonstrate the ability of β cells to regenerate in cases of type 1 diabetes once the autoimmunity is ameliorated.

In the above-mentioned treatment regimen CFA was used to induce TNF-α (tumor necrosis factor-α) production. TNF-α can induce apoptosis in T cells thereby removing the autoreactive T cell population. At the same time, splenocytes can induce negative selection of autoreactive T cells [92]. Because of its toxicity CFA cannot be used in humans. In humans the functional equivalent of CFA is BCG (Bacillus Calmette-Guérin) vaccine that can also induce TNF-α production. Previous clinical trials with BCG have mostly not shown promising results in the treatment of type 1 diabetes [94–96], however, one study reported a beneficial effect too [97]. Considering the possibility that the BCG dosing was not optimal in previous trials, Dr. Faustman has planned to repeat them. Currently, phase I trials have been completed and funding for phase II is being raised. Phase I trials showed no adverse effects in tested individuals and a transient rise in C-peptide levels and dead autoreactive T cells [98].

Other potential approaches to achieve a beneficial immunomodulation for the treatment of type 1 diabetes include HSCT (hematopoietic stem cell transplantation), UCB (umbilical cord blood) transfusion, and more recently the use of GABA (γ-aminobutyric acid). It has been shown in many studies that autologous or allogeneic HSCT can possibly induce tolerance in cases of autoimmune disorders [99]. Hematopoietic stem cells after transplantation did not give rise to pancreatic β cells [100], however, an HSCT prevented diabetes in NOD mice [101]. Therefore, HSCT can have an immunomodulatory effect but may not contribute to regeneration directly. Voltarelli et al. [102] carried out autologous

nonmyeloablative hematopoietic stem cell transplantation in 15 patients with newly diagnosed type 1 diabetes. This treatment was combined with cyclophosphamide and rabbit antithymocyte globulin to reset the immune system memory and allow for the endogenous regeneration to take place. In this study 14 out of 15 patients achieved an insulin-free state for variable periods of time.

The umbilical cord blood (UCB) contains an immunomodulatory and regenerative potential because of its stem cell and regulatory T cell content [103]. Treatment with human umbilical cord blood cells improved glycemia in both type 1 and type 2 diabetic mouse models and also improved the diabetes-associated nephropathy and neuropathy [104, 105]. Considering this beneficial effect, Haller et al. [106] carried out autologous UCB transfusion in seven children with recent-onset type 1 diabetes. A 6-month follow-up showed reduced insulin requirement and lowered HbA1c levels; however, no patient became insulin-free.

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the central nervous system. However, the GABAergic system has been identified in many of the non-neuronal tissues as well including the pancreas, suggesting its role in those tissues [107]. Recently, Soltani et al. [108] demonstrated that GABA treatment can preserve the β cell mass by activating a survival pathway in β cells and at the same time reduce inflammatory cytokine production. In diabetic mice, GABA treatment restored β cell mass and reversed hyperglycemia. Considering the β cell protective and immunoinhibitory effect of GABA, it may prove to be a potential candidate for the treatment of type 1 diabetes. If successful, GABA-based therapy would be much easier to utilize because GABA as a dietary supplement already exists.

9.5 In Vitro Differentiation and Expansion of β Cells

As mentioned in the first section, one of the main obstacles in the β cell replacement therapy is the shortage of available material, i.e., whole pancreas or islets. While one part of the regenerative research is focused on the promotion of endogenous β cell regeneration, the other part is related to the generation of β cells or islet-like structures in vitro, which can then be transplanted instead of the real donor islets. In order to replace the real donor islets such ex vivo generated endocrine cells should be functionally mature which means that they are able to secrete insulin after glucose stimulation and at a level that is physiologically acceptable. In this section, the sources and possible strategies involved in the ex vivo generation of β cells are briefly described.

9.5.1 β Cell Replication

Regarding the expansion of β cells, the first source that comes to mind is the β cell itself. It would be an ideal approach to expand β cells in vitro and transplant them back to treat diabetes. Proliferation of β cells is well known to occur in the adult mouse pancreas under normal conditions as well as during the regeneration of pancreas [25, 32]. Beta cell replication was also shown to be the primary mechanism involved in the early postnatal expansion of β cell mass in human individuals [109]. However, the replication of β cells in vitro is difficult to analyze as they gradually dedifferentiate and lose insulin expression [110, 111]. This makes it difficult to determine whether the replicating cells in the culture are β cells that have now lost their phenotype or they belong to some other cell type in the pancreas. To confirm the dedifferentiation and replication of β cells in vitro, Weinberg et al. [112] cultured β cells isolated from transgenic mice that permanently expressed YFP (yellow fluorescent protein) in β cells only. Indeed, it was found that these labeled β cells gradually lost insulin expression while they remained positive for YFP. However, under the culture conditions used the replication of β cells was rare even after they lose their phenotype. In another study by Parnaud et al. [113], proliferation of β cells isolated from adult humans and rats was compared. While rat β cells were able to proliferate under the culture conditions used, human β cells did not show this capacity. However, a lineage tracing approach was not used leaving a doubt that some dedifferentiated β cells might have proliferated in the culture. Russ et al. [114] used a lineage tracing approach to efficiently study the in vitro proliferation of β cells isolated from adult human islets. They used lentivirus vectors to label insulin-positive β cells with GFP (green fluorescent protein) and traced these cells in the culture. As found in other studies, these cells lost insulin expression but kept on proliferating in the culture up to 16 population doublings. It was also found that the presence of pancreatic non-β cells or a medium conditioned by pancreatic non-β cells was required for active proliferation of these GFP-labeled cells. However, consistent with the previous study by Weinberg et al. [112], mouse β cells showed much lower replication under similar culture conditions. Collectively, these studies show that β cells dedifferentiate when cultured in vitro and the proliferation of dedifferentiated β cells depends on the species and culture conditions used. Furthermore, the capacity of dedifferentiated β cells to expand in vitro is of therapeutic importance as well. Such dedifferentiated β cells are thought to retain some of the β cell characteristics that can make it easier to redifferentiate them into functional β cells for therapeutic use. Future studies will focus on the possible ways to redifferentiate these cells and to determine the functional maturity of β cells generated in this way.

9.5.2 Stem Cells as a Source of β cells

Stem cells are specialized cells that are defined by their ability to constantly renew themselves and differentiate into other types of cells in the living organism. They are divided into different categories depending on their differentiation potency. ES are pluripotent as they can give rise to cells belonging to all of the three primary germ layers, i.e., ectoderm, mesoderm, and endoderm. On the other hand, adult stem cells are only multipotent or unipotent as they can give rise to only a few or just one type of cells [115]. Both embryonic and adult stem cells have been investigated for their ability to generate β cells.

9.5.2.1 Embryonic/Induced Pluripotent Stem Cells to β Cells

ES are derived from the inner cell mass (ICM) of the embryo at blastocyst stage. Due to their pluripotency and unlimited ability of self-renewal, ES cells can be an excellent source for the in vitro differentiation of β cells. First of all they can be expanded in culture to a higher cell mass and then can be converted into β cells or other types of islet cells if specific inductive factors are sequentially applied. However, their origin from the embryo raises some ethical issues as well. This controversy around the use of ES cells can be resolved by the use of induced pluripotent stem cells (iPS). iPS are typically generated from adult somatic cells, e.g., fibroblasts, by forced expression of certain pluripotency-associated genes [116]. iPS cells have the same pluripotency as ES cells but pose no ethical concerns as they are derived from adult cells. iPS technology further allows the generation of β cells from the same individual that can improve the therapeutic outcomes by reducing the need of extensive immunosuppression.

For the successful generation of islets/β cells in vitro it is important to recapitulate the signaling pathways that play a role in the normal embryonic development of pancreas. This includes three main steps: inducing definitive endoderm from ES/iPS cells, promoting the specification of pancreatic fate, and finally generating mature islets. It has been shown that signaling through the nodal pathway plays an important role in the generation of definitive endoderm, which is further specified toward a pancreatic fate under the action of WNT, fibroblast growth factor (FGF) , bone morphogenetic protein (BMP), and retinoic acid (RA) signaling pathways [117]. Successful generation of pancreatic tissue depends on the activation or inhibition of these pathways in a stage-specific and time-dependent manner.

The knowledge gained from the investigation of early pancreas development has helped researchers to generate protocols for in vitro differentiation of β cells and other cells of pancreatic lineage. In this context D' Amour et al. [118] reported the generation of definitive endoderm from human ES, followed by further differentiation into hormone-expressing pancreatic endocrine cells. Their protocol was based on the induction of various signaling cascades sequentially starting from

the ES cells to the level of mature endocrine cells. Afterwards, many research groups have demonstrated the in vitro generation of insulin-producing cells from human ES (hES) or human iPS (hiPS) cells using various modified protocols [117, 119]. However, the efficiency of these protocols is quite low with only a small percentage of insulin or C-peptide-positive cells being developed. Furthermore, such in vitro-derived cells mostly produced insulin at a very low level or they did not respond to glucose stimulation. Finally, very few studies were able to show the decrease in hyperglycemia after transplantation of such in vitro-derived cells into diabetic mouse/rat models [120–122].

To generate fully functional β cells that can mimic the physiology of normal β cells, the existing protocols, therefore, need further refinement. On one hand, it is important to activate various signaling cascades in a strict time-dependent fashion to achieve a microenvironment that closely resembles the in vivo situation. On the other hand, we need to understand the events involved in the final maturity of β cells that takes place from late embryonic to early postnatal period, and then to apply this knowledge to our in vitro differentiation protocols to generate β cells that can match the physiology of normal β cells.

9.5.2.2 Adult Stem Cells to β Cells

Stem cells are also known to exist in the adult organism in many different tissues, e.g., skin, liver, brain, bone marrow. These adult stem cells are mainly tissue specific and therefore have a reduced plasticity compared to ES. They are involved in tissue regeneration and repair, and under normal conditions differentiate into the same tissue type to which they belong [115]. However, some of them may differentiate into a few other cell types especially when they are cultured in vitro under modified conditions to induce pluripotency. There are three main advantages of using adult stem cells instead of ES cells. First of all, because of their reduced plasticity they present a reduced risk of tumor formation following transplantation. In case of ES cells, even a few undifferentiated cells remaining in the culture can lead to teratocarcinoma formation in the host following transplantation. Second, adult stem cells provide an autologous source of stem cells. Transplantation of a tissue generated from such autologous stem cells requires no special immunosuppression. Finally, one critical hindrance in the use of ES is ethical considerations that can be avoided by the use of adult stem cells. The potential of some adult stem cells to generate β cells is discussed here.

Pancreatic Stem Cells

As discussed in the previous sections, neogenesis of β cells can take place in the adult pancreas under certain physiological or pathological conditions, pointing to the fact that a stem/progenitor cell-like population possibly resides in the pancreas. Many possible candidates have been pointed out in the pancreas; however,

conclusive evidence is missing because of the lack of a specific pancreatic stem cell marker.

Pancreatic duct cells

Pancreatic endocrine cells are generated from ductal progenitors during embryonic development and it has also been shown that ductal cells can give rise to β cells under certain conditions even in the adult pancreas [27]. Therefore pancreatic ductal population presents as a probable source for the existence of stem cells. Indeed, Ramiya et al. [123] reported the generation of islet-like clusters, containing α, β, and δ cells, from the long-term culture of pancreatic ductal cells from the adult pre-diabetic NOD mice. These in vitro-generated islets were glucose responsive and were able to reverse diabetes when transplanted into diabetic NOD mice. In another study, Bonner-Weir et al. [124] showed the generation of insulin-positive cells from human adult pancreatic ductal cells grown in a monolayer and overlaid with a thin layer of matrigel. More recently, Yamamoto et al. [125] and Noguchi et al. [126] also showed the isolation of pancreatic stem/progenitor cells from mouse ductal rich population that were able to generate hormone producing cells under specific conditions. These putative stem cells were able to divide actively beyond the population doubling level of 300. However, a similar approach was not successful with cells from human ductal rich population [127]. Although, these studies provide evidence in favor of the existence of stem cells in ductal population, there still remains an element of doubt. It is also possible that ductal cells just dedifferentiate to lose their ductal phenotype before converting into endocrine cells. This problem exists due to the absence of known markers to specify pancreatic stem cells.

Nestin-positive cells

Nestin-positive cells are considered as another candidate for adult pancreatic stem cells. Nestin is an intermediate filament protein that has been shown as a marker for neural stem cells [128]. In a study by Zulewski et al. [129], nestin-positive cells were isolated from rat and human islets. These cells showed extended proliferative capacity during in vitro culture (nearly 8 months) and were able to differentiate into pancreatic endocrine, exocrine, and hepatic phenotypes. Such nestin-positive cells did not express any of the islet hormones or cytokeratin 19 (CK-19), a marker of the ductal cells, indicating that they exist as a separate population in the pancreas. However, nestin-positive cells are found in many other tissues as well, making nestin a poor marker for the identification of pancreatic stem cells [130]. Therefore, in spite of their potential to contribute toward islet differentiation, their role as the true pancreatic stem cells remains elusive.

Small cells and oval cells

Using in vitro culture of human and canine islets, Petropavlovskaia and Rosenberg [131] identified a cluster of small cells that were positive for pancreatic endocrine hormones but were negative for nestin, CK-19, or amylase (a marker of acinar cells). Such small cells had an immature morphology and secreted insulin in response to glucose stimulation. However, these cells were extremely quiescent and difficult to expand in culture. Therefore, even if they contribute toward islet growth, they are not a useful source for the in vitro generation of β cells. Another

population of small oval cells, originally identified in the liver, has been found in the pancreas as well. In some studies, these cells were shown to contribute toward the hepatic lineage instead of the pancreatic one [130]. In contrast, Yang et al. [132] demonstrated the conversion of hepatic oval cells into pancreatic endocrine cells in vitro under high glucose culture condition. These cells secreted insulin in response to glucose stimulation and were able to correct hyperglycemia in NOD-severe combined immunodeficiency (SCID) mice. It is possible that the oval cells identified in pancreas are not the same as those in liver and, therefore, behave in a different way. Irrespective of that, the hepatic oval cells are not an excellent source for the generation of β cells as they appear in the liver only after severe liver injury.

Other possible stem cells in the pancreas

Seaberg et al. [133] isolated putative pancreatic progenitors by culturing both nestin-positive and nestin-negative cells from adult mouse pancreatic islet and ductal compartment. These cells called pancreatic multipotent progenitors (PMPs) by the authors, were able to proliferate in vitro and formed clonal aggregates expressing neural and pancreatic precursor markers. Such PMPs were able to differentiate along the neural as well as pancreatic lineages. They can serve as a possible source for the generation of β cells in vitro, provided that their self-renewal capacity can be increased and their differentiation into β cells can be selectively promoted.

Telomerase expression is associated with the failure of cells to undergo senescence, a common characteristic of most stem cells. Based on this property, Breault et al. [134] generated transgenic mice expressing GFP under the control of mouse telomerase reverse transcriptase (mTert). In these mice the expression of GFP was confirmed in the male germ cells and hematopoietic stem cells, showing the reliability of the system to detect the presence of stem/progenitor cells in the body. Using this system GFP-positive cells were detected in the non-endocrine portion of the pancreas following treatment with GLP-1 analog exendin-4 [135]. This has suggested the presence of certain resident cells in the pancreas that can be activated to act like stem cells under specific conditions.

The advantage of using pancreatic stem cells is the reduced in vitro manipulation required to obtain functional β cells. However, further research is required to identify pancreatic stem cells that have an efficient self-renewal capacity and differentiation ability to generate sufficient β cell mass to be used in the clinical therapies.

Intestinal and Hepatic Stem Cells

Pancreas shares its embryonic origin with liver and gastrointestinal tract. Especially the liver is a more closely related organ that is also indicated by the presence of a similar glucose-sensing system in the hepatocytes and pancreatic β cells [136]. Transdifferentiation of liver into pancreas or pancreas into liver is known to occur under certain pathological conditions [137, 138]. It has also been shown that in the

absence of transcriptional regulator Ptf1a the pancreatic progenitors change their fate and develop into the intestinal epithelium [139]. Therefore, it can be suggested that adult stem/progenitor cells from these tissues might be easier to differentiate toward a pancreatic lineage.

Apart from hepatic oval cells discussed above, there are some other stem/progenitor-like cells in the liver that have the potential to generate insulin-producing cells. Yamada et al. [140] demonstrated the conversion of hepatic stem-like cells (HSLs) into pancreatic endocrine cells following treatment with sodium butyrate and betacellulin. In another study, Nakajima et al. [141] showed that small hepatocytes (SHCs), a type of adult hepatic progenitor cells, could differentiate into cells producing insulin. The gastrointestinal tract contains a large population of stem cells that can be utilized for therapeutic approaches. However, their potential for the generation of β cells is largely unexplored. GLP-$1^{(1-37)}$ treatment was shown to induce insulin production in fetal and adult intestinal epithelial cells both in vitro and in vivo. Such in vitro-generated cells were able to reverse hyperglycemia following transplantation into diabetic mice [142].

Stem Cells from Adult Bone Marrow

Adult bone marrow contains a population of multipotent progenitor stem cells that have the potential to differentiate into hematopoietic cells as well as into cells of some non-hematopoietic tissues [143]. As discussed in the previous section, hematopoietic stem cells from bone marrow have a beneficial immunomodulatory effect in the treatment of type 1 diabetes. However, whether they directly transdifferentiate to generate β cells is still controversial. Lanus et al. [143] detected donor bone marrow-derived β cells in recipient pancreas within 4–6 weeks of transplantation. They used a Cre/loxP system with EGFP (enhanced GFP) reporter to confirm the origin of newly generated β cells. Furthermore, they confirmed that these new β cells were resulting from transdifferentiation of donor bone marrow cells and not by any cell fusion. In another study Hess et al. [144] showed that the transplantation of adult bone marrow-derived stem cells into streptozotocin-induced diabetic mice resulted in the reduction of hyperglycemia. In this case, donor cells promoted endogenous pancreas regeneration and only a few of them converted into β cells. In contrast, some other studies did not find any significant evidence of bone marrow transdifferentiation into β cells following transplantation [145, 146]. Using a different strategy, Oh et al. [147] showed the in vitro transdifferentiation of bone marrow-derived cells into insulin-producing cells when cultured under specific conditions. From these studies the in vivo generation of β cells from transplanted bone marrow-derived cells seems to be infrequent. However, bone marrow-derived cells can still be a potential source of adult stem cells for the in vitro generation of β cells.

Dental Pulp Stem Cells

Adult multipotent mesenchymal stem cells named as dental pulp stem cells (DPSCs) have been isolated from human dental pulp tissue or exfoliated teeth [148]. These cells have the capacity of self-renewal and the ability to differentiate into adipogenic, chondrogenic, and osteogenic lineages and are comparable to the mesenchymal progenitor cells from the bone marrow. Govindasamy et al. [149] showed that such DPSCs could also be induced to differentiate into cells of the pancreatic lineage forming islet-like cell aggregates (ICAs) under specified culture conditions. These ICAs were positive for many endocrine markers and were able to release insulin in a glucose-dependent manner in vitro. The use of DPSCs for the generation of β cells can be very useful because it is easier to obtain these cells from the exfoliated teeth and, at the same time, can also provide an autologous source of β cells.

Germline Stem Cells

Adult stem cells with multipotent potential also exist in the germline, called germline stem cells (GSCs). The spermatogonial stem cells (SSCs), that are male germline stem cells, can acquire embryonic stem cell-like properties under specific culture conditions and are able to generate derivatives of the three embryonic germ layers [150]. These cells can then be used to obtain many different tissues including the pancreatic tissue [151]. Recently, researchers from Georgetown University reported the generation of insulin-producing cells from human SSCs. When transplanted into diabetic mice these cells were able to reduce hyperglycemia. This may provide an autologous source of β cells for male diabetics, generated from their own stem cells. Researchers hope that they would be able to apply this technique to female germline stem cells too. However, these results are just preliminary and further work is required to establish the procedure [152].

9.5.3 Reprogramming of Adult Cells into β Cells and the Prospects of Gene Therapy

Another way to generate functional β cells in vitro or in vivo is through transdifferentiation of mature adult cells that is mostly achieved by forced expression of certain β cell-associated transcription factors or in some cases by treatment with various signaling molecules. For this approach, the best starting population belongs to the pancreatic non-β cells (e.g., acinar cells) or non-pancreatic cells (like those from liver and intestine). These cells would be comparatively easier to reprogram because of their related developmental origin, as mentioned in the previous section.

9.5.3.1 Reprogramming of Liver Cells

Pdx1 (also known as IPF1) is one of the most important transcription factors involved in the development of pancreas and function of β cells. It has been shown by loss-of-function studies that mice lacking a functional Pdx1 allele, fail to develop the pancreas [153, 154]. Keeping this in view, Ferber et al. [155] hypothesized that Pdx1 might be able to convert hepatocytes into insulin-producing cells. Indeed, they were able to induce insulin expression in hepatocytes following recombinant-adenovirus-mediated delivery of Pdx1 into the mouse liver. Insulin produced in this way, was biologically active and was able to reverse the streptozotocin-induced hyperglycemia. The ability of Pdx1 to convert hepatocytes into insulin-producing cells has also been shown in vitro. However, it was found that Pdx1 alone can only generate β cell precursors that need some additional factors like activation of Pax4 transcription factor or exposure to high glucose in vitro or in vivo (e.g., in a diabetic mice) for further maturation into β-like cells [136, 156].

Some other transcription factors downstream of Pdx1 have also been tested for generating insulin-producing cells in this way. Kojima et al. [157] showed islet neogenesis and reversal of diabetes following helper-dependent-adenovirus-(HDAD) mediated delivery of NeuroD and betacellulin (a growth factor for β cells). In another study Kaneto et al. [158] also used the adenovirus-mediated gene delivery to overexpress Pdx1 together with NeuroD or Ngn3 in the mouse liver. They found an increased expression of insulin in hepatocytes that transiently reduced the streptozotocin-induced hyperglycemia. Using a similar strategy, Sapir et al. [159] demonstrated the in vitro transdifferentiation of human fetal and adult liver cells into insulin-producing cells following the Pdx-1 overexpression. The isolated liver cells were able to proliferative in vitro for up to 20 passages and after reprogramming expressed a wide range of β cell and other islet associated factors. These cells also ameliorated hyperglycemia for a long period of time following transplantation into diabetic (NOD-SCID) mice. Based on these studies, it looks promising to transdifferentiate liver cells into β cells in vitro or to carry this out in vivo as a gene therapy approach. However, more studies are required to confirm the maturity and functional stability of such reprogrammed cells over a long period of time.

9.5.3.2 Reprogramming of Gut Cells

Because of its large stem cell population and comparatively good accessibility for gene delivery, intestinal epithelium is also a promising target organ for gene/cell-based therapies [160]. Stable transfection of rat intestinal crypt-like cells (IEC-6) to overexpress Pdx1 and Isl1 resulted in the expression of insulin. However, Pdx1 alone was not sufficient to induce insulin expression in these cells. It required either treatment with betacellulin or combined expression of Isl1 to induce the expression of insulin. While these cells were able to reduce hyperglycemia in diabetic rats, they did not show increased insulin secretion in response to glucose stimulation [161, 162].

Following a different strategy, based on the resemblance between pancreatic β cells and the enteroendocrine cells of the gut, some groups have tried to ectopically express insulin in these cells [163]. Cheung et al. [164] transfected GIP-producing GTC-1 cells with a construct expressing human preproinsulin under rat GIP promoter. Transfected cells processed and released human insulin in a glucose responsive way. Furthermore, transgenic mice expressing the same construct were able to recover from streptozotocin-induced hyperglycemia. In another study Han et al. [165] used a similar strategy to generate insulin-secreting cells from intestinal STC-1 cells. These cells were able to restore normoglycemia after transplantation into streptozotocin-treated NOD-SCID mice. However, later on these mice developed hypoglycemia due to excessive insulin secretion. The cells generated in this way are just intestinal cells that are forced to produce insulin while they keep most of their intestinal characteristics. Therefore, they can reduce hyperglycemia due to insulin production but cannot integrate into the whole metabolic homeostasis as they lack the rest of β cell signaling machinery. One way to deal with this problem is to engineer gut cells that can produce insulin in the presence of an external signal and stop it when the signal is removed. Based on this idea, Unniappan et al. [166] engineered GTC-1 cells in which insulin expression was dependent on mifepristone induction in a dose-dependent manner. When transplanted into streptozotocin-treated diabetic mice and induced with mifepristone, these cells ameliorated hyperglycemia. However, in spite of this induced situation the treated mice exhibited transient hypoglycemia. It is important to note that the cell lines used in these studies were tumor derived and, therefore, may not behave like natural enteroendocrine cells. To make it therapeutically applicable, it would be important to design a strategy to deliver insulin expression cassette to the native gut cells in a safe and efficient way.

9.5.3.3 Reprogramming of Pancreatic Non-β Cells

Acinar Cells

Among the pancreatic non-β cells the exocrine portion of the pancreas is an excellent target for reprogramming into β cells. One reason for this is the relative abundance of acinar cells in the pancreas and their close developmental relationship to β cells. Second, any reprogrammed β cell arising from an acinar cell would reside in the native pancreatic environment, and, therefore, has a better chance of long-term survival and/or maturation. Many in vitro studies have demonstrated that acinar cells can transdifferentiate to produce insulin-secreting cells when cultured in the presence of EGF along with nicotinamide or leukemia inhibitory factor (LIF) [167, 168]. However, in vivo in the adult pancreas, acinar cells can give rise to either acinar or ductal cells, but not endocrine cells, under normal conditions as well as upon injury-induced pancreatic regeneration [22, 169]. For in vivo reprogramming of acinar cells into β cells Zhou et al. [170] used adenovirus-mediated gene transfer to express a combination of three transcription

factors (Pdx1, Ngn3, and MafA) in exocrine cells. A combination of these three factors successfully converted acinar cells into β cells that were indistinguishable from normal β cells. These newly generated cells expressed β cell-specific markers and were able to reduce hyperglycemia in streptozotocin-induced diabetic mice; however, a complete normoglycemia was not achieved. One possible reason could be the insufficient number of β cells generated in this way. Second, the new β cells failed to organize into islet structures that might have limited their effectiveness. Regardless of this, conversion of acinar cells into insulin-producing cells in vivo is an important advance that has the potential to treat diabetes.

Alpha Cells

Direct conversion of glucagon producing α cells into β cells can take place under certain conditions, e.g., during injury-induced β cell regeneration in rodent models [33, 171]. In humans, a small number of glucagon cells co-positive for insulin were found in the pancreata of acute pancreatitis patients, however, their conversion into mature β cells was not confirmed [171]. Conversion of α into β cells is, therefore, not a frequent or easily induced process. Loss-of-function studies in mice have identified Pax4 and Arx as two important transcription factors that play a role in the specification of β and α cells respectively [172, 173]. Additionally, it was found that Pax4 and Arx have opposing roles: a misexpression of Arx in β cells can convert them into α cells and that of Pax4 in α cells converts them into β cells [174, 175]. In this study by Collombat et al. [175], a Cre/loxP system was used to overexpress Pax4 in glucagon cells that led to their transdifferentiation into β cells. The resulting decrease in the level of glucagon was compensated by the generation of new glucagon cells from the ductal progenitors. This continuous cycle of α cell generation and conversion into β cells led to the formation of mega-islets. In spite of these oversized islets with increased insulin content, older mice developed hyperglycemia that might be due to the acquired insulin resistance. However, these newly generated β cells did restore normoglycemia in younger (less than 4 week old) mice that were treated with streptozotocin to kill β cells. Although, currently far from clinical application, such transdifferentiation of α to β cells, holds great potential for the treatment of diabetes. Future studies will be focused on finding alternative ways to easily induce this process in a clinical setting, e.g., treatment with some biomolecules instead of genetic manipulation.

9.6 Xenotransplantation: Pig Islets as an Alternative Source of β Cells

Xenotransplantation of pig islets, if made possible, can circumvent the shortage of available donor pancreas/islets. As compared to whole organs, transplantation of pig islets into non-human primates has shown more promising results [176].

Cardona et al. [177] demonstrated reversal of diabetes in rhesus macaques following transplantation of neonatal pig islets. Similar results were shown by Hering et al. [178], using adult porcine islets in cynomolgus macaques. However, the immunosuppression approach used in these studies is not clinically applicable to humans. To improve the xenotransplantation outcomes, two main strategies are under investigation. First, the generation of transgenic pigs that lacks certain pig-specific antigens (e.g., galactose-α-1,3-galactose) or expresses human complement regulatory proteins (e.g., CD46) to reduce the immune response. Second, the encapsulation of pig islets to protect them from immune attack while keeping them alive and functional. Indeed, the transplantation of transgenic or encapsulated islets showed improved results in non-human primates [179, 180]. Moreover, Elliott et al. [181] reported transient reduction in insulin requirement in a type 1 diabetic patient, following transplantation of encapsulated porcine islets. Further studies are required to investigate the long-term survival and efficacy of wild-type/transgenic pig islets and the encapsulation materials in non-human primates. At the same time, human clinical trials of pig islet transplantation for type 1 diabetes treatment are expected to increase in future [182].

Future perspectives

The only cure to the problem of diabetes is the replacement of functional β-cell mass. As discussed in the chapter, there are many different strategies currently being investigated by researchers to achieve this goal. However, most of the approaches are at experimental level, tested either in vitro or in vivo in animal models. Furthermore, in every approach there are issues that still need to be resolved and, therefore, require further studies. On one hand future studies will focus on improving the current techniques. On the other hand, it would be important to understand the human pancreas development in greater detail and to link this knowledge to the one obtained from animal studies. This would then help in transferring the techniques developed for animal models to the human subjects. Finally, considering the variety of methods being investigated for the treatment of diabetes one can hope that the time is not far away when an insulin-free diabetes treatment would be available to diabetic patients.

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References

1. http://www.who.int/mediacentre/factsheets/fs312/en/index.html
2. http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0002194/
3. Ludwig B, Ludwig S, Steffen A, Saeger HD, Bornstein SR (2010) Islet versus pancreas transplantation in type 1 diabetes: competitive or complementary? Curr Diab Rep 10(6):506–511

4. Kelly KD, Lillehei RC, Merkel FK, Idezuki Y, Goetz FC (1967) Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy. Surgery 61(6):827–837
5. http://surgery.arizona.edu/unit/center/international-pancreas-transplant-registry
6. Richter A, Lerner S, Schröppel B (2011) Current state of combined kidney and pancreas transplantation. Blood Purif 31(1–3):96–101
7. Tzakis AG, Ricordi C, Alejandro R, Zeng Y, Fung JJ, Todo S, Demetris AJ, Mintz DH, Starzl TE (1990) Pancreatic islet transplantation after upper abdominal exenteration and liver replacement. Lancet 336(8712):402–405
8. Brendel M, Hering B, Schulz A, Bretzel R (1999) International islet transplant registry report. University of Giesen, Giesen, pp 1–20
9. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 343(4):230–238
10. Shapiro AM, Ricordi C, Hering B (2003) Edmonton's islet success has indeed been replicated elsewhere. Lancet 362(9391):1242
11. Sorenson RL, Brelje TC (1997) Adaptation of islets of langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones. Horm Metab Res 29(6):301–307
12. Parsons JA, Brelje TC, Sorenson RL (1992) Adaptation of islets of Langerhans to pregnancy: increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. Endocrinology 130(3):1459–1466
13. Rieck S, White P, Schug J, Fox AJ, Smirnova O, Gao N, Gupta RK, Wang ZV, Scherer PE, Keller MP, Attie AD, Kaestner KH (2009) The transcriptional response of the islet to pregnancy in mice. Mol Endocrinol 23(10):1702–1712
14. Parsons JA, Bartke A, Sorenson RL (1995) Number and size of islets of Langerhans in pregnant, human growth hormone-expressing transgenic, and pituitary dwarf mice: effect of lactogenic hormones. Endocrinology 136(5):2013–2021
15. Scaglia L, Smith FE, Bonner-Weir S (1995) Apoptosis contributes to the involution of beta cell mass in the post partum rat pancreas. Endocrinology 136(12):5461–5468
16. Catalano PM, Tyzbir ED, Roman NM, Amini SB, Sims EA (1991) Longitudinal changes in insulin release and insulin resistance in non-obese pregnant women. Am J Obstet Gynecol 165(6 Pt 1):1667–1672
17. Homko C, Sivan E, Chen X, Reece EA, Boden G (2001) Insulin secretion during and after pregnancy in patients with gestational diabetes mellitus. J Clin Endocrinol Metab 86(2):568–573
18. Van Assche FA, Aerts L, De Prins F (1978) A morphological study of the endocrine pancreas in human pregnancy. Br J Obstet Gynaecol 85(11):818–820
19. Butler AE, Cao-Minh L, Galasso R, Rizza RA, Corradin A, Cobelli C, Butler PC (2010) Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. Diabetologia 53(10):2167–2176
20. Kim C, Newton KM, Knopp RH (2002) Gestational diabetes and the incidence of type 2 diabetes: a systematic review. Diabetes Care 25(10):1862–1868
21. Baiu D, Merriam F, Odorico J (2011) Potential pathways to restore β-cell mass: pluripotent stem cells, reprogramming, and endogenous regeneration. Curr Diab Rep 11(5):392–401
22. Pan FC, Wright C (2011) Pancreas organogenesis: from bud to plexus to gland. Dev Dyn 240(3):530–565
23. Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE (1993) A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. Diabetes 42(12):1715–1720
24. Page BJ, Du Toit DF, Muller CJ, Mattysen J, Lyners R (2000) An immuno-cytochemical profile of the endocrine pancreas using an occlusive duct ligation model. JOP 1(4):191–203
25. Dor Y, Brown J, Martinez OI, Melton DA (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 429(6987):41–46
26. Teta M, Rankin MM, Long SY, Stein GM, Kushner JA (2007) Growth and regeneration of adult beta cells does not involve specialized progenitors. Dev Cell 12(5):817–826

27. Inada A, Nienaber C, Katsuta H, Fujitani Y, Levine J, Morita R, Sharma A, Bonner-Weir S (2008) Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. Proc Natl Acad Sci U S A 105(50):19915–19919
28. Xu X, D'Hoker J, Stange G, Bonne S, De Leu N, Xiao X, Van de Casteele M, Mellitzer G, Ling Z, Pipeleers D, Bouwens L, Scharfmann R, Gradwohl G, Heimberg H (2008) Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell 132(2):197–207
29. Lee CS, Leon DD, Kaestner KH, Stoffers DA (2006) Regeneration of pancreatic islets after partial pancreatectomy in mice does not involve the reactivation of neurogenin-3. Diabetes 55(2):269–272
30. Solar M, Cardalda C, Houbracken I, Martin M, Maestro MA, De Medts N, Xu X, Grau V, Heimberg H, Bouwens L, Ferrer J (2009) Pancreatic exocrine duct cells give rise to insulin-producing beta cells during embryogenesis but not after birth. Dev Cell 17(6):849–860
31. Kopp JL, Dubois CL, Schaffer AE, Hao E, Shih HP, Seymour PA, Ma J, Sander M (2011) Sox9 + ductal cells are multipotent progenitors throughout development but do not produce new endocrine cells in the normal or injured adult pancreas. Development 138(4):653–665
32. Nir T, Melton DA, Dor Y (2007) Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 117(9):2553–2561
33. Thorel F, Nepote V, Avril I, Kohno K, Desgraz R, Chera S, Herrera PL (2010) Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 464(7292):1149–1154
34. Cnop M, Igoillo-Esteve M, Hughes SJ, Walker JN, Cnop I, Clark A (2011) Longevity of human islet α- and β-cells. Diabetes Obes Metab 13(Suppl. 1):39–46
35. Perl S, Kushner JA, Buchholz BA, Meeker AK, Stein GM, Hsieh M, Kirby M, Pechhold S, Liu EH, Harlan DM, Tisdale JF (2010) Significant human beta-cell turnover is limited to the first three decades of life as determined by in vivo thymidine analog incorporation and radiocarbon dating. J Clin Endocrinol Metab 95(10):234–239
36. Meier JJ, Bhushan A, Butler AE, Rizza RA, Butler PC (2005) Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? Diabetologia 48(11):2221–2228
37. Lavine JA, Attie AD (2010) Gastrointestinal hormones and the regulation of β-cell mass. Ann NY Acad Sci 1212(1):41–58
38. Perfetti R, Zhou J, Doyle ME, Egan JM (2000) Glucagon-like peptide-1 induces cell proliferation and pancreatic-duodenum homeobox-1 expression and increases endocrine cell mass in the pancreas of old, glucose-intolerant rats. Endocrinology 141(12):4600–4605
39. Zhang J, Tokui Y, Yamagata K, Kozawa J, Sayama K, Iwahashi H, Okita K, Miuchi M, Konya H, Hamaguchi T, Namba M, Shimomura I, Miyagawa JI (2007) Continuous stimulation of human glucagon-like peptide-1 (7–36) amide in a mouse model (NOD) delays onset of autoimmune type 1 diabetes. Diabetologia 50(9):1900–1909
40. Drucker DJ, Nauck MA (2006) The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 368(9548):1696–1705
41. Wang Q, Brubaker PL (2002) Glucagon-like peptide-1 treatment delays the onset of diabetes in 8 week-old db/db mice. Diabetologia 45(9):1263–1273
42. Green BD, Lavery KS, Irwin N, O'Harte FP, Harriott P, Greer B, Bailey CJ, Flatt PR (2006) Novel glucagon-like peptide-1 (GLP-1) analog (Val8)GLP-1 results in significant improvements of glucose tolerance and pancreatic beta-cell function after 3 week daily administration in obese diabetic (ob/ob) mice. J Pharmacol Exp Ther 318(2):914–921
43. Mu J, Petrov A, Eiermann GJ, Woods J, Zhou YP, Li Z, Zycband E, Feng Y, Zhu L, Roy RS, Howard AD, Li C, Thornberry NA, Zhang BB (2009) Inhibition of DPP-4 with sitagliptin improves glycemic control and restores islet cell mass and function in a rodent model of type 2 diabetes. Eur J Pharmacol 623(1–3):148–154
44. Zhou J, Pineyro MA, Wang X, Doyle ME, Egan JM (2002) Exendin-4 differentiation of a human pancreatic duct cell line into endocrine cells: involvement of PDX-1 and HNF3beta transcription factors. J Cell Physiol 192(3):304–314

45. Zhou J, Wang X, Pineyro MA, Egan JM (1999) Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42 J cells into glucagon- and insulin-producing cells. Diabetes 48(12):2358–2366
46. Gallwitz B (2011) GLP-1 agonists and dipeptidyl-peptidase IV inhibitors. Handb Exp Pharmacol 203:53–74
47. Herbach N, Goeke B, Schneider M, Hermanns W, Wolf E, Wanke R (2005) Overexpression of a dominant negative GIP receptor in transgenic mice results in disturbed postnatal pancreatic islet and beta-cell development. Regul Pept 125(1–3):103–117
48. Marenah L, McCluskey JT, Abdel-Wahab YH, O'Harte FP, McClenaghan NH, Flatt PR (2006) A stable analogue of glucose-dependent insulinotropic polypeptide, GIP (LysPAL16), enhances functional differentiation of mouse embryonic stem cells into cells expressing islet-specific genes and hormones. Biol Chem 387(7):941–947
49. Irwin N, Clarke GC, Green BD, Greer B, Harriott P, Gault VA, O'Harte FP, Flatt PR (2006) Evaluation of the antidiabetic activity of DPP IV resistant N-terminally modified versus mid-chain acylated analogues of glucose-dependent insulinotropic polypeptide. Biochem Pharmacol 72(6):719–728
50. Kuntz E, Pinget M, Damge P (2004) Cholecystokinin octapeptide: a potential growth factor for pancreatic beta cells in diabetic rats. JOP 5(6):464–475
51. Lavine JA, Raess PW, Stapleton DS, Rabaglia ME, Suhonen JI, Schueler KL, Koltes JE, Dawson JA, Yandell BS, Samuelson LC, Beinfeld MC, Davis DB, Hellerstein MK, Keller MP, Attie AD (2010) Cholecystokinin is up-regulated in obese mouse islets and expands beta-cell mass by increasing beta-cell survival. Endocrinology 151(8):3577–3588
52. Ahren B, Holst JJ, Efendic S (2000) Antidiabetogenic action of cholecystokinin-8 in type 2 diabetes. J Clin Endocrinol Metab 85(3):1043–1048
53. Rooman I, Lardon J, Bouwens L (2002) Gastrin stimulates beta-cell neogenesis and increases islet mass from transdifferentiated but not from normal exocrine pancreas tissue. DiabetesDiabetes 51(3):686–690
54. Wang TC, Bonner-Weir S, Oates PS, Chulak M, Simon B, Merlino GT, Schmidt EV, Brand SJ (1993) Pancreatic gastrin stimulates islet differentiation of transforming growth factor alpha-induced ductular precursor cells. J Clin Invest 92(3):1349–1356
55. Rooman I, Bouwens L (2004) Combined gastrin and epidermal growth factor treatment induces islet regeneration and restores normoglycaemia in C57Bl6/J mice treated with alloxan. Diabetologia 47(2):259–265
56. Brand SJ, Tagerud S, Lambert P, Magil SG, Tatarkiewicz K, Doiron K, Yan Y (2002) Pharmacological treatment of chronic diabetes by stimulating pancreatic beta-cell regeneration with systemic co-administration of EGF and gastrin. Pharmacol Toxicol 91(6):414–420
57. Massague J, Chen YG (2000) Controlling TGF-beta signaling. Genes Dev 14(6):627–644
58. Kim SK, Hebrook M, Li E, Oh SP, Schrewe H, Harmon EB, Lee JS, Melton DA (2000) Activin receptor patterning of foregut organogenesis. Genes Dev 14(15):1866–1871
59. Furukawa M, Eto Y, Kojima I (1995) Expression of immunoreactive activin A in fetal rat pancreas. Endocr J 42(1):63–68
60. Shiozaki S, Tajima T, Zhang YQ, Furukawa M, Nakazato Y, Kojima I (1999) Impaired differentiation of endocrine and exocrine cells of the pancreas in transgenic mouse expressing the truncated type II activin receptor. Biochim Biophys Acta 1450(1):1–11
61. Zhang YQ, Zhang H, Maeshima A, Kurihara H, Miyagawa J, Takeuchi T, Kojima I (2002) Up-regulation of the expression of activins in the pancreatic duct by reduction of the β cell mass. Endocrinology 143(9):3540–3547
62. Moriya N, Komazaki S, Takahashi S, Yokota C, Asashima M (2000) In vitro pancreas formation from Xenopus ectoderm treated with activin and retinoic acid. Dev Growth Differ 42(6):593–602
63. Shing Y, Christofori G, Hanahan D, Ono Y, Sasada R, Igarashi K, Folkman J (1993) Betacellulin: a mitogen from pancreatic β-cell tumors. Science 259(5101):1604–1607

64. Mashima H, Ohnishi H, Wakabayashi K, Mine T, Miyagawa J, Hanafusa T, Seno M, Yamada H, Kojima I (1996) Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42 J cells into insulin-secreting cells. J Clin Invest 97(7):1647–1654
65. Li L, Seno M, Yamada H, Kojima I (2001) Promotion of β-cell regeneration by betacellulin in ninety percent pancreatectomized rats. Endocrinology 142(12):5379–5385
66. Li L, Yi Z, Seno M, Kojima I (2004) Activin A and betacellulin. Effect of regeneration of pancreatic β cells in neonatal streptozotocin treated rats. Diabetes 53(3):608–615
67. Umezawa K, Hiroki A, Kawakami M, Naka H, Takei I, Ogata T, Kojima I, Koyano T, Kowithayakorn T, Pang HS, Kam TS (2003) Induction of insulin production in rat pancreatic acinar carcinoma cells by conophylline. Biomed Pharmacother 57(8):341–350
68. Ogata T, Li L, Yamada S, Yamamoto Y, Tanaka Y, Takei I, Umezawa K, Kojima I (2004) Promotion of β-cell differentiation by conophylline in fetal and neonatal rat pancreas. Diabetes 53(10):2596–2602
69. Kojima I, Umezawa K (2006) Conophylline: a novel differentiation inducer for pancreatic beta cells. Int J Biochem Cell Biol 38(5–6):923–930
70. Vasavada RC, Gonzalez-Pertusa JA, Fujinaka Y, Fiaschi-Taesch N, Cozar-Castellano I, Garcia-Ocaña A (2006) Growth factors and beta cell replication. Int J Biochem Cell Biol 38(5–6):931–950
71. Tsuda H, Iwase T, Matsumoto K, Ito M, Hirono I, Nishida Y, Yamamoto M, Tatematsu M, Matsumoto K, Nakamura T (1992) Immunohistochemical localization of hepatocyte growth factor protein in pancreas islet A-cells of man and rats. Jpn J Cancer Res 83(12):1262–1266
72. Otonkoski T, Cirulli V, Beattie M, Mally MI, Soto G, Rubin JS, Hayek A (1996) A role for hepatocyte growth factor/scatter factor in fetal mesenchyme-induced pancreatic beta-cell growth. Endocrinology 137(7):3131–3139
73. Bulotta A, Di Cesare E, Ponte E, Falchi M, Sciacchitano S, Cucinotta D, Taruscio D, Di Mario U, Anastasi E (2001) Increased c-met expression during ductal beta cell neogenesis in experimental autoimmune diabetes. Growth Factors 19(4):259–267
74. Garcia-Ocana A, Takane KK, Syed MA, Philbrick WM, Vasavada RC, Stewart AF (2000) Hepatocyte growth factor overexpression in the islet of transgenic mice increases beta cell proliferation, enhances islet mass, and induces mild hypoglycemia. J Biol Chem 275(2):1226–1232
75. Dai C, Li Y, Yang J, Liu Y (2003) Hepatocyte growth factor preserves beta cell mass and mitigates hyperglycemia in streptozotocin-induced diabetic mice. J Biol Chem 278(29):27080–27087
76. Lopez-Talavera JC, Garcia-Ocana A, Sipula I, Takane KK, Cozar-Castellano I, Stewart AF (2004) Hepatocyte growth factor gene therapy for pancreatic islets in diabetes: reducing the minimal islet transplant mass required in a glucocorticoid-free rat model of allogeneic portal vein islet transplantation. Endocrinology 145(2):467–474
77. Sorenson RL, Brelje TC (1997) Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones. Horm Metab Res 29(6):301–307
78. Brelje TC, Sorenson RL (1991) Role of prolactin versus growth hormone on islet β-cell proliferation in vitro: implications for pregnancy. Endocrinology 128(1):45–57
79. Brelje TC, Scharp DW, Lacy PE, Ogren L, Talamantes F, Robertson M, Friesen HG, Sorenson RL (1993) Effect of homologous placental lactogens, prolactins, and growth hormones on islet β-cell division and insulin secretion in rat, mouse, and human islets: implication for placental lactogen regulation of islet function during pregnancy. Endocrinology 132(2):879–887
80. Vasavada RC, Garcia-Ocana A, Zawalich WS, Sorenson RL, Dann P, Syed M, Ogren L, Talamantes F, Stewart AF (2000) Targeted expression of placental lactogen in the beta cells of transgenic mice results in beta cell proliferation, islet mass augmentation, and hypoglycemia. J Biol Chem 275(20):15399–15406
81. Freemark M, Avril I, Fleenor D, Driscoll P, Petro A, Opara E, Kendall W, Oden J, Bridges S, Binart N, Breant B, Kelly PA (2002) Targeted deletion of the PRL receptor: effects on islet development, insulin production, and glucose tolerance. Endocrinology 143(4):1378–1385

82. Liu JL, Coschigano KT, Robertson K, Lipsett M, Guo Y, Kopchick JJ, Kumar U, Liu YL (2004) Disruption of growth hormone receptor gene causes diminished pancreatic islet size and increased insulin sensitivity in mice. Am J Physiol Endocrinol Metab 287(3):405–413
83. Yamada K, Nonaka K, Hanafusa T, Miyazaki A, Toyoshima H, Tarui S (1982) Preventive and therapeutic effects of large-dose nicotinamide injections on diabetes associated with insulitis. DiabetesDiabetes 31(9):749–753
84. Shima K, Zhu M, Kuwajima M (1998) A role of nicotinamide-induced increase in pancreatic beta-cell mass on blood glucose control after discontinuation of the treatment in partially pancreatectomized OLETF rats. DiabetesDiabetes Res Clin Pract 41(1):1–8
85. Otonkoski T, Beattie GM, Mally MI, Ricordi C, Hayek A (1993) Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells. J Clin Invest 92(3):1459–1466
86. Vague P, Picq R, Bernal M, Lassmann-Vague V, Vialettes B (1989) Effect of nicotinamide treatment on the residual insulin secretion in type 1 (insulin-dependent) diabetic patients. Diabetologia 32(5):316–321
87. Mendola G, Casamitjana R, Gomis R (1989) Effect of nicotinamide therapy upon β-cell function in newly diagnosed type 1 (insulin-dependent) diabetic patients. Diabetologia 32(3):160–162
88. Lewis CM, Canafax DM, Sprafka JM, Barbosa JJ (1992) Double-blind randomized trial of nicotinamide on early-onset diabetes. DiabetesDiabetes Care 15(1):121–123
89. Elliott RB, Chase HP (1991) Prevention or delay of type 1 (insulin-dependent) diabetes mellitus in children using nicotinamide. Diabetologia 34(5):362–365
90. Yoshino J, Mills KF, Yoon MJ, Imai S (2011) Nicotinamide mononucleotide, a key NAD(+) intermediate, treats the pathophysiology of diet- and age-induced diabetes in mice. Cell Metab 14(4):528–536
91. Couri CE, Foss MC, Voltarelli JC (2006) Secondary prevention of type 1 diabetes mellitus: stopping immune destruction and promoting beta-cell regeneration. Braz J Med Biol Res 39(10):1271–1280
92. Kodama S, Kühtreiber W, Fujimura S, Dale EA, Faustman DL (2003) Islet regeneration during the reversal of autoimmune diabetes in NOD mice. Science 302(5648):1223–1227
93. Chong AS, Shen J, Tao J, Yin D, Kuznetsov A, Hara M, Philipson LH (2006) Reversal of diabetes in non-obese diabetic mice without spleen cell-derived beta-cell regeneration. Science 311(5768):1774–1775
94. Dahlquist G, Gothefors L (1996) The cumulative incidence of childhood diabetes mellitus in Sweden unaffected by BCG-vaccination. Diabetologia 38(7):500–502
95. Allen HF, Klingensmith GJ, Jensen P, Simoes E, Hayward A, Chase HP (1999) Effect of Bacillus Calmette-Guerin vaccination on new-onset type 1 diabetes. DiabetesDiabetes Care 22(10):1703–1707
96. Huppmann M, Baumgarten A, Ziegler AG, Bonifacio E (2005) Neonatal Bacille Calmette-Guerin vaccination and type 1 diabetes. DiabetesDiabetes Care 28(5):1204–1206
97. Shehadeh N, Calcinaro F, Bradley BJ, Bruchim I, Vardi P, Lafferty KJ (1994) Effect of adjuvant therapy on development of diabetes in mouse and man. Lancet 343(8899):706–707
98. http://www.faustmanlab.org/
99. Burt RK, Slavin S, Burns WH, Marmont AM (2002) Induction of tolerance in autoimmune diseases by hematopoietic stem cell transplantation: getting closer to a cure? Blood 99(3):768–784
100. Butler AE, Huang A, Rao PN, Bhushan A, Hogan WJ, Rizza RA, Butler PC (2007) Hematopoietic stem cells derived from adult donors are not a source of pancreatic-cells in adult non-diabetic humans. DiabetesDiabetes 56(7):1810–1816
101. Kang EM, Zickler MM, Burns S, Langemeijer SM, Brenner S, Phang OA, Patterson N, Harlan D, Tisdale JF (2005) Hematopoietic stem cell transplantation prevents diabetes in NOD mice but does not contribute to significant islet cell regeneration once disease is established. Exp Hematol 33(6):699–705

102. Voltarelli JC, Couri CEB, Stracieri AB, Oliveira MC, Moraes DA, Pieroni F, Coutinho M, Malmegrim KC, Foss-Freitas MC, Simões BP, Foss MC, Squiers E, Burt RK (2007) Autologous non-myeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 297(14):1568–1576
103. Couri CE, Voltarelli JC (2008) Potential role of stem cell therapy in type 1 diabetes mellitus. Arq Bras Endocrinol Metabol 52(2):407–415
104. Ende N, Chen R, Reddi AS (2004) Effect of human umbilical cord blood cells on glycemia and insulitis in type 1 diabetic mice. Biochem Biophys Res Commun 325(3):665–669
105. Ende N, Chen R, Reddi AS (2004) Transplantation of human umbilical cord blood cells improves glycemia and glomerular hypertrophy in type 2 diabetic mice. Biochem Biophys Res Commun 321(1):168–171
106. Haller MJ, Viener H, Brusko T, Wasserfall C, Mcgrail K, Staba S, Cogle C, Atkinson M, Schatz DA, Gainesville FL (2007) Insulin requeirements, HbA1c, and stimulated C-peptide following autologous umbilical cord blood transfusion in children with T1D. American Diabetes Association 67th Scientific sessions. American Diabetes Association Publisher, Chicago, A82 p
107. Tillakaratne NJ, Medina-Kauwe L, Gibson KM (1995) Gamma-Aminobutyric acid (GABA) metabolism in mammalian neural and nonneural tissues. Comp Biochem Physiol A Physiol 112(2):247–263
108. Soltani N, Qiu H, Aleksic M, Glinka Y, Zhao F, Liu R, Li Y, Zhang N, Chakrabarti R, Ng T, Jin T, Zhang H, Lu W, Feng Z, Prud'homme GJ, Wang Q (2011) GABA exerts protective and regenerative effects on islet beta cells and reverses diabetes. Proc Natl Acad Sci U S A 108(28):11692–11697
109. Meier JJ, Butler AE, Saisho Y, Monchamp T, Galasso R, Bhushan A, Rizza RA, Butler PC (2008) Beta cell replication is the primary mechanism subserving the postnatal expansion of beta cell mass in humans. Diabetes 57(6):1584–1594
110. Beattie GM, Itkin-Ansari P, Cirulli V, Leibowitz G, Lopez AD, Bossie S, Mally MI, Levine F, Hayek A (1999) Sustained proliferation of PDX-1 positive cells derived from human islets. Diabetes 48(5):1013–1019
111. Ouziel-Yahalom L, Zalzman M, Anker-Kitai L, Knoller S, Bar Y, Glandt M, Herold K, Efrat S (2006) Expansion and redifferentiation of adult human pancreatic islet cells. Biochem Biophys Res Commun 341(2):291–298
112. Weinberg N, Ouziel-Yahalom L, Knoller S, Efrat S, Dor Y (2007) Lineage tracing evidence for in vitro dedifferentiation but rare proliferation of mouse pancreatic beta-cells. Diabetes 56(5):1299–1304
113. Parnaud G, Bosco D, Berney T, Pattou F, Kerr-Conte J, Donath MY, Bruun C, Mandrup-Poulsen T, Billestrup N, Halban PA (2008) Proliferation of sorted human and rat beta cells. Diabetologia 51(1):91–100
114. Russ HA, Bar Y, Ravassard P, Efrat S (2008) In vitro proliferation of cells derived from adult human beta cells revealed by cell-lineage tracing. Diabetes 57(6):1575–1583
115. Raff M (2003) Adult stem cell plasticity: fact or artifact? Annu Rev Cell Dev Biol 19:1–22
116. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872
117. Nostro MC, Sarangi F, Ogawa S, Holtzinger A, Corneo B, Li X, Micallef SJ, Park IH, Basford C, Wheeler MB, Daley GQ, Elefanty AG, Stanley EG, Keller G (2011) Stage-specific signaling through TGFβ family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells. Development 138(5):861–871
118. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE (2006) Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 24(11):1392–1401
119. Baiu D, Merriam F, Odorico J (2011) Potential pathways to restore β-cell mass: pluripotent stem cells, reprogramming, and endogenous regeneration. Curr Diab Rep 11(5):392–401

120. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, Young H, Richardson M, Smart NG, Cunningham J, Agulnick AD, D'Amour KA, Carpenter MK, Baetge EE (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol 26(4):443–452
121. Matveyenko AV, Georgia S, Bhushan A, Butler PC (2010) Inconsistent formation and nonfunction of insulin-positive cells from pancreatic endoderm derived from human embryonic stem cells in athymic nude rats. Am J Physiol Endocrinol Metab 299(5):713–720
122. Mao GH, Chen GA, Bai H, Song T, Wang Y (2009) The reversal of hyperglycaemia in diabetic mice using PLGA scaffolds seeded with islet-like cells derived from human embryonic stem cells. Biomaterials 30(9):1706–1714
123. Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG (2000) Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. Nat Med 6(3):278–282
124. Bonner-Weir S, Taneja M, Weir GC, Tatarkiewicz K, Song KH, Sharma A, O'Neil JJ (2000) In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci U S A 97(14):7999–8004
125. Yamamoto T, Yamato E, Taniguchi H, Shimoda M, Tashiro F, Hosoi M, Sato T, Fujii S, Miyazaki JI (2006) Stimulation of cAMP signalling allows isolation of clonal pancreatic precursor cells from adult mouse pancreas. Diabetologia 49(10):2359–2367
126. Noguchi H, Oishi K, Ueda M, Yukawa H, Hayashi S, Kobayashi N, Levy MF, Matusmoto S (2009) Establishment of mouse pancreatic stem cell line. Cell Transplant 18(5):563–571
127. Noguchi H, Naziruddin B, Jackson A, Shimoda M, Ikemoto T, Fujita Y, Chujo D, Takita M, Kobayashi N, Onaca N, Hayashi S, Levy MF, Matsumoto S (2010) Characterization of human pancreatic progenitor cells. Cell Transplant 19(6):879–886
128. Strojnik T, Røsland GV, Sakariassen PO, Kavalar R, Lah T (2007) Neural stem cell markers, nestin and musashi proteins, in the progression of human glioma: correlation of nestin with prognosis of patient survival. Surg Neurol 68(2):133–144
129. Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, Müller B, Vallejo M, Thomas MK, Habener JF (2001) Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. Diabetes 50(3):521–533
130. Bonner-Weir S, Sharma A (2002) Pancreatic stem cells. J Pathol 197(4):519–526
131. Petropavlovskaia M, Rosenberg L (2002) Identification and characterization of small cells in the adult pancreas: potential progenitor cells? Cell Tissue Res 310(1):51–58
132. Yang L, Li S, Hatch H, Ahrens K, Cornelius JG, Petersen BE, Peck AB (2002) In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci U S A 99(12):8078–8083
133. Seaberg RM, Smukler SR, Kieffer TJ, Enikolopov G, Asghar Z, Wheeler MB, Korbutt G, van der Kooy D (2004) Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat Biotechnol 22(9):1115–1124
134. Breault DT, Min IM, Carlone DL, Farilla LG, Ambruzs DM, Henderson DE, Algra S, Montgomery RK, Wagers AJ, Hole N (2008) Generation of mTert-GFP mice as a model to identify and study tissue progenitor cells. Proc Natl Acad Sci U S A 105(30):10420–10425
135. Aguayo-Mazzucato C, Bonner-Weir S (2010) Stem cell therapy for type 1 diabetes mellitus. Nat Rev Endocrinol 6(3):139–148
136. Yang LJ (2006) Liver stem cell-derived beta-cell surrogates for treatment of type 1 diabetes. Autoimmun Rev 5(6):409–413
137. Rao MS, Dwivedi RS, Yeldandi AV, Subbarao V, Tan X, Usman MI, Thangada S, Nemali MR, Kumar S, Scarpelli DG, Reddy JK (1989) Role of periductal and ductular epithelial cells of the adult rat pancreas in pancreatic hepatocyte lineage. A change in the differentiation commitment. Am J Pathol 134(5):1069–1086
138. Terada T, Nakanuma Y (1993) An immunohistochemical survey of amylase isoenzymes in chalangiocarcinoma and hepatocellular carcinoma. Arch Pathol Lab Med 117(2):160–162

139. Kawaguchi Y, Cooper B, Gannon M, Ray M, MacDonald RJ, Wright CV (2002) The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet 32(1):128–134
140. Yamada S, Terada K, Ueno Y, Sugiyama T, Seno M, Kojima I (2005) Differentiation of adult hepatic stem-like cells into pancreatic endocrine cells. Cell Transplant 14(9):647–653
141. Nakajima-Nagata N, Sakurai T, Mitaka T, Katakai T, Yamato E, Miyazaki J, Tabata Y, Sugai M, Shimizu A (2004) In vitro induction of adult hepatic progenitor cells into insulin-producing cells. Biochem Biophys Res Commun 318(3):625–630
142. Suzuki A, Nakauchi H, Taniguchi H (2003) Glucagon-like peptide 1 (1–37) converts intestinal epithelial cells into insulin-producing cells. Proc Natl Acad Sci U S A 100(9):5034–5039
143. Ianus A, Holz GG, Theise ND, Hussain MA (2003) In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J Clin Invest 111(6):843–850
144. Hess D, Li L, Martin M, Sakano S, Hill D, Strutt B, Thyssen S, Gray DA, Bhatia M (2003) Bone marrow-derived stem cells initiate pancreatic regeneration. Nat Biotechnol 21(7):763–770
145. Choi JB, Uchino H, Azuma K, Iwashita N, Tanaka Y, Mochizuki H, Migita M, Shimada T, Kawamori R, Watada H (2003) Little evidence of transdifferentiation of bone marrow-derived cells into pancreatic beta cells. Diabetologia 46(10):1366–1374
146. Lechner A, Yang YG, Blacken RA, Wang L, Nolan AL, Habener JF (2004) No evidence for significant transdifferentiation of bone marrow into pancreatic β cells in vivo. Diabetes 53(3):616–623
147. Oh SH, Muzzonigro TM, Bae SH, LaPlante JM, Hatch HM, Petersen BE (2004) Adult bone marrow-derived cells transdifferentiating into insulin-producing cells for the treatment of type I diabetes. Lab Invest 84:607–617
148. Suchánek J, Soukup T, Ivancaková R, Karbanová J, Hubková V, Pytlík R, Kucerová L (2007) Human dental pulp stem cells–isolation and long-term cultivation. Acta Medica (Hradec Kralove) 50(3):195–201
149. Govindasamy V, Ronald VS, Abdullah AN, Nathan KR, Ab Aziz ZA, Abdullah M, Musa S, Kasim NH, Bhonde RR (2011) Differentiation of dental pulp stem cells into islet-like aggregates. J Dent Res 90(5):646–652
150. Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G (2006) Pluripotency of spermatogonial stem cells from adult mouse testis. Nature 440(7088):1199–1203
151. Mardanpour P, Guan K, Nolte J, Lee JH, Hasenfuss G, Engel W, Nayernia K (2008) Potency of germ cells and its relevance for regenerative medicine. J Anat 213(1):26–29
152. Saraswathula, A., Yamaguchi, S., Zakaria, A., Gallicano, G.I (2010) Functional, insulin-secreting pancreatic endoderm derived from human spermatogonial stem cells. Presented Sunday 12 Dec 2010 at the American Society for Cell Biology 50th Annual Meeting, Philadelphia. Abstract ID# 703/B1113
153. Jonsson J, Carlsson L, Edlund T, Edlund H (1994) Insulin-promoter-factor-1 is required for pancreas development in mice. Nature 371(6498):606–609
154. Offield MF, Jetton TL, Labosky PA, Ray M, Stein RW, Magnuson MA, Hogan BL, Wright CV (1996) PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development 122(3):983–995
155. Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seijffers R, Kopolovic J, Kaiser N, Karasik A (2000) Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptzotocin-induced hyperglycemia. Nat Med 6(5):568–572
156. Cao LZ, Tang DQ, Horb ME, Li SW, Yang LJ (2004) High glucose is necessary for complete maturation of Pdx1-VP16-expressing hepatic cells into functional insulin-producing cells. Diabetes 53(12):3168–3178
157. Kojima H, Fujimiya M, Matsumura K, Younan P, Imaeda H, Maeda M, Chan L (2003) NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. Nat Med 9(5):596–603

158. Kaneto H, Nakatani Y, Miyatsuka T, Matsuoka TA, Matsuhisa M, Hori M, Yamasaki Y (2005) PDX-1/VP16 fusion protein, together with NeuroD or Ngn3, markedly induces insulin gene transcription and ameliorates glucose tolerance. Diabetes 54(4):1009–1022
159. Sapir T, Shternhall K, Meivar-Levy I, Blumenfeld T, Cohen H, Skutelsky E, Eventov-Friedman S, Barshack I, Goldberg I, Pri-Chen S, Ben-Dor L, Polak-Charcon S, Karasik A, Shimon I, Mor E, Ferber S (2005) Cell-replacement therapy for diabetes: generating functional insulin-producing tissue from adult human liver cells. Proc Natl Acad Sci U S A 102(22):7964–7969
160. Cairnie AB, Lamerton LF, Steel GG (1965) Cell proliferation studies in the intestinal epithelium of the rat. II. Theoretical aspects. Exp Cell Res 39(2–3):539–553
161. Kojima H, Nakamura T, Fujita Y, Kishi A, Fujimiya M, Yamada S, Kudo M, Nishio Y, Maegawa H, Haneda M, Yasuda H, Kojima I, Seno M, Wong NC, Kikkawa R, Kashiwagi A (2002) Combined expression of pancreatic duodenal homeobox 1 and islet factor 1 induces immature enterocytes to produce insulin. Diabetes 51(5):1398–1408
162. Yoshida S, Kajimoto Y, Yasuda T, Watada H, Fujitani Y, Kosaka H, Gotow T, Miyatsuka T, Umayahara Y, Yamasaki Y, Hori M (2002) PDX-1 induces differentiation of intestinal epithelioid IEC-6 into insulin producing cells. Diabetes 51(8):2505–2513
163. Tudurí E, Kieffer TJ (2011) Reprogramming gut and pancreas endocrine cells to treat diabetes. Diabetes Obes Metab 13(Suppl 1):53–59
164. Cheung AT, Dayanandan B, Lewis JT, Korbutt GS, Rajotte RV, Bryer-Ash M, Boylan MO, Wolfe MM, Kieffer TJ (2000) Glucose-dependent insulin release from genetically engineered K cells. Science 290(5498):1959–1962
165. Han J, Lee HH, Kwon H, Shin S, Yoon JW, Jun HS (2007) Engineered enteroendocrine cells secrete insulin in response to glucose and reverse hyperglycemia in diabetic mice. Mol Ther 15(6):1195–1202
166. Unniappan S, Wideman RD, Donald C, Gunn V, Wall JL, Zhang QX, Webber TD, Cheung AT, Kieffer TJ (2009) Treatment of diabetes by transplantation of drug-inducible insulin-producing gut cells. J Mol Med 87(7):703–712
167. Baeyens L, De Breuck S, Lardon J, Mfopou JK, Rooman I, Bouwens L (2005) In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells. Diabetologia 48(1):49–57
168. Minami K, Okuno M, Miyawaki K, Okumachi A, Ishizaki K, Oyama K, Kawaguchi M, Ishizuka N, Iwanaga T, Seino S (2005) Lineage tracing and characterization of insulin-secreting cells generated from adult pancreatic acinar cells. Proc Natl Acad Sci U S A 102(42):15116–15121
169. Kopinke D, Murtaugh LC (2010) Exocrine-to-endocrine differentiation is detectable only prior to birth in the uninjured mouse pancreas. BMC Dev Biol 10:38
170. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455(7213):627–632
171. Chung CH, Levine F (2010) Adult pancreatic alpha-cells: a new source of cells for beta-cell regeneration. Rev Diabet Stud 7(2):124–131
172. Collombat P, Mansouri A, Hecksher-Sorensen J, Serup P, Krull J, Gradwohl G, Gruss P (2003) Opposing actions of Arx and Pax4 in endocrine pancreas development. Genes Dev 17(20):2591–2603
173. Collombat P, Hecksher-Sørensen J, Broccoli V, Krull J, Ponte I, Mundiger T, Smith J, Gruss P, Serup P, Mansouri A (2005) The simultaneous loss of Arx and Pax4 genes promotes a somatostatin-producing cell fate specification at the expense of the alpha- and beta-cell lineages in the mouse endocrine pancreas. Development 132(13):2969–2980
174. Collombat P, Hecksher-Sørensen J, Krull J, Berger J, Riedel D, Herrera PL, Serup P, Mansouri A (2007) Embryonic endocrine pancreas and mature beta cells acquire alpha and PP cell phenotypes upon Arx misexpression. J Clin Invest 117(4):961–970
175. Collombat P, Xu X, Ravassard P, Sosa-Pineda B, Dussaud S, Billestrup N, Madsen OD, Serup P, Heimberg H, Mansouri A (2009) The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into alpha and subsequently beta cells. Cell 138(3):449–462

176. Ekser, B., Ezzelarab, M., Hara, H., van der Windt, D.J., Wijkstrom, M., Bottino, R., Trucco, M., Cooper, D.K.: Clinical xenotransplantation: the next medical revolution? Lancet [Epub ahead of print] Doi:10.106/S0140-6736(11)61090-X (2011)
177. Cardona K, Korbutt GS, Milas Z, Lyon J, Cano J, Jiang W, Bello-Laborn H, Hacquoil B, Strobert E, Gangappa S, Weber CJ, Pearson TC, Rajotte RV, Larsen CP (2006) Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nat Med 12(3):304–306
178. Hering BJ, Wijkstrom M, Graham ML, Hårdstedt M, Aasheim TC, Jie T, Ansite JD, Nakano M, Cheng J, Li W, Moran K, Christians U, Finnegan C, Mills CD, Sutherland DE, Bansal-Pakala P, Murtaugh MP, Kirchhof N, Schuurman HJ (2006) Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nat Med 12(3):301–303
179. Dufrane D, Goebbels RM, Gianello P (2010) Alginate macroencapsulation of pig islets allows correction of streptozotocin-induced diabetes in primates up to 6 months without immunosuppression. Transplantation 90(10):1054–1062
180. van der Windt DJ, Bottino R, Casu A, Campanile N, Smetanka C, He J, Murase N, Hara H, Ball S, Loveland BE, Ayares D, Lakkis FG, Cooper DK, Trucco M (2009) Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. Am J Transplant 9(12):2716–2726
181. Elliott RB, Escobar L, Tan PL, Muzina M, Zwain S, Buchanan C (2007) Live encapsulated porcine islets from a type 1 diabetic patient 9.5 year after xenotransplantation. Xenotransplantation 14(2):157–161
182. Hering BJ, Cooper DKC, Cozzi E, Schuurman HJ, Korbutt GS, Denner J, O'Connell PJ, Vanderpool HY, Pierson RN 3rd (2009) The international xenotransplantation association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—executive summary. Xenotransplantation 16(4):196–202

Chapter 10
Regenerative Therapies for Liver Diseases

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Abstract The liver responds to injury or tissue loss by rapid restoration of the original cell mass. The high regenerative capacity is sufficient to restore normal volume and function in most forms of acute liver injury and medical interventions are not required. For the development of regenerative therapies a fundamental understanding of these regenerative principles in the liver is required. In this chapter, we discuss the emerging medical approaches for acute liver failure, chronic liver failure, and hereditary liver diseases, which are based on technologies, such as (stem) cell therapy, tissue engineering, bio-artificial devices or gene therapies.

10.1 Principles of Liver Regeneration

The liver comprises about one-fiftieth of the total adult body weight [1], receives approximately 25 % of cardiac output [2] and consists of an exceptional anatomical structure in both biliary system and vasculature. The biliary system,

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an exocrine system in the liver, connects the apical surface of hepatocytes to the intestine through bile canaliculi, which drain into the canals of Hering and finally into bile ducts [3]. Two afferent vessels, the hepatic artery and the portal vein provide the blood supply for the liver. Their terminal branches enter the liver sinusoids, which are characterized by fenestrated and discontinuous endothelium [1]. No basement membrane lines the sinusoid, which allows higher permeability and direct transfer of particles less than 100 nm from the vessels to the basolateral surface of the hepatocytes.

The adult liver is a quiescent organ and as few as one out of 3,000 hepatocytes divides at a given time point to maintain the physiological liver mass. In acute liver damage or through surgical loss of liver mass, however, cell proliferation can be extensively accelerated until the tissue mass has been restored [4]. In only 7 days up to 75 % surgically removed liver mass can be regenerated in rodents [5]. Although the term "liver regeneration" is commonly used, restoration of the liver mass after partial hepatectomy is actually a form of compensatory growth of the remaining liver (hyperplasia).

In the regenerative phase after acute liver injury or tissue loss the liver immediately induces more than 100 genes, which are not expressed in normal liver [6, 7]. The early changes in gene expression reflect both the entry of hepatocytes into the cell cycle as well as the orchestration of specific adjustments that hepatocytes have to make, so that they can deliver all essential hepatic functions while going through cell proliferation. The extensive "reprogramming" of hepatic gene expression requires activation of multiple signaling pathways involving matrix remodeling proteins, growth factors, cytokines, paracrine signals, and neuroendocrine factors.

Small non-coding RNAs, mainly microRNAs (miRNAs), provide an additional level of regulation in liver regeneration. Global loss of miRNAs leads to the impairment of hepatocyte proliferation at the G1-S stage of cell cycle. In particular, miR-21, one of the upregulated miRNAs in HCCs, has been shown to increase the proliferation of hepatoma cells by targeting Pten and Btg2. As of now, data are limited and mainly restricted to the initiation phase of liver regeneration. Importantly, in vivo functions of individual miRNAs during liver regeneration have not yet been identified.

The newborn liver contains mostly diploid hepatocytes, but polyploidization and binuclearity occur rapidly after birth. In perivenous areas hepatocytes are more often polyploid and serve different liver functions when compared to cells of the periportal region ("metabolic zonation") [8, 9]. The gradient of less complex cells with higher proliferation potential (in vitro) in periportal areas and more mature hepatocytes in perivenous areas has been interpreted as evidence for the existence of a physiological niche for cell renewal [10] in the periportal region. Recent experimental studies of hepatocytes with acquired mitochondrial mutations in the cytochrome c oxidase gene have also provided arguments for the periportal region as the "regenerative niche" in normal liver [11]. The "streaming liver hypothesis" postulating that the liver lobule is organized similar to the intestinal crypt and contains a stem/progenitor cell pool arising form the periportal area, however, has been disputed [12].

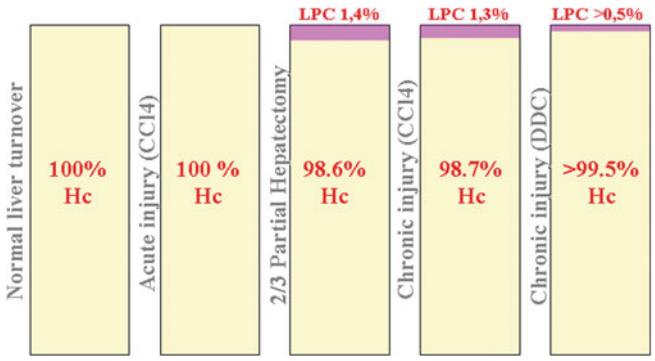

Fig. 10.1 Relative contribution of primary mouse hepatocytes and liver progenitor cells to liver regeneration (Adapted from [21])

Regenerative responses and cell types involved differ depending on the severity and chronicity of liver injury. Although it is not debatable that mature hepatocytes and cholangiocytes represent the first and most important resource for tissue repair [13, 14], a liver stem/progenitor cell compartment is likely to be involved in the repair of injured livers.

The first evidence for the existence and activation of a resident hepatic stem/progenitor cells compartment was provided by various murine animal models of "oval cell" proliferation [15–19]. The general principle underlying "oval cell" activation is based on the combination of a liver injury and the inability of hepatocytes to proliferate in response to the damage. The extent, to which liver stem/progenitor cells contribute to parenchymal liver regeneration in various forms of liver injury was lately controversely discussed. Furuyama et al. reported that liver progenitor cells residing in bile ducts are the predominant source of new hepatocytes in mouse liver homeostasis as well as in liver injury [20]. The data were challenged by a new hepatocyte fate tracing model, which demonstrated only a minor role of stem/progenitor cells for the generation of hepatocytes and biliary epithelial cells in normal and injured liver [21] (Fig. 10.1).

In parallel to what we know from rodent models, also in human liver diseases the inhibition of mature hepatocyte replication favors the proliferation of cell populations with stem/progenitor phenotypes. Activation of these cells has been associated with a variety of liver diseases, and, the numbers have been related to severity of the disease [22, 23]. It has recently been shown that hepatocytes become senescent, owing at least partially to telomere shortening, in the cirrhotic stage of a wide variety of chronic human liver diseases [24, 25]. Replicative exhaustion and senescence of the mature hepatocytes as a result of ongoing proliferation during 20–30 years of chronic liver disease has been linked to the emergence of these stem/progenitor cells and finally with the evolution of hepatocarcinoma and cholangiocarcinoma [26, 27]. However, it is unclear whether these cells are simply a marker of carcinogenic disease states or whether the stem/progenitor cells are at particular risk for transformation.

10.2 Liver Disease States as Targets for Regenerative Therapies

The acute and self-limiting liver diseases (e.g. due to acute viral disease, toxins, transient ischemia) normally result in complete regeneration and "restitutio ad integrum". More massive injuries may temporarily exhaust the regenerative capacity of the liver and result in "acute liver failure", a clinical syndrome, which is characterized by progressive loss of hepatic function and multiorgan failure. Persistent injuries to the liver also induce regenerative responses but eventually result in scarring and excess deposition of extracellular matrix components including collagen. Fibrosis and cirrhosis are the end result of chronic inflammatory reactions induced by a variety of stimuli including persistent infections, autoimmune reactions, allergic responses, chemical insults, radiation, and tissue injury. Although current treatments for fibrotic diseases, such as idiopathic pulmonary fibrosis, systemic sclerosis, and liver fibrosis/cirrhosis typically target the inflammatory response, there is accumulating evidence that the mechanisms driving liver fibrogenesis are distinct from those regulating inflammation. The key cellular mediator of fibrosis is the myofibroblast, which, once activated, serves as the primary collagen-producing cell. Myofibroblasts are generated from a variety of sources including resident mesenchymal cells (Ito cells) and circulating fibroblast-like cells called fibrocytes that are derived from bone marrow stem cells. Myofibroblasts are activated by paracrine signals derived from lymphocytes to macrophages, autocrine factors secreted by myofibroblasts, and pathogen-associated molecular patterns (PAMPS) produced by pathogenic organisms that interact with pattern recognition receptors (i.e. TLRs) on fibroblasts.

The liver is central to many metabolic activities with hundreds of genes involved in their regulation. In recent years, the genetic basis for more than 100 liver diseases involving malfunction of the organ has been clarified. Hereditary liver diseases usually result from point mutations, deletions, or other genetic defects in single or multiple genes, which are normally expressed in the liver and can cause acute and chronic liver diseases. The liver also secrets many proteins, which deliver functions for other organ systems, and a state of protein deficiency may not affect the liver function itself. For most of the hereditary liver diseases liver organ transplantation cures the disease or the state of protein deficiency and has become the most important therapeutic approach. Conceptually, many of these disorders, for which organ transplantation is effective, can be principally cured by cell- or gene therapies.

10.3 Cells for the Treatment of Liver Diseases

Many of the regenerative technologies generated or envisioned to treat liver diseases are based on cellular substrates, which are either transplanted/injected into recipients or utilized in extracorporeal devices. The primary adult hepatocyte is

still the most important cellular resource in clinical situations, in which parenchymal liver functions need to be reconstituted.

Hepatocytes isolated from pig or human livers as well as immortalized human hepatocytes have been tested in extracorporeal liver devices. Transplanted human hepatocytes have been shown to engraft in the recipient liver and to respond to growth stimuli in vivo [28–30]. Despite a high proliferative capacity of hepatocytes, which can undergo more than 69 cell doublings or a 7.3×10^{20}-fold expansion [31] in vivo, the proliferation capacity in cell culture is limited.

This lack of in vitro expansion protocols has stimulated the search for alternative cell sources, which can either expand in cell culture or can be easily harvested from the body in large quantities. Immortalized hepatocytes derived from adult and fetal tissue are restricted to ex vivo applications and have been applied in extracorporal liver devices. Human fetal liver derived hepatoblasts have been applied in a small number of patients with acute liver failure [32] and recently in one patient with hereditary bilirubinemia [33]. These cells are also being tested as a cellular substrate for bioartificial liver devices [34]. Although the isolation of clinical grade stem/progenitor cells from human adult livers has been described, clinical applications were not yet reported.

It has been proposed that (subpopulations of) adult hematopoietic stem cells (HSC), mesenchymal stromal cells (MSC), and cord blood stem cells (CBSC) can transdifferentiate into hepatocytes after transplantation, but the efficacy, by which these cells spontaneously form hepatocytes and liver tissue in animal experiments, still seems questionable [35–39]. As an alternative concept, HSC, MSC, and CBSC are being applied in patients with chronic liver disease with the therapeutic aim to protect resident hepatocytes from injury, induce liver regeneration, and to initiate tissue remodeling.

High therapeutic expectations have been attributed to embryonic stem (ES) cells and, more recently, iPS cells. These cells can be maintained in a state of pluripotency for long periods of time, grown in large quantities [40–45], and differentiated into virtually all cell types of the body. ES-like cells have been generated by transfer and simultaneous expression of four genes and termed induced pluripotent stem (iPS) cells. The direct transcription factor-mediated conversion of fibroblasts into hepatic cells, which could at least temporarily rescue a murine model of metabolic liver failure, was recently demonstrated by two independent groups [46, 47]. To date, it remains speculative, whether direct "trans-programming" of adult stem cells or fibroblasts into the desired phenotype by forced expression of sets of transcription factors represents an alternative approach and may circumvent the state of pluripotency, which is associated with teratoma formation in transplanted recipients.

10.3.1 Modes of Therapeutic Activity

Various modes of therapeutic activity have been proposed for transplanted cells. Transplanted primary hepatocytes, fetal hepatoblasts, and adult liver progenitor cells engraft in the recipient liver and function as parenchymal liver cells. In vitro

hepatic differentiation protocols for stem cells, in particular embryonic and iPS cells, aim to generate cell phenotypes compatible with long-term engraft in the liver and hepatocyte functionality.

Several ways to obtain therapeutic activity have been proposed for transplanted unmodified stem cells. For example, the injection of hematopoietic stem cells (HSC) isolated from adult bone marrow or cord blood was shown to generate hepatocytes at therapeutically significant levels in animal models [35–37]. Initial studies suggested that those extrahepatic stem cells transdifferentiate from hematopoietic to hepatic lineage in the recipient organ, but more recent work has demonstrated fusion of stem cells with hepatocytes as the main mechanism [48]. In an alternative concept HSC and mesenchymal stromal cells have been injected in animals and humans with acute and chronic liver disease in order to prevent apoptosis, to induce regeneration, or to remodel the recipient liver without forming hepatocytes. Paracrine signaling of the transplanted cells and direct cell–cell contact have been proposed as main mechanisms in this setting.

10.3.2 Hepatocytes from ES/iPS Cells

Hepatocytes derived from ES cells may serve as an unlimited cell source for in vitro and in vivo applications. In order to generate hepatocytes from pluripotent stem cells, various differentiation protocols have been published, which usually mimic the events occurring during embryonic development of the liver. In one type of protocol the pluripotent ES cells are differentiated into the hepatocyte state by initial formation of embryoid bodies, followed by the induction of definitive endoderm using instructive cytokines such as Activin A. The endoderm cell population can be further instructed to develop toward the hepatocyte lineage by exposure to bone morphogenetic protein (BMP) 4 and fibroblast growth factor (FGF) 2, both important signals from the cardiac mesoderm in early liver embryogenesis [49]. Assessment of the hepatic phenotype is commonly based on hepatocyte-specific gene expression profiles and metabolic activities, such as cytochrome p450 activity, glycogen storage, or urea synthesis, which determine the efficacy of the differentiation protocol. At the end of a differentiation process it is important to remove contaminating undifferentiated ES cells from the heterogeneous cell culture to minimize the risk of teratoma formation. This can be achieved by various FACS/MACS sorting techniques or by the transfer of cell type-specific expression of antibiotic resistance genes [50].

Transplantation of ES derived hepatic cells to the liver results in engraftment as both, mature hepatocytes and bile duct epithelial cells [49, 51]. The level of liver repopulation obtained with hepatocyte-differentiated ESCs is very low, but can be increased somewhat when cells are transplanted in a favorable liver environment, which allows positive selection of the transplanted cells. To date, most published ESC differentiation protocols generate hepatocyte-like cells, but not the fully

functional, mature, and transplantable equivalents of hepatocytes that are isolated from adult livers.

The pioneering work of Yamanaka and colleagues has paved the way for generation of embryonic stem cell-like cells from almost any postnatal organ, such as skin, liver, and blood. These cells have been named induced pluripotent stem (iPS) cells. The iPS cells are generated from somatic cells by transduction with viral vectors expressing the stem cell genes *oct4*, *sox2*, *c-myc*, and *klf4* [44, 45]. The combination of these four transcription factors was identified from initially 24 different transcription factors. It was shown that these 4 factors were sufficient to also induce somatic cell reprogramming in human cells. Subsequently, iPS cells were generated without viral integration [52] to avoid integrated virus associated genotoxicity. iPS cells resemble ES cells as they possess self-renewal capacity, the ability to differentiate into cells of ectoderm, mesoderm and endoderm, and teratoma formation after transplantation in mice. Similar to mouse ES cells, hepatocyte differentiation of mouse and human iPS cells has been documented [53–56].

10.4 Liver Tissue Engineering

Liver tissue engineering is an emerging research field and aims to create functional liver structures using isolated hepatocytes and/or other cells types. It has been demonstrated in animal models, that sheets of liver tissue can be grown under the renal capsule or under the skin [57, 58]. Alternatively, hepatic tissues could be engineered ex vivo to produce therapeutic effects allowing this approach to become an effective modality for the treatment of acute liver failure. Three dimensional liver bioreactors, which are the main component of cell-based liver support devices, may be considered as a “tissue engineering” approach. A much more complex and not yet achieved task will be the generation of transplantable liver tissue with a functional blood supply and biliary system.

10.5 Therapeutic Concepts in Liver Disease

Regenerative therapies or treatments involving regenerative technologies are currently being developed for liver diseases of diverse etiologies. In acute liver failure syndromes, acute on chronic liver failure and non-function of transplanted livers the therapeutic approaches aim to substitute liver function (synthesis of proteins, metabolism, and detoxification) either by extracorporal support devices, transplantation of liver cells, or by engineering and transplantation of functional liver tissue. In chronic liver diseases conventional drugs, cytokines, stem cell therapy, and gene transfer techniques are being employed to specifically interfere with the inflammatory and profibrotic pathways. For hereditary metabolic liver disease the experimental and clinical approaches focus on substitution of defective

genes and proteins by allogeneic transplantation of hepatocytes or by gene therapy. Therapies involving regenerative technologies, such as (stem) cell therapies and gene transfer protocols, which emerge for liver cancer, viral infections, and immune-mediated liver diseases, are beyond the scope of this book chapter and have been reviewed elsewhere.

10.5.1 Acute Liver Failure

Acute liver failure (ALF) is a syndrome of diverse etiology, in which patients without previously recognized liver disease sustain a liver injury that results in rapid loss of hepatic function. Depending on the etiology and severity of the insult, some patients undergo rapid hepatic regeneration and spontaneously recover. However, nearly half of the patients with ALF require and undergo orthotopic liver transplantation or die. Even with optimal early management many patients with ALF develop a cascade of complications often presaged by the systemic inflammatory response syndrome, which involves failure of nearly every organ system. No satisfactory treatment exists for those patients other than liver transplantation. However, the number of donor livers available is limited and the outcome of liver transplantation for ALF is significantly lower than transplantation for chronic liver disease. Furthermore, many ALF patients are not placed on the transplant list due to exclusion criteria, such as sepsis, psychiatric illness, and multi-system organ failure. Specialized treatment algorithms for the intensive care of patients with liver failure and the introduction of antioxidative drug treatments have already significantly improved the survival of affected patients in the past. Trials of plasmapheresis and hypothermia from European consortia are near completion and drugs that facilitate the excretion of ammonia, such as L-ornithine phenylacetate [59], may provide a neuroprotective bridge to orthotopic liver transplantation.

Future therapies for ALF would ideally maintain the patient's clinical stability long enough to allow liver regeneration to occur, which would obviate the need for orthotopic liver transplantation. Realistically, the goal of such therapies will be to serve as a bridge to orthotopic liver transplantation. Extracorporeal liver support devices have been developed to achieve the goal of "bridging" by temporarily supporting liver detoxification function. Artificial liver support refers to purely mechanical devices including albumin dialysis, while bioartificial liver support refers to devices with a cellular component. Artificial systems remove toxins by filtration or adsorption while bioartificial liver systems perform these functions along with biotransformation and synthetic functions of biochemically active hepatocytes.

The molecular adsorbent recirculating system (MARS™; Gambro, Lund, Sweden) is the most frequently used type of albumin dialysis [60]. The key feature to the function of albumin dialysis is the concentration gradient of low-molecular-weight substrates between the patient's blood and the 20 % albumin in the secondary circuit. This concentration gradient allows diffusible low-molecular-weight

substrates to flow down their gradient over the membrane where they are transiently bound by albumin in the secondary circuit [61].The low-molecular-weight substrate is then removed from the system by conventional dialysis and hemodiafiltration within the secondary circuit.

The initial clinical study described a series of 13 patients who underwent treatment after failure of response to best medical therapy for acute-on-chronic hepatic failure. In this series, the overall survival was 69 % and the authors cited that all patients showed a positive response to therapy [62]. Other encouraging case reports and small studies eventually led to more widespread use of the system. To date, roughly 7,500 patients have been treated with MARS for various hepatic diseases, including acute liver failure patients. A meta-analysis assessing the use of MARS looked at four randomized controlled trials including a total of 67 patients and two selected nonrandomized trials including 61 patients [63]. Patients had either acute or acute-on-chronic liver failure. Primary meta-analysis did not show a statistically significant survival benefit. Recently, the results of a large multicenter randomized trial of MARS in patients with ALF fulfiling high-urgency liver-transplant criteria in France were presented [64]. The data showed a trend toward better surivival in the MARS treatment group, but the difference did not reach significance. The transplant-free 6 month survival, however, was significantly prolonged in those patients treated with at least three sessions of MARS. "Prometheus™, which employs fractionated plasma separation, is a close variant of MARS. While MARS is a two-circuit system separated by an albumin impermeable membrane, Prometheus utilizes a membrane with a 250 kDa cutoff between circuits, thereby making the membrane permeable to albumin and hence albumin-bound toxins. While a large portion of the toxins, which accumulate during liver failure are water soluble, many are still bound by albumin. Therefore fractionated plasma separation may be advantageous in regard to toxin removal. Other factors that distinguish Prometheus from MARS include the fact that while MARS is prefilled with 120 g of exogenous human albumin, the patient's endogenous albumin loads the secondary circuit in Prometheus. Because Prometheus is loaded with the patient's albumin, there may be a drop in the patient's albumin levels during treatment [65, 66].

Most of the clinical data involving Prometheus are either uncontrolled or retrospective. A controlled trial, published as an abstract, looking at the effect of fractionated plasma separation on hepatic encephalopathy, demonstrated that a 6 h treatment course improved clinical grade and sensory-evoked potentials [67]. Multiple case series describe both, acute and acute-on-chronic liver failure patients being treated with Prometheus. Only recently, the results of a controlled randomized multicenter clinical trial in 145 patients with acute on chronic liver failure were reported in abstract form [68]. Survival rates after 1 and 3 month were not significantly different in the treated versus the control group. However, patients with hepatorenal syndrome type I and MELD score of >30 showed a significant survival benefit. Currently available data thus illustrate a need for new prospective randomized controlled trials to clarify indications and clinical impact of extracorporeal artificial liver support devices.

It is unlikely that the complex mechanism, by which the liver ensures homeostasis, can be replaced by means of nonbiologic detoxification alone. A bioartificial liver, which incorporates hepatocytes from various sources, has the theoretical advantage of not only providing blood purification through dialysis, but also providing the hepatocyte-specific functions which are lost with ALF. The first biologically based liver assist device to be tested in FDA-approved phase II/III trial was HepatAssist™ by Arbios (formerly Circe, Waltham, MA). The device employed a hollow fiber extracorporeal bioreactor loaded with cryopreserved primary porcine hepatocytes. A randomized, controlled, multicenter phase II/III clinical trial was conducted in patients with fulminant/subfulminant liver failure and primary graft nonfunction [69]. The study demonstrated favorable safety, but failed to demonstrate improved 30-day survival in the overall study population. Although subgroups of the study population showed significant survival benefits, HepatAssist is not yet approved by the FDA. The extracorporeal liver assist device (ELAD™) developed by Vital Therapies (San Diego, CA) utilizes hollow fiber cartridges loaded with cells from the C3A human hepatoblastoma cell line. The most current model also contains a conventional hemodialysis unit. An early randomized controlled trial of 24 patients with acute alcoholic hepatitis demonstrated that therapy with ELAD produced reduced levels of ammonia and bilirubin along with improvement in hepatic encephalopathy when compared to controls [70]. However, a statistically significant survival advantage was not demonstrated. The modular extracorporeal liver support system (MELS™; Charité, Berlin, Germany) is a hepatocyte based liver support therapy composed of four independently functioning hollow fiber capillary cell compartments. A phase I study in 2003 including eight patients with ALF demonstrated safety, with all eight patients being successfully bridged to transplantation [71]. Clinical experience with MELS has been limited by the infrequent and unpredictable supply of human hepatocytes and concerns of xenozoonosis involving pig hepatocytes which are prevalent in Europe. The bioartificial liver support system (BLSS™) by Excorp medical (Minneapolis, MN,U.S.A) is a system that utilizes $\sim$100 g of primary porcine hepatocytes in a single hollow fiber cartridge. Venovenous bypass is used to circulate the patient's blood through the system. A phase I trial, in which four patients were treated with BLSS, demonstrated safety [72]. Currently, a phase II/III study is underway, and results will further define the role of this device. The Amsterdam medical Center bioartificial liver (AMC-BALTM; AMC, Amsterdam, The Netherlands) uses 100 g of primary porcine hepatocytes bound to a spiral-shaped polyester fabric with integrated hollow fibers. During treatment, the bioreactor is perfused with the patient's plasma. A phase I study of the system examined seven patients with ALF who underwent multiple treatments with AMC-BAL [73]. Six were successfully bridged to transplantation, and one patient recovered liver function without transplantation. Improvements were observed in both clinical and biochemical parameters including a decrease in both bilirubin and ammonia. No adverse events were associated with treatment. While preliminary results were encouraging, larger randomized, controlled trials are needed to determine the role of AMC-BAL.

In acute liver failure hepatocyte transplantation may act as a bridge to recovery and regeneration of the injured native liver or alternatively to orthotopic liver transplantation once an organ becomes available. The procedure may also be used in patients who are not candidates for organ transplantation. A major advantage of hepatocyte transplantation is the immediate availability of cryopreserved cells. Sufficient cell mass (approximately 10–15 % of liver cell mass) is needed to provide enough metabolic function [74]. The mass of cells, which can be transplanted into the liver, is, however, limited. Other options include intrasplenic or intraperitoneal transplantation, which allow a larger volume of cells. The spleen has been used successfully as injection site in animals [75, 76] and human transplantation [77]; however, in view of the number of immunologically active cells located in the spleen, rejection or destruction of the non-native cells needs consideration. Hepatocyte transplantation in patients with ALF has resulted in a reduction in ammonia and bilirubin with improvements in hepatic encephalopathy and cardiovascular instability [77, 78]. In the absence of any randomized controlled trials, it is difficult to comment on the true efficacy of the intervention.

There are a few studies on liver cell therapy for treatment of acute liver failure in humans with the intention to bridge the patients to orthotopic liver transplantation or recovery. Main challenges for future applications are the appropriate timing of cell transplantation, the restricted uptake capacity of the recipient liver, the availability of cells, and the need for immunosuppression to prevent the rejection of the transplanted cells. The latter point may become more important than previously considered, since the liver failure gives a high risk for septic complications itself, which will be aggravated by immunosuppressive drugs.

Extended liver resections have been associated with significant morbidity and mortality due to hepatic dysfunction or hepatic failure in the postoperative period. Autologous bone marrow stem cell therapies may offer the potential to enhance hepatic regeneration in this setting, perhaps increasing the safety of the procedure. Preclinical models and initial translational studies have suggested that autologous bone marrow stem cell administration can facilitate hepatic regeneration following both acute and chronic liver disease [79]. Infusion of HSC in three patients after extended liver resection demonstrated the therapeutic potential, however, more and controlled clinical trial data are needed [80].

10.5.2 Chronic Liver Disease and Liver Fibrosis

Chronic injury and inflammation triggers a gradual loss of liver function and deposition of extracellular matrix components, which leads to fibrosis and finally to cirrhosis of the liver. Although acute injury does activate mechanisms of fibrogenesis, more sustained signals associated with chronic liver diseases lead to a fibrogenic response which engages several different cell types. Cirrhosis of the liver as a clinical endpoint of the fibrogenic process is probably an irreversible condition and the only long-term therapeutic solution for end-stage chronic liver

disease today is liver organ transplantation. However, experimental and clinical data indicate that earlier events of the perpetuated fibrogenic process in the liver can be stopped or even reversed.

There is now experimental evidence that several endogenous factors/cytokines play important roles in regulation of liver fibrogenesis. The use of interferon alpha -2α and -2β is nowadays the main therapeutic strategy for the treatment of chronic viral hepatitis and compensated viral liver cirrhosis [81, 82]. In addition to decreasing viremia in HBV and HCV infections, it also may lead to reduced liver fibrosis.

New therapeutic targets interfering with fibrogenesis are emerging from translational research and have been recently addressed in clinical trials. Interferon-gamma1β (IFN-γ1β) is a pleiotropic cytokine that displays antifibrotic, antiviral, and antiproliferative activity. Initial studies conducted in patients with HCV-related liver diseases have shown a fibrosis reduction in some of the patients [83]. In particular, patients with elevated interferon-inducible T cell-alpha chemoattractant (ITAC) levels in their blood and, perhaps less advanced disease stages, may best be suited for IFN-gamma1β based therapy [84].

Interleukin-10 (IL-10) was first described as a cytokine synthesis inhibitory factor for T lymphocytes produced from T helper 2 cell clones. In fact various cell populations produce IL-10 in the body, including T cell subsets, monocytes, macrophages, and also various other cell types present in organs such as the liver. IL-10 gene polymorphisms are possibly associated with liver disease susceptibility or severity. Recombinant human IL-10 is currently tested in clinical trials in patients not responding to standard Peg-IFN α therapy.

PDGF is the most potent mitogen for hepatic stellate cell-derived myofibroblasts and levels of the growth factor have been shown to increase in liver diseases. Autocrine signaling by PDGF was the first cytokine loop discovered in hepatic stellate cell activation and is among the most potent ones [85]. Hepatic PDGF-α overexpression using the CRP-gene promoter was accompanied by a significant increase in hepatic procollagen III mRNA expression as well as TGF-β1 expression. Liver histology showed increased deposition of extracellular matrix in transgenic but not in wildtype mice. These results point to a mechanism of fibrosis induction by PDGF-α via the TGF-β1 signaling pathway [86]. On the other hand, Dominant-negative soluble PDGF receptor beta is currently investigated as a possible new antifibrogenic target.

TGFβ1 remains, however, the classic fibrogenic cytokine. TGF β1 activates stellate cells via the SMAD proteins pathway and also stimulates collagen expression in stellate cells through a hydrogen peroxide and C/EBPβ -dependent mechanism. There is experimental evidence that hepatocyte-specific overexpression of TGFβ1 in transgenic mice increases fibrosis in vivo, and that soluble TGFβ receptor type II treatment inhibits fibrosis in vivo. Also, it has been shown that adenovirus encoding antisense TGFβ mRNA inhibits fibrogenesis in vivo.

More experimental strategies aim to reduce extracellular matrix deposition by overexpression of MMP's. Siller-Lopez et al. have used an extrahepatic human neutrophil collagenase complementary MMP-8 DNA cloned in an adenovirus

vector (AdMMP8) as a therapeutic agent in cirrhosis using CCl_4 and bile duct–ligated cirrhotic rats models. Liver fibrosis in bile duct–ligated cirrhotic animals was decreased by 45 % along with reduced hydroxyproline levels in AdMMP8 treated animals. Treatment in both models correlated with improvements in ascites, functional hepatic tests, and gastric varices indicating diminished portal hypertension in animals injected with AdMMP8 [87].

Alternative treatment concepts aim to protect existing hepatocytes and/or to increase the hepatocyte mass. Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes [88–91] has mitogenic and morphogenic activities for a wide variety of cells [92, 93] and also plays an essential role in the development and regeneration of the liver [94]. The protein has also been shown to have antiapoptotic activity in hepatocytes [95]. Transduction of the HGF gene has suppressed the increase of transforming growth factor-β1 (TGF-β1), which plays an essential part in the progression of liver cirrhosis and inhibited fibrogenesis and hepatocyte apoptosis leading to complete resolution of fibrosis in the cirrhotic liver in a rat model [96].

10.5.2.1 Stem Cell Therapy of Chronic and Acute of Chronic Liver Disease

Although the concept of stem cell therapy for various diseases is principally accepted, the practical approach in humans remains difficult. Bone marrow derived mononucleated cells, hematopoietic stem and progenitor cells, mesenchymal stem (stromal) cells and sinusoidal endothelial cells are currently being investigated. There are several proposed mechanisms by which stem and progenitor cells might support regeneration in targeted organs including the liver: intercellular signaling through cell–cell contacts, paracrine signaling (growth factors, cytokines, and hormones) or cell fate change in the target organ.

The concept of stem/progenitor cell infusions exerting a paracrine regenerative effect on the liver is gaining support and is backed up by both rodent and human studies, although the latter are small and uncontrolled. Endothelial precursor cells (EPC) have been shown in rodent models to promote angiogenesis and the degradation of liver scar tissue thereby contributing to liver regeneration [97–99]. By participation in neovascularization and by the expression of multiple growth factors, transplanted EPCs significantly accelerate liver regeneration. This is achieved by enhancing proliferative activity of hepatocytes leading to improved survival after chemically induced liver injury [97].

Sakaida et al. have demonstrated that transplanted bone marrow cells degrade extracellular matrix in carbon tetrachloride (CCl_4)-induced liver fibrosis, with a significantly improved survival rate in this animal model. Their findings suggest that transplanted bone marrow cells can degrade collagen fibers and reduce liver fibrosis by strong expression of MMPs, especially MMP-9 [100].

Other groups have raised concerns about the role of certain subtypes of bone marrow stem cells in chronic liver injury [101]. It has been shown that bone marrow-derived myofibroblasts significantly contributed to fibrogenesis in a

chronic liver injury model in mice. They originated predominantly from bone marrow cells enriched for mesenchymal progenitor cells. These cells were located in the region of hepatic scarring and actively expressed collagen. The data suggest that an axis of recruitment from the bone marrow to the liver does exist in chronic injury and that the therapeutic application of certain subsets of bone marrow-derived cells may contribute to, rather than resolve scarring of the liver tissue. The choice of the transplanted bone marrow cell type might thus be important with regard to supporting liver regeneration or fibrogenesis.

Taken together the infusion of stem cells might provide an array of factors supporting not only liver regeneration, but also the remodeling of impaired liver architecture by interfering with fibrogenesis. Important experimental findings, however, suggest that infused bone marrow cells may also contribute to fibrogenesis [102, 103] giving some cautious notes for the uncritical use of stem cells for chronic liver disease outside of controlled clinical trials [100, 104–106].

Several clinical trials already investigated the effect of bone marrow (stem) cells in patients with liver disease. They were mainly uncontrolled, with only small numbers of patients enrolled and have provided heterogeneous results. The trials can be categorized in 4 groups according to the main endpoint and source of cells: [1] effects of granulocyte colony–stimulating factor (G-CSF) mobilized bone marrow cells in advanced chronic liver disease, [2] effects of infusion of autologous mononuclear cells collected from bone marrow in advanced chronic liver disease, [3] effects of collection (with or without ex vivo manipulation), and infusion of mobilized bone marrow cells in advanced chronic liver disease and [4] effects of bone marrow infusions on liver regeneration (after selective portal venous embolization) prior to extended hepatectomy for liver tumors [107–118].

The trials are quite heterogeneous with regard to the source of stem cells used and the number of patients included. The following stem cells sources have been used: bone marrow from iliac crest (50–400 ml), G-CSF mobilization only, G-CSF mobilization followed by leukapheresis and CD-34+ selection and reinfusion. All but one trial were non-randomized. The stem cells were administered by peripheral vein infusions (3 studies), by hepatic artery infusions (5 studies), or portal vein infusions (2 studies). The largest study conducted so far by Lyra et al. was also the only randomized one and included 30 patients.

Eight out of 11 trials have shown a moderate improvement in liver function (albumin, INR, bilirubin, Child-Pugh score, MELD score) and the follow-up period has ranged from 2 to 12 months.

In one recent study safety and efficacy of hepatic artery administration of mobilized autologous and ex vivo expanded adult $CD34^+$ hematopoietic stem cells in patients with alcoholic cirrhosis (ALC) was assessed [118]. This study reported one of the largest numbers of CD34 positive stem cells infused in cirrhotic patients so far. Nine patients with biopsy-proven ALC and abstinence from alcohol for at least 6 months were included in the study and all patients tolerated the procedure well, with no treatment-related side effects or toxicities observed. Significant improvement in liver function was shown by decrease in serum bilirubin levels,

serum alanine transaminase, and aspartate transaminase. The Child-Pugh score improved in 7 out of 9 patients and in 5 patients ascites production had declined.

Two studies so far aimed to ameliorate acute on chronic liver disease by administration of granulocyte—colony stimulating factor (G-CSF) treatment. In contrast to an earlier study by Campli et al. [119] a more recent study from India showed profound effects on short-term survival, which was associated with a marked increase of CD34 stem cells in the liver of recipients [120].

10.5.3 Hereditary Liver Disease

Liver organ transplantation can be viewed as a form of gene therapy for inherited liver diseases since the procedure substitutes a defective gene with a normal copy from a healthy donor. Animal studies have shown that for most monogenetic liver diseases partial substitution of a missing or defective protein is able to reverse the clinical phenotype and can result in complete remission of the disease. This redundancy opens the possibility to apply minimally invasive therapies such as cell and gene therapies to correct an existing gene defect. Although many hurdles still exist, feasibility has been proven unequivocally in animal models and therapeutic protocols are now emerging in the clinical arena.

10.5.3.1 Transplantation of Mature (Adult) Hepatocytes

In recent years the interest in liver cell therapy has been increasing continuously, since the demand for whole liver transplantations in human beings far outweighs the supply [121]. From the clinical point of view, transplantation of hepatocytes or hepatocyte-like cells may represent an alternative to orthotopic liver transplants for the correction of genetic disorders resulting in metabolically deficient states. The aim of hepatocyte transplantation in metabolic disease is to partially replace the missing function without the need to replace the whole organ. Almost 30 children and adults who received liver cell therapy for metabolic liver disease are reported in literature [122, 123]. Clinical therapies up to now have been performed by infusing fresh or cryopreserved primary hepatocyte suspensions isolated from donated organs. The availability of high quality liver tissue for cell isolation, however, has slowed the widespread application of this therapy. Furthermore, the clinical situation of target patients is rarely immediately life threatening and often acceptable conventional therapies are available. Therefore, the potential benefit must be carefully weighed against any possible complications, such as side effect from immunosuppression, hepatocyte embolization of the pulmonary vascular system, sepsis or hemodynamic instability.

Objective parameters such as laboratory data (i.e. bile acid, clotting factors, etc.) can be determined to unequivocally assess the efficacy of the treatment. The results of hepatocyte transplantation for many metabolic liver diseases have been

encouraging with demonstrable, although short-term correction of metabolic deficiency in the majority of cases. Therapeutic benefit has been reported in a girl with Crigler–Najjar Syndrome Type I, which is a recessively inherited metabolic disorder characterized by severe unconjugated hyperbilirubinemia [124]. Isolated hepatocytes were infused through the portal vein and partially corrected plasma bilirubin levels for more than 11 months. Similarly, a 9 year-old boy received 7.5×10^9 hepatocytes, infused via the portal vein, which resulted in a decrease in bilirubin level from 530 ± 38 μmol/L (mean ± SD) before to 359 ± 46 μmol/L [125]. Hughes et al. also reported a 40 % reduction in bilirubin levels in a Crigler–Najjar Syndrome Type I patient following transplantation of hepatocytes [126]. Although these data demonstrate efficacy and safety, a single course of cell application seems not sufficient to correct Crigler–Najjar Syndrome Type I completely.

Sustained response was reported in a patient with argininosuccinate lyase deficiency after repeated hepatocyte transplantation. Engraftment of the transplanted cells was analyzed in repeated liver biopsies for more than 12 months by fluorescence in situ hybridization for the Y-chromosome and by measurement of tissue enzyme activity [127]. Promising results have also been obtained in a 47-year-old woman suffering from glycogen storage disease type 1a, an inherited disorder of glucose metabolism resulting from mutations in the gene encoding the hepatic enzyme glucose-6-phosphatase [128]. 2×10^9 ABO-compatible hepatocytes were infused into the portal vein. Nine months after cell transplantation, her metabolic situation had clearly improved. Successful hepatocyte transplantation has also been achieved in a 4-year-old girl with infantile Refsum disease, an inborn error of peroxisome metabolism, leading to increased levels of serum bile acids and the formation of abnormal bile acids [129]. A total of 2×10^9 hepatocytes from a male donor were given during eight separate intraportal infusions. Abnormal bile acid production (for instance pipecholic acid) had decreased by 40 % after 18 months. Recently, hepatocyte transplantation has been used successfully to treat inherited factor VII deficiency [130]. Two brothers (aged 3 months and 3 years) received infusions of 1.1 and 2.2×10^9 ABO-matched hepatocytes into the inferior mesenteric vein. Transplantation clearly improved the coagulation defect and decreased the necessity for exogenous factor VII to approximately 20 % of that prior to cell therapy. As with the other metabolic liver diseases, hepatocyte transplantation has been shown to provide a partial correction of urea cycle defects. Patients showed clinical improvement, reduced ammonia levels, and increased production of urea [131–135].

10.5.3.2 Transplantation of Stem Cells

In the last few years, many reports have suggested that extrahepatic stem cells participate in liver regeneration and may be useful for treating many diseases [136–140]. However, subsequent work by several independent groups has clearly shown that hepatocyte replacement levels after injection of extrahepatic stem cells

or by bone marrow transplantation are low (<0.01 %), unless those bone marrow-derived hepatocytes have a selective growth advantage [141–143]. Furthermore, in most of the cases, fusion with host hepatocytes rather than transdifferentiation of extrahepatic cells, has been described as the underlying mechanism [144–147].

So far no convincing evidence has yet been provided in animal models that stem cells including HSC, MSC, iPS, or cells derived from cord blood or the amnion can generate therapeutically significant numbers of hepatocytes for the correction of hereditary metabolic liver diseases. Consequently, no credible data on the use of stem cells in patients with hereditary liver disease have been published.

10.5.3.3 Gene Therapy

The liver is involved in the synthesis of serum proteins, regulation of metabolism, and maintenance of homeostasis and thus provides a variety of opportunities for gene therapeutic corrections. Gene therapy is the treatment of an inherited or acquired disease through the manipulation of a patients' genetic status or sequence in selected cells by introducing various types of genetic materials, such as virally bound nucleic acids, plasmid DNAs, antisense oligonucleotides, and short interference RNAs. Both viral and non-viral methods have been developed for effective gene delivery. Currently, only viral vectors have transduction efficacies needed for liver-based gene therapy of inherited metabolic diseases in humans.

Viral Vectors

Viral gene delivery employs replication deficient viruses as a carrier to bring genetic materials into cells through their natural infection mechanism. Viral vectors are created using molecular biological techniques by which portion of the viral genome is replaced with a gene of interest. Major drawbacks of viral vectors are their genetic and immunologic toxicities, which are mainly associated with an arbitrary recombination with genomic DNA of the target cells and acute immune stimulation, respectively. Because adult humans have already developed immunity against several types of viruses from which viral vectors are developed, an exposure of the viral vectors to patients often results in strong immunological reaction, and consequently disables efficient gene delivery and long-term gene expression.

Viral vectors frequently used in gene therapy studies are derived from retrovirus, adenovirus, and adeno-associated virus. Retroviruses, enveloped RNA viruses with a particle size of approximately 100 nm, only infect dividing cells and are capable of integrating reverse transcribed DNA into the host genome at an unpredictable location [148]. Viral integration has led to leukemia development as revealed by recent gene therapy trial on X-linked SCID [149]. The requirement of hepatectomy ($\sim$70 %) to stimulate hepatic proliferation is generally considered as a drawback for retrovirus-mediated gene delivery to the liver [150, 151]. Lentiviruses, a subclass of retroviruses including human immunodeficiency virus, can

transduce non-dividing as well as dividing cells. The lentivirus preintegration complex is able to pass the intact nuclear membrane, which allows it to integrate into the host genome without cell division [152]. It was reported that animals can be repeatedly infected with lentiviral vectors [153].

Adenoviruses are double-stranded DNA viruses with a diameter of approximately 110 nm. Adenoviruses infect both replicating and non-replicating cells, have a relatively large genome, and are unable to integrate into the host genome [154]. A number of serotypes has been used to create adenoviral vectors and employed in 24.8 % of clinical trials till the end of March, 2008 [155]. These vectors exhibit a broad range of liver tropism with serotype 5 as the most commonly used to date [156]. Adenoviral vector is the first proven gene carrier for the treatment of cancer [157]. Because this virus is a natural human pathogen, preexisting immunity against adenovirus can cause severe allergic reaction and inactivation of viral vectors [158]. The current strategy in avoiding these problems is to use a serotype which the patients have no immunity against [156]. If the immunogenic drawbacks can be overcome in the future, adenoviral vectors will probably find a great diversity of clinical applications.

Adeno-associated virus (AAV) belongs to the Parvoviridae virus family and is approximately 26 nm in diameter without envelope [159]. It requires a helper virus for replication such as adenovirus. It is non-pathogenic and can infect quiescent cells. AAV is currently classified into 12 serotypes, and the liver is known to be a preferential target especially for AAV-8 [160]. It was reported that this virus can insert its genome at a defined site on chromosome 19 termed AAVS1 with nearly 100 % certainty [161]. The site-directed integration is controlled by viral Rep proteins [162], which are often deleted in recombinant AAV vectors in favor of more space for the exogenous gene to be packaged into the tiny viral particle. Cotransfection of plasmids coding for Rep protein was reported to restore capability of the site-directed integration and enable a long-term expression of the transgene without inducing insertional mutagenesis [163]. Results from a number of animal studies also indicate that AAV is less immunogenic when compared to adenoviruses [164].

Preclinical Evaluation

Feasability of gene therapies has been demonstrated in a wide variety of animal models. Long-Evans cinnamon rats are a model of Wilson disease and transfer of the *ATP7B* gene to hepatocytes ameliorates both biochemical and histological pathologies [165]. Transgene products released into blood circulation after successful gene transfer into the hepatocytes corrected pathological manifestation both inside and/or outside of the liver in glycogen storage diseases (type Ia, [166] Ib [167] and II [167], mucopolysaccharidosis type I [168], IIIB [169] and VII [170], hereditary tyrosinemia type I [171], UDP glucuronyltransferase deficiency (Crigler-Najjar type I) [172], and hemophilia [173–175].

A complete and persistent phenotypic correction of phenylketonuria in mice was reported after hydrodynamic gene delivery of murine phenylalanine hydroxylase cDNA with the help of phiBT1 phage integrase for long-term gene expression [176]. Further, the efficacy of adenovirus-mediated in vivo gene therapy for ornithine transcarbamylase deficiency was reported in mice and non-human primates [177]. Hyperlipidemia was not only effectively treated in the respective genetic mouse models through delivery of apolipoprotein B [178] or E [179] genes but also in wild type mice treated with a high-fat diet. A reciprocal pathophysiological condition of hypoalphalipoproteinemia was effectively reversed by adenoviral transduction of human apolipoprotein A-I gene in model mice as well [180].

Liver-Directed Gene Therapy in Humans

Gene therapy has the potential to offer a definitive cure for monogenic diseases by achieving a long-term correction of pathology. Monogenic diseases in the liver are divided into two groups depending on whether cell damage in the liver is involved or not. For example, hemophilia, familiar hypercholesterolemia, and phenylketonuria show systemic manifestations without significant liver cell damage, and have the least risk for hepatotoxicity in orthotopic gene delivery. In fact phase I/II clinical trials for hemophilia B were completed with promising results [181]. Unfortunately, however, the development of inhibitory antibodies against the exogenous factor IX and/or components of viral vectors diminished a persistent phenotypic correction [181, 182]. One possible solution to avoid antibody development against exogenous gene products is gene delivery into the fetal liver to induce tolerance to the exogenous gene products [172, 175, 183] or alternative injection routes [184]. It is important to point out that significant difference exists between animal studies and human clinical trials with respect to immunological reactions [185, 186].

In case of the monogenic liver diseases with substantial hepatocellular damage, gene therapy should not be a primary indication unless gene delivery can be completed in all hepatocytes in the liver. Successful delivery of human fumarylacetoacetate hydrolase gene into hepatocytes protected $FAH^{(-/-)}$ -mice mimicking hereditary tyrosinemia type 1 disease from fulminant liver failure by restoring the enzyme activity [187, 188]. However, hepatocellular damage continued in the rest of hepatocytes that had not received the transgene and resulted in the frequent development of hepatocellular carcinoma. Liver transplantation should be a primary option for the diseases in this category at this moment.

10.6 Future Directions

Regenerative therapies involving various types of cells as well as gene therapies are currently being investigated in research laboratories around the world and more and more find the way into therapeutic algorithms in the clinic. Bioartificial liver

support systems and cell therapies are currently limited by the availability of good quality hepatocytes. A renewable source of highly metabolically competent hepatocytes will be essential for any successful bioartificial liver system. To date porcine hepatocytes are most commonly being used with limited acceptance due to ongoing concerns of xenozoonosis. Immortalized human hepatocytes have not shown expression of prerequisite hepatocyte function including ammonia detoxification. Other limitations of first-generation bioartificial liver systems, which need to be solved, include excess device complexity, insufficient number of hepatocytes to support a failing liver, early hepatocyte death, and absence or loss of differentiated function.

The application of stem cells in liver cell therapies seems to be a promising approach for the treatment of liver diseases. However, several issues still have to be addressed to fulfil this promise. We need to identify, both inside and outside of the liver, the stem cell candidates that are able to form mature hepatocytes in vitro and functional liver tissue after transplantation in vivo. The fundamental molecular pathways involved in the differentiation of hepatocytes and cholangiocytes from stem/progenitor cells, the factors that are responsible for in vitro differentiation of various stem cells into hepatocytes, the mechanisms involved in the fusion of stem cells and hepatocytes and the aspects that can potentially enhance these mechanisms need to be studied in more detail. With future progress in stem cell research, the various stem cell sources including hepatic stem/progenitor cells, embryonic, and adult extrahepatic stem cells should provide great opportunities for the treatment of liver disorders.

Additional work is also needed in the development of an ideal gene delivery system. The efficacy of delivery and the level of transgene expression achieved by the current methods have resulted in phenotypic correction of various hereditary liver diseases in animal models. The most efficient vehicles for gene delivery to the liver developed so far are viral vectors. Among the viral vectors applicable to liver gene delivery, lentiviral vectors appear to have great advantage because of their ability to transduce the liver cells at resting state and generate persistent gene expression. Gene toxicity by insertional mutagenesis with the transactivation of potentially harmful genes and interactions of the host immune system with the viral proteins and the therapeutic product need to be studied in more detail. Active participation of hepatologists in gene therapy research will accelerate the process in turning gene therapy into a common practice for the treatment of various diseases through the liver.

In summary, advanced approaches in regenerative hepatology will cover strategies to improve endogenous liver regeneration, to correct monogenetic liver diseases by gene therapy, and to support organ function with additional hepatic cells, either in extracorporal devices or as cell transplants. For the latter aspect, improved cell isolation and propagation techniques to utilize cells from donor organs or advanced stem cell-differentiation protocols become of utmost importance to ensure the supply of functional hepatic cells.

References

1. Sherlock S, Dooley J (2002) Disease of the liver and biliary system. Blackwell Publishing Company, Oxford
2. Schiff ER, Sorrell MF, Maddrey WC (2007) Schiff's diseases of the liver. Lippincott Williams and Wilkins, Philadelphia
3. Burt AD, Portmann BC, Ferrell LD (2007) MacSween's pathology of the liver. Elsevier, Philadelphia
4. Fausto N, Campbell JS, Riehle KJ (2006) Liver regeneration. Hepatology 43:S45–S53
5. Michalopoulos GK, DeFrances MC (1997) Liver regeneration. Science 276:60–66
6. Taub R (1996) Transcriptional control of liver regeneration. FASEB J 10(4):413–427
7. Taub R (2004) Liver regeneration: from myth to mechanism. Nat Rev Mol Cell Biol 10:836–847
8. Gorla GR, Malhi H, Gupta S (2001) Polyploidy associated with oxidative injury attenuates proliferative potential of cells. J Cell Sci 114(16):2943–2951
9. Jungermann K, Kietzmann T (2000) Oxygen: modulator of metabolic zonation and disease of the liver. Hepatology 31(2):255–260
10. Sigal SH, Gupta S, Gebhard DF, Holst P, Neufeld D, Reid LM (1995) Evidence for a terminal differentiation process in the rat liver. Differentiation 59:35–42
11. Fellous TG, Islam S, Tadrous PJ, Elia G, Kocher HM, Bhattacharya S, Mears L, Turnbull DM, Taylor RW, Greaves LC, Chinnery PF, Taylor G, McDonald SA, Wright NA, Alison MR (2009) Locating the stem cell niche and tracing hepatocyte lineages in human liver. Hepatology 49(5):1655–1663
12. Bralet MP, Branchereau S, Brechot C, Ferry N (1994) Cell lineage study in the liver using retroviral mediated gene transfer: evidence against the streaming of hepatocytes in normal liver. Am J Pathol 144:896–905
13. Quante M, Wang TC (2009) Stem cells in gastroenterology and hepatology. Nat Rev Gastroenterol Hepatol 6(12):724–737
14. Duncan AW, Dorrell C, Grompe M (2009) Stem cells and liver regeneration. Gastroenterology 137(2):466–481
15. Grisham JW, Hartroft WS (1961) Morphologic identification by electron microscopy of "oval" cells in experimental hepatic degeneration. Lab Invest 10:317–332
16. Suzuki A, Zheng YW, Kaneko S, Onodera M, Fukao K, Nakauchi H, Taniguchi H (2002) Clonal identification and characterization of self-renewing pluripotent stem cells in the developing liver. J Cell Biol 156:173–184
17. Fausto N (2004) Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. Hepatology 39:1477–1487
18. Thorgeirsson SS (1996) Hepatic stem cells in liver regeneration. Faseb J 10:1249–1256
19. Fausto N, Campbell JS (2003) The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech Dev 120:117–130
20. Furuyama K, Kawaguchi Y, Akiyama H, Horiguchi M, Kodama S, Kuhara T, Hosokawa S, Elbahrawy A, Soeda T, Koizumi M, Masui T, Kawaguchi M, Takaori K, Doi R, Nishi E, Kakinoki R, Deng JM, Behringer RR, Nakamura T, Uemoto S (2003) Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. Nat Genet 43(1):34–41
21. Malato Y, Naqvi S, Schürmann N, Ng R, Wang B, Zape J, Kay MA, Grimm D, Willenbring H (2011) Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. J Clin 121(12):4850–4860
22. Roskams T, Yang SQ, Koteish A, Durnez A, DeVos R, Huang X, Achten R, Verslype C, Diehl AM (2003) Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. Am J Pathol 163:1301–1311
23. Roskams T (2006) Liver stem cells and their implication in hepatocellular and cholangiocarcinoma. Oncogene 25(27):3818–3822

24. Marshall A, Rushbrook S, Davies SE, Morris LS, Scott IS, Vowler SL, Coleman N, Alexander G (2005) Relation between hepatocyte G1 arrest, impaired hepatic regeneration, and fibrosis in chronic hepatitis C virus infection. Gastroenterology 128:33–42
25. Wiemann SU, Satyanarayana A, Tsahuridu M, Tillmann HL, Zender L, Klempnauer J, Flemming P, Franco S, Blasco MA, Manns MP, Rudolph KL (2002) Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. Faseb J 16:935–942
26. Alison MR, Lovell MJ (2005) Liver cancer: the role of stem cells. Cell Prolif 38:407–421
27. Mishra L, Banker T, Murray J, Byers S, Thenappan A, He AR, Shetty K, Johnson L, Reddy EP (2009) Liver stem cells and hepatocellular carcinoma. Hepatology 49(1):318–329
28. Dandri M, Burda MR, Török E, Pollok JM, Iwanska A, Sommer G, Rogiers X, Rogler CE, Gupta S, Will H, Greten H, Petersen J (2001) Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. Hepatology 33(4):981–988
29. Bissig KD, Le TT, Woods NB, Verma IM (2007) Repopulation of adult and neonatal mice with human hepatocytes: a chimeric animal model. Proc Natl Acad Sci USA 104(51):20507–20511
30. Haridass D, Yuan Q, Becker PD, Cantz T, Iken M, Rothe M, Narain N, Bock M, Nörder M, Legrand N, Wedemeyer H, Weijer K, Spits H, Manns MP, Cai J, Deng H, Santo Di JP, Guzman CA, Ott M (2009) Repopulation efficiencies of adult hepatocytes, fetal liver progenitor cells, and embryonic stem cell-derived hepatic cells in albumin-promoter-enhancer urokinase-type plasminogen activator mice. Am J Pathol 175(4):1483–1492
31. Overturf K (1997) al-Dhalimy, M., Ou, C.N., Finegold, M., and Grompe, M.: Serial transplantation reveals the stem-cell-like regenerative potential of adult mouse hepatocytes. Am J Pathol 151:1273–1280
32. Habibullah CM, Syed IH, Qamar A, Taher-Uz Z (1994) Human fetal hepatocyte transplantation in patients with fulminant hepatic failure. Transplantation 58(8):951–952
33. Khan AA, Parveen N, Mahaboob VS, Rajendraprasad A, Ravindraprakash HR, Venkateswarlu J, Rao P, Pande G, Narusu ML, Khaja MN, Pramila R, Habeeb A, Habibullah CM (2008) Treatment of Crigler-Najjar Syndrome type 1 by hepatic progenitor cell transplantation: a simple procedure for management of hyperbilirubinemia. Transplant Proc 40(4):1148–1150
34. Poyck PP, van Wijk AC, van der Hoeven TV, de Waart DR, Chamuleau RA, van Gulik TM, Oude Elferink RP, Hoekstra R (2008) Evaluation of a new immortalized human fetal liver cell line (cBAL111) for application in bioartificial liver. J Hepatol 48(2):266–275
35. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP (1999) Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170
36. Alison MR, Poulsom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, Novelli M, Prentice G, Williamson J, Wright NA (2000) Hepatocytes from non-hepatic adult stem cells. Nature 406:257
37. Newsome PN, Johannessen I, Boyle S, Dalakas E, McAulay KA, Samuel K, Rae F, Forrester L, Turner ML, Hayes PC, Harrison DJ, Bickmore WA, Plevris JN (2003) Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. Gastroenterology 124:1891–1900
38. Aurich I, Mueller LP, Aurich H, Luetzkendorf J, Tisljar K, Dollinger MM, Schormann W, Walldorf J, Hengstler JG, Fleig WE, Christ B (2007) Functional integration of hepatocytes derived from human mesenchymal stem cells into mouse livers. Gut 56(3):405–415
39. Cantz T, Sharma AD, Jochheim-Richter A, Arseniev L, Klein C, Manns MP, Ott M (2004) Reevaluation of bone marrow-derived cells as a source for hepatocyte regeneration. Cell Transplant 13:659–666
40. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292(5819):154–156
41. Rathjen J, Rathjen PD (2001) Mouse ES cells: experimental exploitation of pluripotent differentiation potential. Curr Opin Genet Dev 11:587–594
42. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282(5391):1145–1147

43. Boiani M, Scholer HR (2005) Regulatory networks in embryo-derived pluripotent stem cells. Nat Rev Mol Cell Biol 6:872–884
44. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
45. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
46. Sekiya S, Suzuki A (2011) Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 475(7356):390–393
47. Huang P, He Z, Ji S, Sun H, Xiang D, Liu C, Hu Y, Wang X, Hui L (2011) Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 475(7356):386–389
48. Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M (2003) Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 422:897–901
49. Gouon-Evans V, Boussemart L, Gadue P, Nierhoff D, Koehler CI, Kubo A, Shafritz DA, Keller G (2006) BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. Nat Biotechnol 24:1402–1411
50. Drobinskaya I, Linn T, Saric T, Bretzel RG, Bohlen H, Hescheler J, Kolossov E (2008) Scalable selection of hepatocyte- and hepatocyte precursor-like cells from culture of differentiating transgenically modified murine embryonic stem cells. Stem Cells 26(9):2245–2256
51. Touboul T, Hannan NR, Corbineau S, Martinez A, Martinet C, Branchereau S, Mainot S, Strick-Marchand H, Pedersen R, Di Santo J, Weber A, Vallier L (2010) Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. Hepatology 51(5):1754–1765
52. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K (2008) Induced pluripotent stem cells generated without viral integration. Science 322:945–949
53. Li W, Wang D, Qin J, Liu C, Zhang Q, Zhang X, Yu X, Lahn BT, Mao FF, Xiang AP (2010) Generation of functional hepatocytes from mouse induced pluripotent stem cells. J Cell Physiol 222:492–501
54. Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA (2010) Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 51:297–305
55. Song Z, Cai J, Liu Y, Zhao D, Yong J, Duo S, Song X, Guo Y, Zhao Y, Qin H, Yin X, Wu C, Che J, Lu S, Ding M, Deng H (2009) Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. Cell Res 19:1233–1242
56. Sullivan GJ, Hay DC, Park IH, Fletcher J, Hannoun Z, Payne CM, Dalgetty D, Black JR, Ross JA, Samue lK, Wang G, Daley GQ, Lee JH, Church GM, Forbes SJ, Iredale JP, Wilmut I (2010) Generation of functional human hepatic endoderm from human induced pluripotent stem cells. Hepatology 51:329–335
57. Ohashi K, Yokoyama T, Yamato M, Kuge H, Kanehiro H, Tsutsumi M, Amanuma T, Iwata H, Yang J, Okano T, Nakajima Y (2007) Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. Nat Med 13(7):880–885
58. Ohashi K, Koyama F, Tatsumi K, Shima M, Park F, Nakajima Y, Okano T (2010) Functional life-long maintenance of engineered liver tissue in mice following transplantation under the kidney capsule. J Tissue Eng Regen Med 4(2):141–148
59. Jalan R, Wright G, Davies NA, Hodges SJ (2007) L-Ornithine phenylacetate (OP): a novel treatment for hyperammonemia and hepatic encephalopathy. Med Hypotheses 69:1064–1069
60. Mitzner SR, Stange J, Klammt S, Koball S, Hickstein H, Reisinger EC (2009) Albumin dialysis MARS: knowledge from 10 years of clinical investigation. ASAJO J 55(5):498–502
61. Steiner C, Sen S, Stange J, Williams R, Jalan R (2004) Binding of bilirubin and bromosulphthalein to albumin: implications for understanding the pathophysiology of liver failure and its management. Liver Transpl 10:1531–1538

62. Stange J, Mitzner S, Risler T (1999) Molecular adsorbent recycling system (MARS): clinical results of a new membrane-based blood purification system for bioartificial liver support. Artif Organs 23:319–330
63. Khuroo MS, Khuroo MS, Farahat KL (2004) Molecular adsorbent recirculating system for acute and acute-on-chronic liver failure: a meta-analysis. Liver Transpl 10:1099–1106
64. Saliba F, Camus C, Durand F, Mathurin B, Delafosse K, Barange PF, Perrigault P, Revel L, Serfaty M, Belnard A, Letierce P, Ichai D (2009) Samuel: predictive factors of transplant free survival in patients with fulminant and subfulminant hepatic failure: results from a randomized controlled multicenter trial. J Hepatol 50(1):S89–S90
65. Santoro A, Faenza S, Mancini E (2006) Prometheus system: a technological support in liver failure. Transplant Proc 38:1078–1082
66. Rifai K, Ernst T, Kretschmer U (2003) Prometheus: a new extracorporeal system for the treatment of liver failure. J Hepatol 39:984–990
67. Kramer L (2000) Clinical experience with artifical liver support in chronic liver failure with encephalopathy. ASAIO J a211
68. Rifai K, Kribben A, Gerken G (2010) Extracorporeal liver support by fractionated plasma separation and absorption (Prometheus®) in patients with acute on chronic liver failure (HELIOS study): a prospective randomized controlled multicenter study. J Hepatol 52(Suppl. 1):S3
69. Demetriou AA, Brown RS Jr, Busuttil RW (2004) Prospective, randomized, multicenter, controlled trial of a bioartificial liver in treating acute liver failure. Ann Surg 239:660–667
70. Ellis AJ, Hughes RD, Nicholl D (1999) Temporary extracorporeal liver support for severe acute alcoholic hepatitis using BioLogic-DT. Int J Artif Organs 22:27–34
71. Sauer IM, Kardassis D, Zeillinger K (2003) Clinical extracorporeal hybrid liver support: phase I study with primary porcine liver cells. Xenotransplantation 10:460–469
72. Mazariegos GV, Kramer DJ, Lopez RC (2001) Safety observations in phase I clinical evaluation of the Excorp Medical bioartificial liver support system after the first four patients. ASAIO J 47:471–475
73. Van De Kerkhove MP, Di Florio E, Scuderi V (2002) Phase I clinical trial with the AMC-bioartificial liver. Int J Artif Organs 25:950
74. Asonuma K, Gilbert JC, Stein JE, Takeda T, Vacanti JP (1992) Quantitation of transplanted hepatic mass necessary to cure the Gunn rat model of hyperbilirubinemia. J Pediatr Surg 27:298–301
75. Cai J, Ito M, Nagata H, Westerman KA, Lafleur D, Chowdhury JR, Leboulch P, Fox IJ (2002) Treatment of liver failure in rats with end-stage cirrhosis by transplantation of immortalized hepatocytes. Hepatology 36:386–394
76. Kobayashi N, Ito M, Nakamura J, Cai J, Gao C, Hammel JM, Fox IJ (2000) Hepatocyte transplantation in rats with decompensated cirrhosis. Hepatology 31:851–857
77. Bilir BM, Guinette D, Karrer F, Kumpe DA, Krysl J, Stephens J, McGavran L, Ostrowska A, Durham J (2000) Hepatocyte transplantation in acute liver failure. Liver Transpl 6:32–40
78. Fisher RA, Strom SC (2006) Human hepatocyte transplantation: worldwide results. Transplantation 82:441–449
79. Stutchfield BM, Rashid S, Forbes SJ, Wigmore SJ (2010) Practical barriers to delivering autologous bone marrow stem cell therapy as an adjunct to liver resection. Stem Cells Dev 19(2):155–162
80. am Esch JS 2nd, Knoefel WT, Klein M, Ghodsizad A, Fuerst G, Poll LW, Piechaczek C, Burchardt ER, Feifel N, Stoldt V, Stockschläder M, Stoecklein N, Tustas RY, Eisenberger CF, Peiper M, Häussinger D, Hosch SB (2005) Portal application of autologous CD133+ bone marrow cells to the liver: a novel concept to support hepatic regeneration. Stem Cells 23(4):463–470
81. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD (2001) Pegylated interferon alpha-2b plus ribavirin compared with interferon alpha-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet 358:958–965

82. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr et al (2002) Peginterferon alpha 2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347:975–982
83. Muir AJ, Sylvestre PB, Rockey DC (2006) Interferon gamma-1b for the treatment of fibrosis in chronic hepatitis C infection. J Viral Hepat 13(5):322–328
84. Pockros PJ, Jeffers L, Afdhal N, Goodman ZD, Nelson D et al (2007) Final results of a double-blind, placebo-controlled trial of the antifibrotic efficacy of interferon-gamma1b in chronic hepatitis C patients with advanced fibrosis or cirrhosis. Hepatology 45(3):569–578
85. Borkham-Kamphorst E, Kovalenko E, van Roeyen CR, Gassler N, Bomble M et al (2008) Platelet-derived growth factor isoform expression in carbon tetrachloride-induced chronic liver injury. Lab Invest 88(10):1090–1100
86. Thieringer F, Maass T, Czochra P, Klopcic B, Conrad I et al (2008) Spontaneous hepatic fibrosis in transgenic mice overexpressing PDGF-A. Gene 423(1):23–28
87. Siller–Lopez F, Sandoval A, Salgado S, Salazar A, Bueno M (2004) Treatment with human metalloproteinase-8 gene delivery ameliorates experimental rat liver cirrhosis. Gastroenterology 126:1122–1133
88. Nakamura T, Nawa K, Ichihara A (1984) Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem Biophys Res Commun 122:1450–1459
89. Russell WE, Mcgowan JA, Bucher NL (1984) Partial characterization of a hepatocyte growth factor from platelets. J Cell Physiol 119:183–192
90. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S (1989) Molecular cloning and expression of human hepatocyte growth factor. Nature 342:440–443
91. Miyazawa K, Tsubouchi H, Naka D, Takahashi K, Okigaki M, Arakaki N, Nakayama H, Hirono S, Sakiyama O, Takahashi K et al (1989) Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. Biochem Biophys Res Commun 163:967–973
92. Boros P, Miller CM (1995) Hepatocyte growth factor: a multifunctional cytokine. Lancet 345:293–295
93. Michalopoulos GK, DeFrances MC (1997) Liver regeneration. Science 276:60–66
94. Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, Gherardi E, Birchmeier C (1995) Scatter factor/hepatocyte growth factor is essential for liver development. Nature 373:699–702
95. Bardelli A, Longati P, Albero D, Goruppi S, Schneider C, Ponzetto C, Comoglio PM (1996) HGF receptor associates with anti-apoptotic protein BAG-1 and prevents cell death. EMBO J 15:6205–6212
96. Ueki T, Kaneda Y, Tsutsui H, Nakanishi K, Sawa Y et al (1999) Hepatocyte growth factor gene therapy of liver cirrhosis in rats. Nature Med 5(2):226–230
97. Taniguchi E, Kin M, Torimura T et al (2006) Endothelial progenitor cell transplantation improves the survival following liver injury in mice. Gastroenterology 130:521–531
98. Nakamura T, Torimura T, Sakamoto M et al (2007) Significance and therapeutic potential of endothelial progenitor cell transplantation in a cirrhotic liver rat model. Gastroenterology 133:91–107
99. Ueno T, Nakamura T, Torimura T et al (2006) Angiogenic cell therapy for hepatic fibrosis. Med Mol Morphol 39:16–21
100. Sakaida I, Terai S, Yamamoto N et al (2004) Transplantation of bone marrow cells reduces CCL4-induced liver fibrosis in mice. Hepatology 40:1304–1311
101. Russo FP, Alison MR, Bigger BW et al (2006) The bone marrow functionally contributes to liver fibrosis. Gastroenterology 130:1807–1821
102. Takezawa R, Watanabe Y, Akaike T (1995) Direct evidence of macrophage differentiation from bone marrow cells in the liver: a possible origin of Kupffer cells. J Biochem (Tokyo) 118:1175–1183
103. Kisseleva T, Uchinami H, Feirt N et al (2006) Bone marrow-derived fibrocytes participate in pathogenesis of liver fibrosis. J Hepatol 45:429–438

104. Fang B, Shi M, Liao L et al (2004) Systemic infusion of FLK1(_) mesenchymal stem cells ameliorate carbon tetrachloride-induced liver fibrosis in mice. Transplantation 78:83–88
105. Zhao DC, Lei JX, Chen R et al (2005) Bone marrow derived mesenchymal stem cells protect against experimental liver fibrosis in rats. World J Gastroenterol 11:3431–3440
106. Oyagi S, Hirose M, Kojima M et al (2006) Therapeutic effect of transplanting HGF-treated bone marrow mesenchymal cells into CCl4- injured rats. J Hepatol 44:742–748
107. Houlihan DD, Newsome PN (2008) Critical review of clinical trials of bone marrow stem cells in liver disease. Gastroenterology 135:438–450
108. Gaia S, Smedile A, Omede P et al (2006) Feasibility and safety of G-CSF administration to induce bone marrow-derived cells mobilization in patients with end stage liver disease. J Hepatol 45:13–19
109. Terai S, Ishikawa T, Omori K et al (2006) Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. Stem Cells 24:2292–2298
110. Mohamadnejad M, Namiri M, Bagheri M et al (2007) Phase 1 human trial of autologous bone marrow-hematopoietic stem cell transplantation in patients with decompensated cirrhosis. World J Gastroenterol 28:3359–3363
111. Mohamadnejad M, Alimoghaddam K, Mohyeddin-Bonab M et al (2007) Phase 1 trial of autologous bone marrow mesenchymal stem cell transplantation in patients with decompensated liver cirrhosis. Arch Iran Med 10:459–466
112. Lyra AC, Soares MB, da Silva LF et al (2007) Feasibility and safety of autologous bone marrow mononuclear cell transplantation in patients with advanced chronic liver disease. World J Gastroenterol 13:1067–1073
113. Lyra AC, Soares MB, da Silva LF et al (2007) A pilot randomised controlled study used to evaluate efficacy of autologous bone marrow mononuclear cells transplantation in patients with advanced chronic liver disease (abstr). Hepatology 46(Suppl 1):271A
114. Gordon MY, Levicar N, Pai M et al (2006) Characterisation and clinical application of human CD34_ stem/progenitor cell populations mobilized into the blood by granulocyte colony-stimulating factor. Stem Cells 24:1822–1830
115. Levicar N, Pai M, Habib NA et al (2008) Long-term clinical results of autologous infusion of mobilized adult bone marrow derived CD34_ cells in patients with chronic liver disease. Cell Prolif 41:115–125
116. Yannaki E, Anagnostopoulos A, Kapetanos D et al (2006) Lasting amelioration in the clinical course of decompensated alcoholic cirrhosis with boost infusions of mobilized peripheral blood stem cells. Exp Hematol 34:1583–1587
117. am Esch JS 2nd, Knoefel WT, Klein M et al. (2005) Portal application of autologous CD133+ bone marrow cells to the liver: a novel concept to support hepatic regeneration. Stem Cells 23:463–470
118. Pai M, Zacharoulis D, Milicevic MN, Helmy S, Jiao LR et al (2008) Autologous infusion of expanded mobilized adult bone marrow-derived CD34+ cells into patients with alcoholic liver cirrhosis. Am J Gastroenterol 103:1952–1958
119. Di Campli MA, Zocco N, Saulnier A, Grieco G, Rapaccini G, Addolorato C, Rumi A, Santoliquido G, Leone G, Gasbarrini A, Gasbarrini C (2007) Safety and efficacy profile of G-CSF therapy in patients with acute on chronic liver failure. Dig Liver Dis 39:1071–1076
120. Garg V, Garg H, Khan A, Trehanpati N, Kumar A, Sharma BC, Sakhuja P, Sarin SK (2011) Granulocyte-colony stimulating factor mobilizes CD34+ cells and improves survival of patients with acute-on-chronic liver failure. Gastroenterology 142(3):505–551
121. Nussler A, Konig S, Ott M et al (2006) Present status and perspectives of cell-based therapies for liver diseases. J Hepatol 45:144–159
122. Fisher RA, Strom SC (2006) Human hepatocyte transplantation: worldwide results. Transplantation 82:441–449
123. Fitzpatrick E, Mitry RR, Dhawan A (2009) Human hepatocyte transplantation: state of the art. J Intern Med 266(4):339–357
124. Fox IJ, Chowdhury JR, Kaufman SS et al (1998) Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N Engl J Med 338:1422–1426

125. Ambrosino G, Varotto S, Strom SC, Guariso G, Franchin E, Miotto D et al (2005) Isolated hepatocyte transplantation for Crigler–Najjar syndrome type 1. Cell Transplant 14:151–157
126. Hughes RD, Mitry RR, Dhawan A (2005) Hepatocyte transplantation for metabolic liver disease: UK experience. J R Soc Med 98:341–345
127. Stephenne X, Najimi M, Sibille C, Nassogne MC, Smets F, Sokal EM (2006) Sustained engraftment and tissue enzyme activity after liver cell transplantation for argininosuccinate lyase deficiency. Gastroenterology 130:1317–1323
128. Muraca M, Gerunda G, Neri D, Vilei MT, Granato A, Feltracco P et al (2008) Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. Lancet 359:317–318
129. Sokal EM, Smets F, Bourgois A, Van Maldergem L, Buts JP, Reding R et al (2003) Hepatocyte transplantation in a 4-year-old girl with peroxisomal biogenesis disease: technique, safety, and metabolic follow-up. Transplantation 76:735–738
130. Dhawan A, Mitry RR, Lehec S, Hughes RD, Terry C, Bansal S et al (2004) Hepatocyte transplantation for inherited factor VII deficiency. Transplantation 78:1812–1814
131. Horslen SP, McCowan TC, Goertzen TC et al (2003) Isolated hepatocyte transplantation in an infant with a severe urea cycle disorder. Pediatrics 111:1262–1267
132. Mitry RR, Dhawan A, Hughes RD, Bansal S, Lehec S, Terry C et al (2004) One liver, three recipients: segment IV from split-liver procedures as a source of hepatocytes for cell transplantation. Transplantation 77:1614–1616
133. Stephenne X, Najimi M, Smets F, Reding R, de Ville de Goyet J, Sokal EM (2005) Cryopreserved liver cell transplantation controls ornithine transcarbamylase deficient patient while awaiting liver transplantation. Am J Transplant 5:2058–2061
134. Dhawan A, Mitry RR, Hughes RD (2005) Hepatocyte transplantation for liver-based metabolic disorders. J Inherit Metab Dis 29:431–435
135. Meyburg J, Das AM, Hoerster F, Lindner M, Kriegbaum H, Engelmann G, Schmidt J, Ott M, Pettenazzo A, Luecke T, Bertram H, Hoffmann GF, Burlina A (2009) One liver for four children: first clinical series of liver cell transplantation for severe neonatal urea cycle defects. Transplantation 87(5):636–641
136. Alison MR, Poulsom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, Novelli M, Prentice G, Williamson J, Wright NA (2000) Hepatocytes from non-hepatic adult stem cells. Nature 406:257
137. Herzog EL, Chai L, Krause DS (2003) Plasticity of marrow-derived stem cells. Blood 102:3483–3493
138. Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M (2000) Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 6:1229–1234
139. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP (1999) Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170
140. Theise ND, Badve S, Saxena R, Henegariu O, Sell S, Crawford JM, Krause DS (2000) Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 31:235–240
141. Cantz T, Sharma AD, Jochheim-Richter A, Arseniev L, Klein C, Manns MP, Ott M (2004) Reevaluation of bone marrow-derived cells as a source for hepatocyte regeneration. Cell Transplant 13:659–666
142. Kanazawa Y, Verma IM (2003) Little evidence of bone marrow-derived hepatocytes in the replacement of injured liver. Proc Natl Acad Sci USA 100(Suppl 1):11850–11853
143. Wagers AJ, Sherwood RI, Christensen JL, Weissman IL (2002) Little evidence for developmental plasticity of adult hematopoietic stem cells. Science 297:2256–2259
144. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A (2003) Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 425:968–973
145. Quintana-Bustamante O, Alvarez-Barrientos A, Kofman AV, Fabregat I, Bueren JA, Theise ND, Segovia JC (2006) Hematopoietic mobilization in mice increases the presence of bone marrow-derived hepatocytes via in vivo cell fusion. Hepatology 43:108–116

146. Vassilopoulos G, Wang PR, Russell DW (2003) Transplanted bone marrow regenerates liver by cell fusion. Nature 422:901–904
147. Willenbring H, Bailey AS, Foster M, Akkari Y, Dorrell C, Olson S, Finegold M, Fleming WH, Grompe M (2004) Myelomonocytic cells are sufficient for therapeutic cell fusion in liver. Nat Med 10:744–748
148. Sinn PL, Sauter SL, McCray PB Jr (2005) Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors—design, biosafety, and production. Gene Ther 12:1089–1098
149. Bey H, Abina S, von Kalle C et al (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 348:255–256
150. Rettinger SD, Ponder KP, Saylors RL, Kennedy SC, Hafenrichter DG, Flye MW (1993) In vivo hepatocyte transduction with retrovirus during in-flow occlusion. J Surg Res 54:418–425
151. Branchereau S, Calise D, Ferry N (1994) Factors influencing retroviral-mediated gene transfer into hepatocytes in vivo. Hum Gene Ther 5:803–808
152. Amado RG, Chen IS (1999) Lentiviral vectors—the promise of gene therapy within reach. Science 285:674–676
153. Kafri T, Blomer U, Peterson DA, Gage FH, Verma IM (1997) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat Genet 17:314–317
154. Ghosh SS, Gopinath P, Ramesh A (2006) Adenoviral vectors: a promising tool for gene therapy. Appl Biochem Biotechnol 133:9–29
155. The Journal of Gene Medicine. The Journal of Gene Medicine Clinical Trial site. Available from URL: http://www.wiley.co.uk/wileychi/genmed/clinical/. 22 Dec 2008
156. Jager L, Ehrhardt A (2007) Emerging adenoviral vectors for stable correction of genetic disorders. Curr Gene Ther 7:272–283
157. Peng Z (2005) Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. Hum Gene Ther 16:1016–1027
158. Marshall E (1999) Gene therapy death prompts review of adenovirus vector. Science 286:2244–2245
159. Grieger JC, Samulski RJ (2005) Adeno-associated virus as a gene therapy vector: vector development, production and clinical applications. Adv Biochem Eng Biotechnol 99:119–145
160. Wu Z, Asokan A, Samulski RJ (2006) Adeno-associated virus serotypes: vector toolkit for human gene therapy. Mol Ther 14:316–327
161. Samulski RJ, Zhu X, Xiao X et al (1991) Targeted integration of adeno-associated virus (AAV) into human chromosome 19. EMBO J 10:3941–3950
162. Young SM Jr, McCarty DM, Degtyareva N, Samulski RJ (2000) Roles of adeno-associated virus Rep protein and human chromosome 19 in site-specific recombination. J Virol 74:3953–3966
163. Howden SE, Voullaire L, Vadolas J (2007) The transient expression of mRNA coding for Rep protein from AAV facilitates targeted plasmid integration. J Gene Med 10:42–50
164. Coura Rdos S, Nardi NB (2007) The state of the art of adeno-associated virus-based vectors in gene therapy. Virol J 4:99
165. Merle U, Encke J, Tuma S, Volkmann M, Naldini L, Stremmel W (2006) Lentiviral gene transfer ameliorates disease progression in Long-Evans cinnamon rats: an animal model for Wilson disease. Scand J Gastroenterol 41:974–982
166. Ghosh A, Allamarvdasht M, Pan CJ et al (2006) Long-term correction of murine glycogen storage disease type Ia by recombinant adeno-associated virus-1-mediated gene transfer. Gene Ther 13:321–329
167. Yiu WH, Pan CJ, Allamarvdasht M, Kim SY, Chou JY (2007) Glucose-6-phosphate transporter gene therapy corrects metabolic and myeloid abnormalities in glycogen storage disease type Ib mice. Gene Ther 14:219–226
168. Kobayashi H, Carbonaro D, Pepper K et al (2005) Neonatal gene therapy of MPS I mice by intravenous injection of a lentiviral vector. Mol Ther 11:776–789
169. Di Natale P, Di Domenico C, Gargiulo N et al (2005) Treatment of the mouse model of mucopolysaccharidosis type IIIB with lentiviral-NAGLU vector. Biochem J 388:639–646

170. Ponder KP, Melniczek JR, Xu L et al (2002) Therapeutic neonatal hepatic gene therapy in mucopolysaccharidosis VII dogs. Proc Natl Acad Sci USA 99:13102–13107
171. Overturf K, Al Dhalimy M, Tanguay R et al (1996) Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinaemia type I. Nat Genet 12:266–273
172. Seppen J, van der Rijt R, Looije N, van Til NP, Lamers WH, Oude Elferink RP (2003) Long-term correction of bilirubin UDP glucuronyltransferase deficiency in rats by in utero lentiviral gene transfer. Mol Ther 8:593–599
173. Miao CH, Thompson AR, Loeb K, Ye X (2001) Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer in vivo. Mol Ther 3:947–957
174. Herzog RW, Yang EY, Couto LB et al (1999) Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. Nat Med 5:56–63
175. Waddington SN, Nivsarkar MS, Mistry AR et al (2004) Permanent phenotypic correction of hemophilia B in immunocompetent mice by prenatal gene therapy. Blood 104:2714–2721
176. Chen L, Woo SL (2005) Complete and persistent phenotypic correction of phenylketonuria in mice by site-specific genome integration of murine phenylalanine hydroxylase cDNA. Proc Natl Acad Sci USA 102:15581–15586
177. Raper SE, Wilson JM, Yudkoff M, Robinson MB, Ye X, Batshaw ML (1998) Developing adenoviral-mediated in vivo gene therapy for ornithine transcarbamylase deficiency. J Inherit Metab Dis 21(Suppl 1):119–137
178. Crooke RM, Graham MJ, Lemonidis KM, Whipple CP, Koo S, Perera RJ (2005) An apolipoprotein B antisense oligonucleotide lowers LDL cholesterol in hyperlipidemic mice without causing hepatic steatosis. J Lipid Res 46:872–884
179. Kim IH, Jozkowicz A, Piedra PA, Oka K, Chan L (2001) Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. Proc Natl Acad Sci USA 98:13282–13287
180. Oka K, Belalcazar LM, Dieker C et al (2007) Sustained phenotypic correction in a mouse model of hypoalphalipoproteinemia with a helper-dependent adenovirus vector. Gene Ther 14:191–202
181. Manno CS, Pierce GF, Arruda VR et al (2006) Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med 12:342–347
182. Mingozzi F, High KA (2007) Immune responses to AAV in clinical trials. Curr Gene Ther 7:316–324
183. Sabatino DE, Mackenzie TC, Peranteau W et al (2007) Persistent expression of hF.IX After tolerance induction by in utero or neonatal administration of AAV-1-F.IX in hemophilia B mice. Mol Ther 15:1677–1685
184. Tominaga K, Kuriyama S, Yoshiji H et al (2004) Repeated adenoviral administration into the biliary tract can induce repeated expression of the original gene construct in rat livers without immunosuppressive strategies. Gut 53:1167–1173
185. Ye P, Thompson AR, Sarkar R et al (2004) Naked DNA transfer of Factor VIII induced transgene-specific, species-independent immune response in hemophilia A mice. Mol Ther 10:117–126
186. Gao G, Lu Y, Calcedo R et al (2006) Biology of AAV serotype vectors in liver-directed gene transfer to nonhuman primates. Mol Ther 13:77–87
187. Overturf K, Al Dhalimy M, Ou CN et al (1997) Adenovirus-mediated gene therapy in a mouse model of hereditary tyrosinemia type I. Hum Gene Ther 8:513–521
188. Grompe M, Overturf K, al Dhalimy M, Finegold M (1998) Therapeutic trials in the murine model of hereditary tyrosinaemia type I: a progress report. J Inherit Metab Dis 21:518–531

Chapter 11
Clinical Studies of Cell Therapy for Liver Cirrhosis

James A. Thomas and Stuart J. Forbes

Abstract Liver cirrhosis is a major cause of mortality worldwide. The current curative treatment for end-stage liver cirrhosis is liver transplant but this is severely limited by organ availability. There is therefore a pressing need to develop alternative strategies for the treatment of liver cirrhosis. Autologous cell therapies for liver cirrhosis have been tested in animal models of liver injury and in clinical trials. Cells tested in clinical studies include haematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and mononuclear cell preparations. Overall, early results suggest that there may be a positive benefit from some of the cell therapies. However, there are risks associated with injecting cells into the blood supply to the liver. Randomised controlled studies are required to prove efficacy of any therapy and such studies are awaited.

Abbreviations

BM	Bone marrow
G-CSF	Granulocyte-colony stimulating factor
HSCs	Haematopoietic stem cells
MSCs	Mesenchymal stem cells
MELD	Model for end-stage liver disease
MNCs	Mononuclear cells
PCNA	Proliferating cell nuclear antigen

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11.1 Introduction

Cirrhosis is the most prevalent form of severe liver disease worldwide. End-stage liver disease with its accompanying complications is the most frequent indication for transplantation. The characteristic disturbed fibrotic architecture with a reduced number of compromised hepatic cells is not an environment conducive to naïve hepatocyte/hepatocyte-like cell engraftment and function. Studies attempting to provide parenchymal support in this manner have been generally unsuccessful. Critically, such an approach does not address the considerable clinical problems of portal hypertension and hepatocellular carcinoma development.

BM stem cells play important roles in organ repair [1] and early data suggested that BM cells could directly contribute to hepatocyte or hepatic progenitor cell populations. This encouraged small Phase 1 human studies of BM cell therapy in cirrhosis. It is now recognised that BM cells do not transdifferentiate into these cell populations in a physiologically relevant manner in chronic liver disease [2, 3]. Nonetheless, the BM does supply non-parenchymal cell types that have important roles in the generation [4] and resolution [5] of fibrosis and support the liver's intrinsic regenerative compartments [6].

Experimental models of chronic liver disease have indicated that a more productive therapeutic strategy might be to modulate the underlying processes of inflammation, fibrosis and regeneration. Even advanced liver scarring has a reversible component [7], therefore realistic goals of cell therapy include causing sufficient regression to allow endogenous regenerative pathways to restore parenchymal mass or independent activation of these pathways. Animal models have demonstrated the positive effects of specific BM fractions [8], endothelial progenitor cells [9] and differentiated macrophages [10] in reducing the burden of fibrotic tissue and improving regeneration in the injured liver. At least, part of the mechanism seems to involve the upregulation of scar degrading matrix metalloproteinase enzymes and paracrine stimulation of regeneration. A better understanding of the appropriate therapeutic cell types and their actions obtained by such preclinical studies will translate into robust clinical trials to test their efficacy in cirrhotic patients.

11.2 Cell Types Used in Human Studies

Clinical studies have largely focused on the therapeutic potential of BM stem cells. This may have originally arisen from the incorrect hypothesis that these cells could treat cirrhosis by directly supplying hepatocytes. Nonetheless, the body of data is suggestive of clinical potential indicating the possibility of alternative mechanisms of action. The two best characterised stem cells within the BM are haematopoietic and MSCs.

HSCs are multipotent stem cells constituting a small subpopulation of the BM. These cells can give rise to all the major blood cell lineages. BM fractions can be enriched for HSCs using cell surface markers such as CD34 and CD133. The subset of CD34+ cells that co-express CD133+ are considered to be more enriched for stem cells [11]. More recently, CD34−/CD133+ cells have been identified as having the capacity to form endothelial progenitor cells [12].

MSCs are a heterogenous population of plastic adherent BM stromal cells characterised by multipotentiality (including along osteocytic, chondrocytic and adipocytic lineages) and a number of positive and negative surface markers [13]. This multipotentiality and relative ease of accessibility in addition to their immunomodulatory properties [14] has made MSCs an attractive candidate for cell therapy. Studies using MSCs have given conflicting results [15–17]. Some studies have shown a reduction in liver fibrosis following MSC delivery whereas other groups have not been able to reproduce these results. Furthermore, there is the risk of the MSCs themselves differentiating into collagen producing myofibroblasts [4]. These studies suggest that MSCs, at least given the current limitations in characterisation and purification, may not be ideal cell populations to treat cirrhosis. This highlights the key principle that the long-term phenotype of donor cells must be well characterised as favourable and stable.

The cost and complexity of cell sorting is a potential barrier to clinical translation. One solution to this problem is the use of less purified cell fractions. MNCs are separated from BM by density gradient centrifugation. CD34+ cells constitute only 1–2 % of this population [18] and the proportion of true stem cells within this fraction is even lower. However, it is conceivable that an "active" component of the therapy may reside in the non-stem cell fraction or be the result of interactions between donor cell types.

Autologous cells for infusion are initially harvested by BM aspiration or leukapheresis. Granulocyte colony-stimulating factor (G-CSF) is routinely used in clinical haematological practice to improve HSC yields from peripheral blood. Studies in which HSCs were obtained from peripheral blood used G-CSF priming to increase stem cell numbers. Rodent data support roles for G-CSF in mobilising BM stem cells and also directly enhancing liver regeneration [19]. To date, G-CSF alone does not seem to cause a consistent improvement in liver function in cirrhotic patients [20].

11.3 Summary of Clinical Trials

11.3.1 Haematopoietic Stem Cells

A phase 1 clinical study of CD34+ cells administration to 5 cirrhotic patients was conducted by Gordon and colleagues [21]. The aetiologies included alcohol, PSC and HBV and HCV. CD34+ cells were mobilised to peripheral blood by G-CSF

administration and subsequently isolated from the mononuclear cell fraction by magnetic cell sorting. These cells were manipulated by plastic adherence and cultured in media containing a number of cytokine (including stem cell factor, GM-SCF, IL-3, and G-CSF. $1 \times 10^6 - 2 \times 10^8$ cells were reinfused via the hepatic artery ($n = 2$) or portal vein ($n = 3$). There was no clear change in liver biochemistry or clinical parameters over the initial 2 month period or on longer term follow up of 12–18 months [22]. The same group also published a report of nine patients with alcoholic cirrhosis who had been abstinent for a minimum of 6 months [23]. CD34+ cells were isolated following G-CSF administration and cultured in similar conditions. A mean dose of 2.3×10^8 cells was injected into the hepatic artery. There were no detected complications whilst liver biochemistry and Child-Pugh score seemed to improve over a 3 month period.

The intraportal delivery of CD133+ BM cells has been examined in the context of hepatic resection in non-cirrhotic patients by Furst et al. [24]. Thirteen patients underwent portal vein embolization to stimulate regeneration prior to extended right hepatectomy for large hepatic malignancy (primary and secondary). Six of these patients additionally received $2.4 - 12.3 \times 10^6$ CD133+ cells that had been positively selected from BM aspirate. This resulted in significantly greater gains of hepatic volume allowing earlier tumour resection. One of the treated patients had liver fibrosis due to HCV infection. In addition to non-cirrhotic liver surgery, it is possible that these pro-regenerative effects could also occur in the pathophysiologically different context of the cirrhosis.

In a study from the Royan Institute, Iran, four patients with decompensated cirrhosis were given $2.5 - 8 \times 10^6$ BM derived CD34+ cells via the hepatic artery in a radiologically guided procedure [25]. One of these patients (autoimmune aetiology) developed progressive renal failure and died of liver failure prompting premature termination of the trial. The adverse event was linked to radiocontrast nephropathy in a patient with advanced liver disease and pre-existing renal impairment. This highlights the critical nature of issues such as patient selection and route of cell administration.

Salama et al. [26] studied the effects of partially differentiated CD34+ cells in 48 cirrhotic patients (36 HCV, 12 autoimmune aetiology). Participants underwent G-CSF mobilisation and leukapheresis. CD34+ cells were isolated, cultured and amplified for a further 7 days in media containing GM-CSF and growth factors derived from buffalo rat liver extract. The culturing process was intended to begin hepatocytic differentiation. RT-PCR demonstrated that these cells were beginning to express the genes for albumin and subsequently α1-antitrypsin during this protocol. 1×10^9 cells were infused via the portal vein (or hepatic artery if hepatofugal flow detected). Three serious complications were noted: haemoperitoneum post-procedure (stabilised with blood transfusion) and two GI bleeds 1 week after infusion (1 controlled endoscopically, 1 fatal). LFTs improved in both the HCV and autoimmune aetiology patients in conjunction with clinical assessment of ascites.

The same authors also conducted a randomised controlled trial of CD34/133+ cells in HCV cirrhosis [27]. Ninety patients received 5 days of G-CSF treatment

followed by BM aspiration and selection of CD34/CD133+ cells. 5×10^7 cells were reinfused via the portal vein. Fifty control subjects received control water injections in lieu of subcutaneous G-CSF. Cell recipients had improved serum albumin levels, transaminases, prothrombin concentration, Childs-Pugh score and clinical status which persisted to the end of the study at 6 months.

11.3.2 Mesenchymal Stem Cells

The first published study of MSC therapy for cirrhosis was from the University of Tehran [28]. Four patients with decompensated cirrhosis (3 cryptogenic, 1 autoimmune aetiology) received 3.1×10^7 BM derived, in vitro cultured MSCs via a peripheral vein. One year later, the subjects had improved quality of life questionnaire scores and three of the four patients had a net reduction in MELD score.

Another study from the Royan Institute, Iran, used MSCs cultured from BM aspirate which then underwent 48 h of a hepatocytic differentiation protocol prior to injection. From cell procurement to delivery, this process took approximately 2 months. $3 - 5 \times 10(7)$ autologous cells were given to eight cirrhotic patients (4 HBV, 1 HCV, 1 alcohol, 2 cryptogenic) via the portal vein (or peripheral vein in two patients when intraportal was not possible). All patients had lower MELD scores 6 months after treatment (from mean 17.9 to 10.7) [29].

The most recent study in the field is also the largest: 52 patients with chronic HBV who consented to autologous MSC therapy were compared to 105 controls matched for age, sex, LFTs and MELD score [30] BM was aspirated and then cultured to generate MSCs which were infused via the hepatic artery. Serum albumin and bilirubin levels were improved within 2 weeks of infusion. By the 3rd week, PT and MELD scores had also improved. These benefits were not sustained after 36 weeks. There were no significant differences in HCC rate or survival.

11.3.3 Unsorted Mononuclear Cells

Terai and colleagues enrolled nine cirrhotic patients (3 HBV, 5 HCV, 1 cryptogenic aetiology) from the Yamaguchi University Hospital in Japan. $2.21 - 8.05 \times 10^9$ BM derived unsorted MNCs were given via a peripheral vein. 2.39 % of infused cells were CD45+/CD34+. Significant improvements in serum albumin and Child's-Pugh score were noted up to 6 months later [31]. Three of these patients also had pre- and post-treatment liver biopsies. These demonstrated an increase in immunohistochemical markers of regeneration (α-fetoprotein and PCNA) 4 weeks after therapy.

Striking improvements were detected in two patients with decompensated alcoholic cirrhosis who received 4 or 6.93×10^6 CD34+ cells/kg in a mononuclear cell fraction [32]. These cells were obtained by leukapheresis following G-

CSF mobilisation and returned via a peripheral route. The clinical and biochemical recovery was maximal 12 months into the 30 month follow-up period.

A randomised controlled trial of autologous MNC therapy for cirrhosis was performed at from the Hospital Sao Rafael, Salvador, Brazil [33]. Fifteen of 30 randomised patients with a variety of aetiologies (mostly HCV, alcohol and cryptogenic) on the liver transplant waiting list received $1.6 - 11.2 \times 10^8$ mononuclear BM cells via the hepatic artery. There were no serious adverse events in the first 3 months after treatment. Three patients in the treatment arm died from complications of cirrhosis at 6, 8 and 10 months compared to two in the control arm (plus another patient receiving a liver transplant due to progressive liver failure). MELD score increased in control patients whilst remaining stable in cell recipients; however, the difference between the groups did not reach statistical significance. Serum albumin levels did improve transiently following MNC infusion; the effect at 3 months was diminished by the 12 month time-point. Similarly serum bilirubin levels also trended towards a short-lived improvement following treatment.

Kim et al. studied 10 patients with HBV cirrhosis who were peripherally reinfused with 1×10^8 BM derived MNCs [34]. Maximal improvement in serum albumin and prothrombin time was at 6 months into the 12 month follow-up period. Childs-Pugh score (but not MELD) also reduced at 6 months along with improvements in subjective indicators of well being and quality of life. Interestingly, analysis of serial biopsies showed a marked increase in the number of liver progenitor cells and a trend towards increased proliferating hepatocytes at the 3 month time-point. This was diminished by 6 months. A 5 % increase in liver volume at MRI was measured at 6 months.

BM derived MNCs were used as an adjunct to Kasai procedure for paediatric patients with extrahepatic biliary atresia in a controlled study from New Delhi, India [35]. 4.4×10^7 MNC were injected into the hepatic artery and/or portal vein during surgery (if not accessible, superior mesenteric vein radicals or transhepatic routes were used). This resulted in improved serum bilirubin, alkaline phosphatase and transaminases in the post-operative period. The reduction in bilirubin was maintained during the following 12 months in conjunction with reduced episodes of cholangitis and improved survival.

Couto and colleagues conducted a Phase 1 study of BM derived MNCs labelled with ^{99}Tc-$SnCl_2$ [36]. Whole body scintigraphy following the delivery of cells via the hepatic artery demonstrated 41 and 32 % hepatic radiotracer retention at 3 and 24 h, respectively. There were transient improvements in serum albumin and bilirubin but not MELD score. Early complications included peri-procedural hepatic artery dissection and transient cardiomyopathy. Other adverse events included eosinophilic fasciitis and HCC development.

A recent study compared intraportally delivered BM derived MNCs against CD133+ cells in six patients with end-stage liver disease [37]. There were no significant differences in measured liver function parameters or adverse events detected. This raises the possibility of potential equivalence of the much simpler procedure of delivering MNCs in comparison to HSC enriched populations—obviously larger randomised controlled trials would be required to formally test this.

11.4 Conclusions from Human Studies and Future Directions

The early small, uncontrolled studies of autologous BM cell therapy for liver diseases have shown that this is both technically feasible and safe—when carried out in carefully chosen patient groups and delivered in a controlled manner. Data from these studies has been encouraging; however, it is not possible to draw firm conclusions in the absence of control participants. Results from controlled trials have provided stronger evidence of benefit paving the way for randomised controlled trials to test the efficacy of cell therapy.

The natural history of chronic liver disease usually involves progression along an overall downward trend. In situations when the injurious stimulus can be lifted, most notably in alcoholic cirrhosis, liver function can dramatically improve. This highlights the importance of considering disease aetiology and patient specific circumstances in trial design. It is critical that such entry criteria, for example regarding abstinence from alcohol or anti-viral therapy, are standardised to limit variability between study arms. To this end, recent trials (e.g. Salama et al. [27]; Peng et al. [30]) have used single disease aetiology in their studies. This allows powerful and specific interrogation of the effects of the candidate cell type in a given context. An alternative approach is for large trials to prospectively subgroup participants by aetiology to allow differential effects to be detected.

Disease stage and stability is a key consideration in terms of the probability of demonstrating therapeutic benefit and ultimately targeting treatment to patient groups. Decompensated cirrhosis inherently involves episodes of illness at unpredictable intervals. In comparison to patients with less advanced disease, larger, well-powered trials would be needed to show a benefit in this setting. Other than liver transplantation, there are limited disease modifying treatment options for patients with decompensated disease. Therefore, though potentially more challenging, improving disease at this stage could for example, result in significantly improved quality of life with fewer hospital admissions and potentially obviate the need for transplantation.

HSC populations have been shown to be safe however there is still no mechanistic understanding of their potential actions. The most plausible scenario is paracrine stimulation of the injured liver activating endogenous repair pathways. The precise purity of the cells reflects a combination of practicality, cost and effectiveness. If MNC therapy proves to have similar or even greater benefit to concentrated stem cells, this could improve the ease of translation to the clinic. The notable caveat concerning MSC use is their potential to form deleterious cell types [4, 15]. The early direction of MSC differentiation (as used by Kharaziha et al. [29]) prior to delivery offers intriguing possibilities regarding manipulation of subsequent donor cell phenotypes. Such techniques could utilise beneficial, perhaps immunomodulatory, MSC phenotypes whilst restricting potentiality thereby avoiding negative effects. The overall effect would be determined by the interaction between transplanted cells and the recipient environment. The long term consequences of delivering such cells would need to be rigorously tested

before entering clinical use. As the cell therapy literature increases, it is conceivable that distinct donor cell specific effects could emerge. The choice of cell type may ultimately be tailored to a patient's aetiology or stage of disease.

The chosen route of administration needs to balance the advantages of concentrated, local cell delivery against the ease and safety of using peripheral access. Fatal complications relating to the invasive nature of injecting into the hepatic artery or portal vein [25, 26, 36] must prompt careful consideration. The tracking of donor cells using techniques such as whole body scintigraphy [36] provides important information regarding the distribution of cells in vivo. Such data will underpin mechanistic insights into cell localisation and longevity in turn informing the practicalities of cell administration. The transient improvements in liver function shown in several of the studies indicate that the repeated delivery of cells may be needed to exert clinically important benefits and substantially modify disease. This in turn will impact upon risk–benefit analyses of the choice of delivery route. The use of inconsistent cell numbers within individual studies hampers detection of effect by increasing variation. Beyond this, comparisons between studies are also hampered by potential dose effects. If specific cell types are shown to have benefit, then further studies will be needed to address this issue.

The most important outcome measures are quality of life and survival. These endpoints are in turn mediated by the rates of complications, HCC development and requirement for transplantation. In addition to these measures, reliable surrogates are needed to provide realistic and achievable goals for clinical trials.

The fluctuating nature of chronic liver disease means that narrow windows of data collection can miss clinically relevant trends. Therefore, serial data collection is needed to detect subtle changes. The most convenient are the routine blood tests (such as prothrombin time and serum albumin, LFTs and creatinine). The Child's-Pugh scoring system has well recognised limitations such as the subjective assessment of encephalopathy and ascites and the effects of clinical events on albumin and ascites levels. Advantages of the MELD score include its objectivity and clinical use for transplant prioritisation and as a correlate of mortality. Liver biopsy has a recognised morbidity and mortality but provides tissue for analysis of both mechanism and effect. The sampling error of liver biopsy necessitates its interpretation in a broader context including in conjunction with biomarkers such as panels of serum markers of liver fibrosis [38]. Non-invasive tests such as indocyanine green clearance and ^{31}P magnetic resonance spectroscopy are increasingly well recognised as representative biomarkers [39]. As the effects of different cell types become better understood, the combination of measures used will depend on the cell type in question, its anticipated effect and the relevant patient population.

In summary, the increasing understanding of the mechanisms of cell therapy emerging from animal work will inform the rational design of human trials. Clinical data concerning candidate cell types and their potential effects are accumulating. The next step is to perform robust randomised controlled trials to test the relevant parameters determining translation to clinical use.

References

1. Korbling M, Estrov Z (2003) Adult stem cells for tissue repair—a new therapeutic concept? N Engl J Med 349:570–582
2. Thorgeirsson SS, Grisham JW (2006) Hematopoietic cells as hepatocyte stem cells: a critical review of the evidence. Hepatology 43:2–8
3. Vig P, Russo FP, Edwards RJ, Tadrous PJ, Wright NA, Thomas HC, Alison MR, Forbes SJ (2006) The sources of parenchymal regeneration after chronic hepatocellular liver injury in mice. Hepatology 43:316–324
4. Russo FP, Alison MR, Bigger BW, Amofah E, Florou A, Amin F, Bou-Gharios G, Jeffery R, Iredale JP, Forbes SJ (2006) The bone marrow functionally contributes to liver fibrosis. Gastroenterology 130:1807–1821
5. Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, Wu S, Lang R, Iredale JP (2005) Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J Clin Invest 115:56–65
6. Tirnitz-Parker JE, Viebahn CS, Jakubowski A, Klopcic BR, Olynyk JK, Yeoh GC, Knight B (2010) Tumor necrosis factor-like weak inducer of apoptosis is a mitogen for liver progenitor cells. Hepatology 52:291–302
7. Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S, Hovell C, Arthur MJ (1998) Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. J Clin Invest 102:538–549
8. Sakaida I, Terai S, Yamamoto N, Aoyama K, Ishikawa T, Nishina H, Okita K (2004) Transplantation of bone marrow cells reduces CCl4-induced liver fibrosis in mice. Hepatology 40:1304–1311
9. Nakamura T, Torimura T, Sakamoto M, Hashimoto O, Taniguchi E, Inoue K, Sakata R, Kumashiro R, Murohara T, Ueno T, Sata M (2007) Significance and therapeutic potential of endothelial progenitor cell transplantation in a cirrhotic liver rat model. Gastroenterology 133:91–107 e1
10. Thomas JA, Pope C, Wojtacha D, Robson AJ, Gordon-Walker TT, Hartland S, Ramachandran P, Van Deemter M, Hume DA, Iredale JP, Forbes SJ (2011) Macrophage therapy for murine liver fibrosis recruits host effector cells improving fibrosis, regeneration, and function. Hepatology 53:2003–2015
11. de Wynter EA, Buck D, Hart C, Heywood R, Coutinho LH, Clayton A, Rafferty JA, Burt D, Guenechea G, Bueren JA, Gagen D, Fairbairn LJ, Lord BI, Testa NG (1998) CD34 + AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors. Stem Cells 16:387–396
12. Friedrich EB, Walenta K, Scharlau J, Nickenig G, Werner N (2006) CD34−/CD133+/VEGFR-2+ endothelial progenitor cell subpopulation with potent vasoregenerative capacities. Circ Res 98:e20–e25
13. Kolf CM, Cho E, Tuan RS (2007) Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthr Res Ther 9:204
14. Ryan JM, Barry F, Murphy JM, Mahon BP (2007) Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol 149:353–363
15. di Bonzo LV, Ferrero I, Cravanzola C, Mareschi K, Rustichell D, Novo E, Sanavio F, Cannito S, Zamara E, Bertero M, Davit A, Francica S, Novelli F, Colombatto S, Fagioli F, Parola M (2008) Human mesenchymal stem cells as a two-edged sword in hepatic regenerative medicine: engraftment and hepatocyte differentiation versus profibrogenic potential. Gut 57:223–231
16. Carvalho AB, Quintanilha LF, Dias JV, Paredes BD, Mannheimer EG, Carvalho FG, Asensi KD, Gutfilen B, Fonseca LM, Resende CM, Rezende GF, Takiya CM, de Carvalho AC, Goldenberg RC

(2008) Bone marrow multipotent mesenchymal stromal cells do not reduce fibrosis or improve function in a rat model of severe chronic liver injury. Stem Cells 26:1307–1314
17. Zhao DC, Lei JX, Chen R, Yu WH, Zhang XM, Li SN, Xiang P (2005) Bone marrow-derived mesenchymal stem cells protect against experimental liver fibrosis in rats. World J Gastroenterol 11:3431–3440
18. Steen R, Morkrid L, Tjonnfjord GE, Egeland T (1994) c-kit ligand combined with GM-CSF and/or IL-3 can expand CD34+ hematopoietic progenitor subsets for several weeks in vitro. Stem Cells 12:214–224
19. Piscaglia AC, Shupe TD, Oh SH, Gasbarrini A, Petersen BE (2007) Granulocyte-colony stimulating factor promotes liver repair and induces oval cell migration and proliferation in rats. Gastroenterology 133:619–631
20. Lorenzini S, Isidori A, Catani L, Gramenzi A, Talarico S, Bonifazi F, Giudice V, Conte R, Baccarani M, Bernardi M, Forbes SJ, Lemoli RM, Andreone P (2008) Stem cell mobilization and collection in patients with liver cirrhosis. Aliment Pharmacol Ther 27:932–939
21. Gordon MY, Levicar N, Pai M, Bachellier P, Dimarakis I, Al-Allaf F, M'Hamdi H, Thalji T, Welsh JP, Marley SB, Davies J, Dazzi F, Marelli-Berg F, Tait P, Playford R, Jiao L, Jensen S, Nicholls JP, Ayav A, Nohandani M, Farzaneh F, Gaken J, Dodge R, Alison M, Apperley JF, Lechler R, Habib NA (2006) Characterization and clinical application of human CD34+ stem/progenitor cell populations mobilized into the blood by granulocyte colony-stimulating factor. Stem Cells 24:1822–1830
22. Levicar N, Pai M, Habib NA, Tait P, Jiao LR, Marley SB, Davis J, Dazzi F, Smadja C, Jensen SL, Nicholls JP, Apperley JF, Gordon MY (2008) Long-term clinical results of autologous infusion of mobilized adult bone marrow derived CD34+ cells in patients with chronic liver disease. Cell Prolif 41(Suppl 1):115–125
23. Pai M, Zacharoulis D, Milicevic MN, Helmy S, Jiao LR, Levicar N, Tait P, Scott M, Marley SB, Jestice K, Glibetic M, Bansi D, Khan SA, Kyriakou D, Rountas C, Thillainayagam A, Nicholls JP, Jensen S, Apperley JF, Gordon MY, Habib NA (2008) Autologous infusion of expanded mobilized adult bone marrow-derived CD34+ cells into patients with alcoholic liver cirrhosis. Am J Gastroenterol 103:1952–1958
24. Furst G, Schulte am Esch J, Poll LW, Hosch SB, Fritz LB, Klein M, Godehardt E, Krieg A, Wecker B, Stoldt V, Stockschlader M, Eisenberger CF, Modder U, Knoefel WT (2007) Portal vein embolization and autologous CD133+ bone marrow stem cells for liver regeneration: initial experience. Radiology 243:171–179
25. Mohamadnejad M, Namiri M, Bagheri M, Hashemi SM, Ghanaati H, Zare Mehrjardi N, Kazemi Ashtiani S, Malekzadeh R, Baharvand H (2007) Phase 1 human trial of autologous bone marrow-hematopoietic stem cell transplantation in patients with decompensated cirrhosis. World J Gastroenterol 13:3359–3363
26. Salama H, Zekri AR, Zern M, Bahnassy A, Loutfy S, Shalaby S, Vigen C, Burke W, Mostafa M, Medhat E, Alfi O, Huttinger E (2010) Autologous hematopoietic stem cell transplantation in 48 patients with end-stage chronic liver diseases. Cell Transplant 19:1475–1486
27. Salama H, Zekri AR, Bahnassy AA, Medhat E, Halim HA, Ahmed OS, Mohamed G, Al Alim SA, Sherif GM (2010) Autologous CD34+ and CD133+ stem cells transplantation in patients with end stage liver disease. World J Gastroenterol 16:5297–5305
28. Mohamadnejad M, Alimoghaddam K, Mohyeddin-Bonab M, Bagheri M, Bashtar M, Ghanaati H, Baharvand H, Ghavamzadeh A, Malekzadeh R (2007) Phase 1 trial of autologous bone marrow mesenchymal stem cell transplantation in patients with decompensated liver cirrhosis. Arch Iran Med 10:459–466
29. Kharaziha P, Hellstrom PM, Noorinayer B, Farzaneh F, Aghajani K, Jafari F, Telkabadi M, Atashi A, Honardoost M, Zali MR, Soleimani M (2009) Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I–II clinical trial. Eur J Gastroenterol Hepatol 21:1199–1205
30 Peng L, Xie DY, Lin BL, Liu J, Zhu HP, Xie C, Zheng YB, Gao ZL (2011) Hepatology. Sep 2;54(3):820–828. doi: 10.1002/hep.24434. [Epub: 14 Jul, 2011]

31. Terai S, Ishikawa T, Omori K, Aoyama K, Marumoto Y, Urata Y, Yokoyama Y, Uchida K, Yamasaki T, Fujii Y, Okita K, Sakaida I (2006) Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. Stem Cells 24:2292–2298
32. Yannaki E, Anagnostopoulos A, Kapetanos D, Xagorari A, Iordanidis F, Batsis I, Kaloyannidis P, Athanasiou E, Dourvas G, Kitis G, Fassas A (2006) Lasting amelioration in the clinical course of decompensated alcoholic cirrhosis with boost infusions of mobilized peripheral blood stem cells. Exp Hematol 34:1583–1587
33. Lyra AC, Soares MB, da Silva LF, Braga EL, Oliveira SA, Fortes MF, Silva AG, Brustolim D, Genser B, Dos Santos RR, Lyra LG (2010) Infusion of autologous bone marrow mononuclear cells through hepatic artery results in a short-term improvement of liver function in patients with chronic liver disease: a pilot randomized controlled study. Eur J Gastroenterol Hepatol 22:33–42
34. Kim JK, Park YN, Kim JS, Park MS, Paik YH, Seok JY, Chung YE, Kim HO, Kim KS, Ahn SH, Kim do Y, Kim MJ, Lee KS, Chon CY, Kim SJ, Terai S, Sakaida I, Han KH (2010) Autologous bone marrow infusion activates the progenitor cell compartment in patients with advanced liver cirrhosis. Cell Transplant 19:1237–1246
35. Sharma S, Kumar L, Mohanty S, Kumar R, Datta Gupta S, Gupta DK (2011) Bone marrow mononuclear stem cell infusion improves biochemical parameters and scintigraphy in infants with biliary atresia. Pediatr Surg Int 27:81–89
36. Couto BG, Goldenberg RC, da Fonseca LM, Thomas J, Gutfilen B, Resende CM, Azevedo F, Mercante DR, Torres AL, Coelho HS, Maiolino A, Alves AL, Dias JV, Moreira MC, Sampaio AL, Sousa MA, Kasai-Brunswick TH, Souza SA, Campos-de-Carvalho AC, Rezende GF (2011) Bone marrow mononuclear cell therapy for patients with cirrhosis: a phase 1 study. Liver Int 31:391–400
37. Nikeghbalian S, Pournasr B, Aghdami N, Rasekhi A, Geramizadeh B, Hosseini Asl SM, Ramzi M, Kakaei F, Namiri M, Malekzadeh R, Vosough Dizaj A, Malek-Hosseini SA, Baharvand H (2011) Autologous transplantation of bone marrow-derived mononuclear and CD133(+) cells in patients with decompensated cirrhosis. Arch Iran Med 14:12–17
38. Rosenberg WM, Voelker M, Thiel R, Becka M, Burt A, Schuppan D, Hubscher S, Roskams T, Pinzani M, Arthur MJ (2004) European liver fibrosis G. Serum markers detect the presence of liver fibrosis: a cohort study. Gastroenterology 127:1704–1713
39. Lim AK, Patel N, Hamilton G, Hajnal JV, Goldin RD, Taylor-Robinson SD (2003) The relationship of in vivo 31P MR spectroscopy to histology in chronic hepatitis C. Hepatology 37:788–794

Chapter 12
Stem Cell Applications for the Treatment of Gastrointestinal System Diseases

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Abstract Gastrointestinal system is formed by organs with complex morphology and functions. Digestive tract is not a sterile medium and is exposed to external environment aggressions. As a result, its diseases are complex and encompass a broad spectrum of pathogenic mechanisms and their treatment is often challenging and frequently incurable or chronicity-tending processes are found. In this scenario, stem cells with their differentiation potential and encouraging properties could be helpful. Nowadays Stem Cells have yielded promising results in preclinical studies, some approaches have started clinical development and registered clinical trials are exponentially increasing every year. In 2011, the most advanced programs, involving Crohn's disease and fistulous disease, have reached phase III of development. Other tested diseases include

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ulcerative colitis, fecal incontinence or celiac disease. If the preliminary results are confirmed, we think that this therapy may become a clinical reality in the near future. In this chapter, we summarize Stem Cells potential benefits, the current experience and the future perspectives in gastrointestinal system diseases.

Abbreviations

SCs	Stem Cells
ESCs	Embryonic Stem Cells
ASs	Adult Stem cells
iPS	Induced Pluripotent Stem
BM-MSCs	Bone Marrow derived Mesenchymal Stem Cells
MAPCS	Multipotent Adult Progenitor Cells
ASCs	Adipose derived Stem Cells
HSCs	Hematopoietic Stem Cells
GISCs	GastroIntestinal Stem Cells
LPCs	Liver Progenitor Cells
EPCs	Endothelial Progenitor Cells
CBC	Cript Base Columnar cells
BMSC	Bone Marrow Stem Cells
CDAI	Crohn's Disease Activity Index
IBDQ	Inflammatory Bowel Disease Questionnaire
BM-MNCs	Bone Marrow Mononuclear Cells
SVF	Stromal Vascular Fraction from lipoaspirate
PDSCs	Placental Derived Stem Cells
U-MSCs	Umbilical derived Mesenchymal Stem Cells

In this chapter, we analyze the potential benefits, state-of-the-art and future perspectives regarding stem cell therapy for gastrointestinal system excluding liver diseases and diabetes, both treated in another chapters.

12.1 General Considerations about Gastrointestinal System

12.1.1 Anatomic Considerations

Gastrointestinal system comprises a tube or tract and solid contiguous viscera (hepatobiliary subsystem and exocrine pancreas). Each tract's segment constitutes a real organ with specific functions and some controlled sphincters separate and control transferring between them.

Histologically, the wall of hollow viscera is organized into specialized layers. *Mucosa* acts as a barrier and as a site for controlled transfer of fluids or nutrients.

Submucosa is a mucosa supporting layer. *Muscular* mediates mixing and propulsion. Many organs are covered by a *serosal* layer providing support.

A rich vascular, lymphatic (including parietal structures) and neuronal networks provide the incoming and outgoing communication for the homeostasis. Embryologically, the vast majority of the structures is of endodermal origin but there are also mesodermal contributions in muscular layers, nervous, lymphatics, and blood supply.

12.1.2 Gastrointestinal Tract Functions

There are two main functions: assimilation of nutrients and elimination of waste. All tract segments and solid contiguous viscera secretions are implicated in nutrients assimilation. Liver also filters and detoxifies substances absorbed in gut prior the passage into the bloodstream and detoxifies circulating toxins and metabolic waste elements eliminated by gut or in urine.

12.1.3 Other Considerations

Mucosal epithelial cells and hepatocytes show a hypersensitivity to some injuries and probably this explains their high renewal rate. In some tract segments, architecture is organized for this purpose in such way that differentiation and apoptosis occur in an ordered fashion along the crypt–villus axis.

12.2 Stem Cell Sourcing and Way of Application

These issues are analyzed in detail in another chapters so we are going to make only some comments.

Stem cells (SCs) can be obtained directly from different adult tissues as Adult Stem Cells (ASs, with variable differentiation potential), as pluripotent SCs derived from adult tissue samples (either by therapeutic cloning or through the creation of Induced Pluripotent cells —iPS), from embryos or from other minor sources (umbilical cord…).

They can be applied for therapy autologously (to avoid possible immunological rejection) or in an allogeneic way. For autologous application, cells could be obtained from adult directly as ASs, by therapeutic cloning or through iPS creation.

SCs can be applied directly (undifferentiated) or after prior differentiation according to therapeutic goals.

12.3 General Stem Cell Properties with Therapeutic Potential

The most important properties are their *proliferation and differentiation potential*. In the last years, some ASs have shown greater plasticity (differentiation potential) than previously thought, including cells from other germ layers (transdifferentiation) as mentioned in another book sections [1–4].

With regard to digestive tract diseases, initial studies suggested that ASs plasticity represented their principal mechanism of action. Nevertheless, more recent data suggest also that certain Bone Marrow derived SCs (BM-SCs) can also fuse (in a variable proportion) with intestinal epithelial cells [5, 6].

Other important potentially therapeutic functions are:

1. *Regulatory:* SCs can modify other cells function by molecular signal mediators or through cell-to-cell interactions. Regarding to digestive diseases, there is an evidence related to liver: SCs stimulate the reduced hepatocytes proliferation capacity through a paracrine way [7, 8].
2. *Immunomodulation:* for example Mesenchymal Stem Cells (MSCs) can inhibit certain immune-cell functions, may modulate dendritic cell activity [9] and have low immunogenicity due to expression of low levels of HLA-I, HLA-II and co-stimulatory molecules [10].
3. *Proangiogenic:* SCs participate in the formation of new vessels [11] either by direct differentiation or through mediator release.
4. *Homing to damaged tissues:* SCs have demonstrated the ability to migrate to damaged tissues by mediator signaling [12, 13].

12.4 Choosing "The Best" Stem Cell to Apply for Digestive Diseases

We are going to describe briefly the main properties of the different stem cell types potentially suitable for digestive tract disease:

12.4.1 Embryonic Stem Cells

May seem the best choice because they are really pluripotent and practically immortal. But there are some specific problems: tumors formation tendency (teratomas appear when applied in vivo–as with iPS-), immunologic barrier (except for therapeutic cloning), and ethical concerns.

12.4.2 Adult Stem Cells

- *SCs from bone marrow*: there are several ASs populations; the most used are hematopoietic stem cells (HSCs), bone-marrow-derived mesenchymal stem cells (BM-MSCs) and endothelial precursor cells (EPCs). Another interesting population is *Multipotent Adult Progenitor Cells* (MAPC) [14] which have the following attractive characteristics: high plasticity (pluripotent) maintained despite multiple culture passages and appear to be immune privileged allowing allogeneic use.
- *Adipose-derived mesenchymal stem cells* (*ASCs*) [15]: isolated from the stromal-vascular fraction of lipoaspirates. Advantages: easy to obtain (adipose tissue is abundant and easy to obtain) and higher yield of stem cell isolation than other tissues.

MSCs possess substantial differentiation and replication potential, anti-proliferative and anti-inflammatory activity and are immune privileged (safe for allogeneic use).

- *Gastrointestinal stem cells* (*GISCs*): GISCs are multipotent SCs housed in specific 'niches' within gastric glands [16] and intestinal crypts [17, 18] able to give rise to various gut cell types through committed precursor cells [19]. Bone marrow derived cells are thought to play an important role in GISC's niche (maybe by their differentiation into intestinal subepithelial myofibroblasts) as it has been seen in experiments with different sex bone marrow transplants [20]. These cells are capable to generate organoids that maintained the basic epithelial architecture of small bowel [21], pyloric epithelium [22] or colon [18].
- *Liver stem cells:* liver has cells called liver progenitor cells (LPCs), facultative liver stem cells or "oval cells" in rodents that can give rise to either hepatocytes or cholangiocytes. They will be commented more extensively in the corresponding chapter.

12.4.3 Induced Pluripotent Stem Cells

These cells were firstly described from murine fibroblasts in 2006 [23] and from human skin fibroblasts in 2007 [24] and are analyzed deeply in another chapter. iPS cells are similar to embryonic stem cells (ESCs) in proliferative and differentiation potentiality but avoid immunological barrier. Related to digestive disease, recently hepatocytes have been obtained from mouse iPS [25].

Considering all these sources, none of them would be clearly preferable for general use in digestive tract diseases given that SCs must rebuild the complex architecture for its organs and no type of SC has been shown to be superior for this "multilayer repopulation". Maybe the only real considerations for selecting the cell type are general ones like origin, tissue accessibility, morbidity of their obtaining, isolation simplicity, yielding (SCs obtained per tissue processed), or

Table 12.1 Comparison between the three main sources of SCs applied for human cell therapy

	HSCs	BM-MSCs	ASCs
Quantity of donor tissue	Abundant	Not very abundant	Very abundant
Accessibility	Not very accessible except by mobilization	Not very accessible (within the bone)	Very accessible (subcutaneous)
Extraction technique	Under local anesthesia including periosteum	Under local anesthesia including periosteum	Local or regional anesthesia
	Higher morbidity	Higher morbidity	Low morbidity
	Greater regional/general anesthesia need	Greater regional/general anesthesia need	
	Peripheral blood leukapheresis		
Mobilization required?	G-CSF infusion (with side effects) to obtain peripheral blood)	Not required	Not required
Conditioning required	Occasionally	Not required	Not required
Yield (SCs/g) of extracted tissue	Less effective	Less effective	More effective (10–100x yield)
Cell	characteristics	Seem to have lower potentiality for non-hematopoietic cells.	High potentiality, replicative capacity, etc.
Identical to BM-MSCs			

another less "scientific" ones as the application experience. Literature analysis highlights the use of bone marrow cells (HSC and BM-MSC) and adipose tissue, the former due to tradition of use and the latter because of the obtaining facilities. Table 12.1 compares these cell types.

12.5 Particular Characteristics of Digestive Diseases with Significance for Possible Stem Cell Applications

In the last decades, advancements in diseases knowledge, pharmaceutical research, surgical and anesthetic techniques and perioperative care have improved digestive disease outcomes. Although, many clinical challenges remain unresolved and SCs might play an important role. These situations are going to be classified, for didactic purposes, into the following categories although each gastrointestinal disease usually combines some of them.

12.5.1 Inflammatory Disease

The immunomodulatory capacity of some SCs might alter their course. Some possible applications could be:

- Fistulizing disease of any location, a chronic inflammation state which, despite complex surgery, recurs frequently and is associated with high morbidity.
- Drug-resistant chronic idiopathic inflammatory bowel disease (IBD), which shows limited response to surgery, with short-term complications and recurrences.
- Abdominal solid organ transplantations rejection amelioration as BM-MSCs injected have shown to reduce acute graft-versus-host disease [26].

12.5.2 Radiation Damage

Acute or chronic intestinal damage occurs in 10–20 % of patients receiving abdominopelvic radiotherapy. It often results in severe mucosal atrophy with enteric dysfunction or bleedings and a risk of mutations. Few therapeutic options are available and a lot of patients need surgery with poor outcomes and low quality of life.

12.5.3 Neoplastic Disease

Tumors remain a controversial topic in SCs therapy; fully conclusive studies to confirm whether SCs might be tumorigenic per se or could modify the course of neoplasms are lacking. Most articles rule out the tumorigenic potential of ASs reported for ESCs. Nevertheless, neoplastic disease, actual or historical, is usually considered an exclusion criterion in stem cell therapy clinical trials.

Actually, the unique realistic application in digestive neoplastic disease would be HSCs transplantation as a rescue from bone marrow ablation caused by aggressive chemotherapy [27, 28].

12.5.4 Ischemic Diseases

They are characterized by the lack of available specific treatments. The most beneficial potential effect would be promoting angiogenesis as it has been found in other ischemic conditions (limb [29], myocardium [30], etc.).

12.5.5 Functional Deficit

Cellular loss or degeneration characterizes functional deficits such as liver cirrhosis, achalasia, Hirschprung's disease, gastroesophageal reflux, and anal incontinence. The differentiation potential or the paracrine modulation of resident SCs niches [31, 32] would be the most useful properties.

12.5.6 Surgical Complications

The following may be amenable to SCs therapy:

Sepsis of abdominal origin: frequently the damage is perpetuated by immunological mechanisms even when the original source of infection is controlled. Immunomodulation properties could be useful.

Anastomotic dehiscences: many digestive surgical procedures (colonic, esophageal, etc.) are taxed with high morbidity and mortality due to anastomotic complications. Immunomodulatory and angiogenic properties might help reduce their incidence.

Peritoneal adherences: are the main cause of bowel obstruction and a frequent cause of infertility and pain. Pathological inflammatory mechanisms play a fundamental role in their origin. Immunomodulatory properties may be beneficial.

12.6 General Considerations about Stem Cell Application and Experience in Gastrointestinal System Disorders

Currently, SCs are considered as drugs and therefore, their application is subjected to strict regulations and in many countries to specific laws (especially for ESCs use). European regulatory agencies consider SCs as Advanced Therapy Medicinal Products (EU Directive no. 1394/2007) and USA agencies as Human Cellular and Tissue-based product. For these reasons, every new SC application should surpass preclinical studies, quality controls and manufacturing recommendations, and carefully designed clinical trials.

During stem cell therapy development, initial application ideas were frequently in desperate conditions so we have contemplated than after the preliminary animal results, SCs have been offered to single patients even before some important issues have been fully clarified. Consequently preclinical and clinical experimentation have run in parallel. Nevertheless we must not forget that SCs must surpass all experimental steps before conclusions are obtained in order to prevent creating society false expectations.

Related to *gastrointestinal system*, most studies to date (June 2011) have been done in animal models and several approaches have been tested in humans (only a few in controlled clinical trials). In the following sections, we will briefly describe and analyze critically some of the most relevant applications.

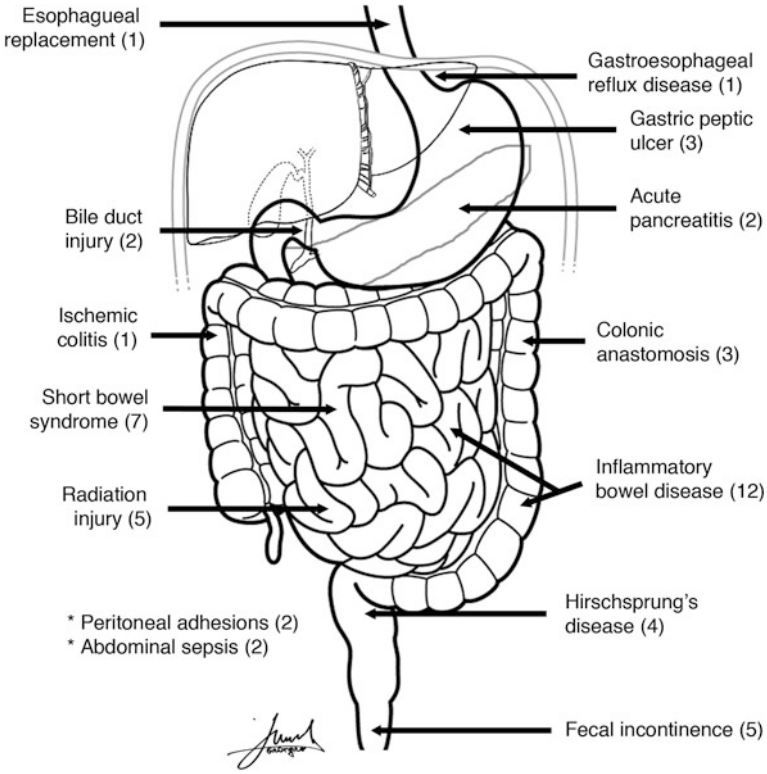

Fig. 12.1 Schematic view of gastrointestinal system diseases (excluded liver and endocrine pancreas) investigated in experimental animal models with stem cell therapy. Approximate numbers of published papers in relevant journals by June 2011 are indicated in *brackets*

12.7 Animal Studies Published on Stem Cell Application for Treatment of Gastrointestinal System Diseases

A schematic representation of the most noteworthy studies published is shown in Fig. 12.1.

12.7.1 Esophagus and Stomach

Esophageal replacement: tissue engineering with biomaterials composed of SCs seeded on biopolymers has been assessed, so far only in in vitro models [33].

Gastroesophageal reflux: studies using muscle and BM-MSCs [34] injected on gastroesophageal junction have showed the formation of muscle neofibers with good results. Nevertheless, recently published articles reveal that BM-SCs could

participate in Barrett's esophagus and adenocarcinoma pathogenesis [35] so clinical studies need to be conducted with caution in this field.

Gastric peptic ulcer: BM-MSCs have been injected directly observing faster healing. The proposed mechanism is proangiogenic factors secretion (VEGF and HGF) [36, 37].

Gastric ethanol-induced ulcers: in a study with bone marrow transplantation in rats prior to ulcers induction, bone marrow derived cells were involved in stomach regeneration [38].

12.7.2 Bile Ducts

One study, in a canine model, applied human amniotic membrane grafts to repair common bile duct lesions; they concluded that grafts alone (in lateral lesions) or supplemented by peritoneomuscular graft (circular lesions) are a promising tool (healing without stenosis) [39]. Lin et al. [40] proposed using autologous BMSCs derived cholangiocytes for postoperative biliary-fistula but this has not been realized yet.

12.7.3 Small Intestine

The most promising advances have been made in epithelial regeneration after radiation. Semont et al. [41] reported that infused human BM-MSCs could be engrafted into rat irradiated intestine and contribute to its regeneration. Okamoto et al. [42] reported that BM-derived cells could promote regeneration of damaged intestinal epithelia in human and that BM-derived epithelial cells significantly appear. BMSCs intestinal mucosa location rate ranges from 0.17 to 2.7 % [41, 43]. Zhang et al. [44] found that MSCs modified by CXCR4 had significantly higher engraftment rate and better results.

Full thickness regeneration has been the goal of several studies. Very interesting results have been obtained using organoids (small samples of intestinal tissue containing GISCs [45–47]), ESCs (three-dimensional intestinal tissue has been recently produced from human ESCs [48]) and ASs. With ASs, Hori et al. [49] achieved regeneration lacking muscle layer by MSC seeding on collagen sponge graft in dogs; Nakase et al. [50] seeded that graft with smooth muscle cells and others got regeneration and implantation from MSCs-enriched collagen-agarose 3D patch on a human or swine aortic fragment [51]. We think that further studies are needed before application in clinical setting.

12.7.4 Large Intestine

Enteric nervous progenitors in embryonic and adult gut was previously described [52]; some researchers thought that could be used in *Hirschsprungs' disease* even in autologous way because disease is segmentary. In one publication, neurospheres

are obtained from human and mouse enteric nervous progenitors and applied to mouse aganglionic bowel locating in aganglionic area, differentiating and restoring normal contraction rate [53].

SCs have been also studied in *ischemic colitis*. In an animal model produced by partial intestinal obstruction and ligation of marginal vessels, BM-MNCs were directly injected on ischemic colonic wall; macroscopic and microscopic colitis severity scores and whole-gut transit time decreased and capillary and myenteric plexus density increased, both significantly compared with controls. Transplanted cells were detected in all intestinal layers but authors could not demonstrate SCs differentiation into vascular or neural cells [54].

Regarding *fecal incontinence* , there are studies on the application of BM-MSCs for surgical damage and repair in rats [55], on muscle SCs for cryolesion in rats [56], on myoblasts transplanted to mice after surgical damage [57] and with human umbilical cord matrix and BM-MSCs from rabbit in rabbits [58]. A recently published paper on a rabbit model of external sphincter surgical damage studied delayed autologous muscle progenitor cells injection without surgical repair. Authors found SCs in the sphincter, regenerated myotubes and a significant decrease in interstitial fibrosis and higher Ki-67-positive cells in SCs group (without cd-34 cells infiltration); manometry and electromyography showed also a statistically significant improvement 4 weeks after cell injection [59]. Our group is working with animal models of this condition with ASCs.

12.7.5 Inflammatory Bowel Disease

12.7.5.1 Crohn's Disease

Several studies have been performed. We highlight three murine models: one uses murine and human ASCs [60], another applies BM-HSCs topically [61] and the third compares HSCs and MSCs [62]. Generally, an improvement has been reported. Studies have focussed on the regenerative and trophic role (providing VEGF and TGF-β1-) [61, 63], and immunoregulation [64] as therapeutic mechanisms. Two good reviews on this topic are provided by Dryden et al. [65] and by Lanzoni et al. [66]. More studies are needed to confirm the results.

12.7.5.2 Ulcerative Colitis

This condition has been tested later than Crohn's provided better treatments results and that experimental models usually reproduce better Crohn's (transmural) than Ulcerative Colitis (mucosal). Recent studies have suggested microvascular dysfunction and endothelial barrier defect or impaired contribution of BM-EPCs (by decreased release and/or impaired homing to colon) as critical pathogenic mechanisms. All are ideas for SCs application [67].

12.7.6 Pancreas

Available treatments for acute pancreatitis are not very effective. In murine models, intravenous human BM-MSCs have shown to reduce acinar-cell degeneration, pancreatic edema, inflammatory cell infiltration and cytokines expression. BM-MSCs reduced CD3+ T cells and increased regulatory T cells [68]. A good analysis on this issue is provided by Schneider et al. [69].

12.7.7 Surgical Complications

12.7.7.1 Anastomotic Dehiscences

In the Stem Cell Therapy Laboratory of "La Paz" Hospital, we have studied whether ASCs might help in reducing colorectal surgery dehiscences. We "wrapped" surgical sutures in ASCs so that, on passing suture through the tissue, ASCs were deposited at the critical point. These are denoted as "biosutures" or "stem-cell-coated sutures". It was observed that the sutures became coated within 24 h culturing and that they could be used in the same way as conventional ones. The animal model used was colic anastomosis with simple stitches in rats. There were no differences between biosutures vs conventional sutures in the incidence of dehiscence, dilation, obstruction, histological inflammatory pattern and rupture pressure; the frequency of adherences was significantly lower in the biosuture group at 4 and 7 days but not later, while anastomotic resistance was maintained [70].

Going further, we studied a model of adhesion-free colonic anastomosis employing intraperitoneal 4 % icodextrin. Biosutures improved significantly the strength of anastomoses at fourth postoperative day [71].

A Turkish group described an animal model of ischemic colic anastomosis (they ligated mesocolic vessels from 2 cm adjacent to cutting ends and made end-to-end anastomosis with interrupted inverted stitches). Authors studied rats at fourth and seventh postoperative day applying allogenic BM-MSCs injected on anastomotic area. They reported that BM-MSCs use significantly increased anastomosis bursting pressure and hydroxyproline levels and significantly accelerated healing parameters (epithelialization, collagen deposition, fibroblastic activity and angiogenesis) except for inflammation and necrosis on the fourth day. On the seventh day, only modified significantly fibroblastic activity, collagen deposition and necrosis [72].

12.7.7.2 Sepsis of Abdominal Origin

In a pioneer study, BM-MSCs were applied intravenously in an animal model of abdominal sepsis before and just after its induction [73]. A decrease in mortality of up to 50 % and an improvement in organic functions were seen in BM-MSCs group. Similar results were achieved with ASCs [74].

12.7.7.3 Peritoneal Adherences

Several studies with animal models have been undertaken to assess whether SCs can be used to prevent their formation [75, 76]. Our group is also working in this research line.

12.8 Ongoing and Published Studies with Human Application of Stem Cells for Gastrointestinal Diseases

Crohn's disease, fistulising disease, ulcerative colitis and fecal incontinence are the main diseases tested so far. In the last years, the number of registered clinical trials with SCs is increasing continuously (more than 500 new the last year) and digestive diseases (included liver) are among the main application fields.

If we review the main official clinical trials registry, provided by the US National Institutes of Health (NIH) in the www.clinicaltrials.gov Web page, we can find by June 2011 3,563 trials with SCs, of which 26 are applied to gastrointestinal tract diseases, excluded liver and endocrine pancreas. We summarize them in Table 12.2. We also represent them schematically in Fig. 12.2. In www.clinicaltrials.gov, every trial is coded as "NCT" followed by a number (for example NCT0027853).

12.8.1 Crohn's Disease

The presumed *therapeutic mechanisms* in the clinical setting are the capacity to reset the native immune system (HSCs) and the immunomodulatory properties (MSCs). Native immune system replacement is obtained because the preconditioning treatments eliminate T cells and naive cells could be generated by HSCs derived hematopoiesis and if HSCs are allogeneic, they also produce a graft-versus-autoreactive response [66].

12.8.1.1 Relevant Published Studies

Preliminary support came from *retrospective analyses of hematological transplantations*. We are going to highlight two case series. In the first, Crohn's remission was observed in four out of five patients (without immunosuppressants at least 6 months) with leukemia after allogeneic bone marrow transplantation [77] with a median follow-up of 4.5–15.3 years. In the second [78], symptoms remission occurred in all patients (six with Crohn's and four with ulcerative colitis) after myeloablative treatment and allogeneic transplantation; the follow-up

Table 12.2 Summary of clinical trials related to gastrointestinal system (excluded liver and endocrine pancreas) listed on www.clinicaltrials.gov in June 2011

Disease	Type of cells	Way of use	Phase I	Phase I/phase II	Phase II	Phase III	Phase IV	Subtotal	Total
Crohn's disease	HSCs	Autologous	1	1	2	1	0	5	12
		Allogeneic	0	1	0	0	0	1	
	BM-MSCs	Autologous	0	0	0	0	0	0	
		Allogeneic	0	0	2	3	0	5	
	ASCs	Autologous	0	0	0	0	0	0	
		Allogeneic	0	0	0	0	0	0	
	PDSCs	Autologous	0	0	0	0	0	0	
		Allogeneic	0	0	1	0	0	1	
Fistulizing disease	HSCs	Autologous	0	0	0	0	0	0	11
		Allogeneic	0	0	0	0	0	0	
	BM-MSCs	Autologous	0	0	0	0	0	0	
		Allogeneic	0	1	0	1*	0	1 ± 1	
	ASCs	Autologous	1	1	4	2	0	8	
		Allogeneic	0	2	0	0	0	2	
Ulcerative colitis	HSCs	Autologous	0	0	0	0	0	0	2
		Allogeneic	0	0	0	0	0	0	
	BM-MSCs	Autologous	0	0	0	0	0	0	
		Allogeneic	0	0	0	0	0	0	
	U-MSCs	Autologous	0	0	0	0	0	0	
		Allogeneic	0	1	0	0	0	1	
	MAPCs	Autologous	0	0	0	0	0	0	
		Allogeneic	0	0	1	0	0	1	
Fecal incontinence	HSCs	Autologous	0	0	0	0	0	0	1
		Allogeneic	0	0	0	0	0	0	
	BM-MSCs	Autologous	0	0	0	0	0	0	
		Allogeneic	0	0	0	0	0	0	
	ASCs	Autologous	1	0	0	0	0	1	
		Allogeneic	0	0	0	0	0	0	
TOTAL			3	7	10	6	0		26

The disease, type of stem cells used, way of use (autologous or allogeneic) and phase of development are indicated. The symbol * represents the Crohńs phase III trial which has among secondary outcomes draining fistula reduction (explained in the text) so it is contemplated twice.

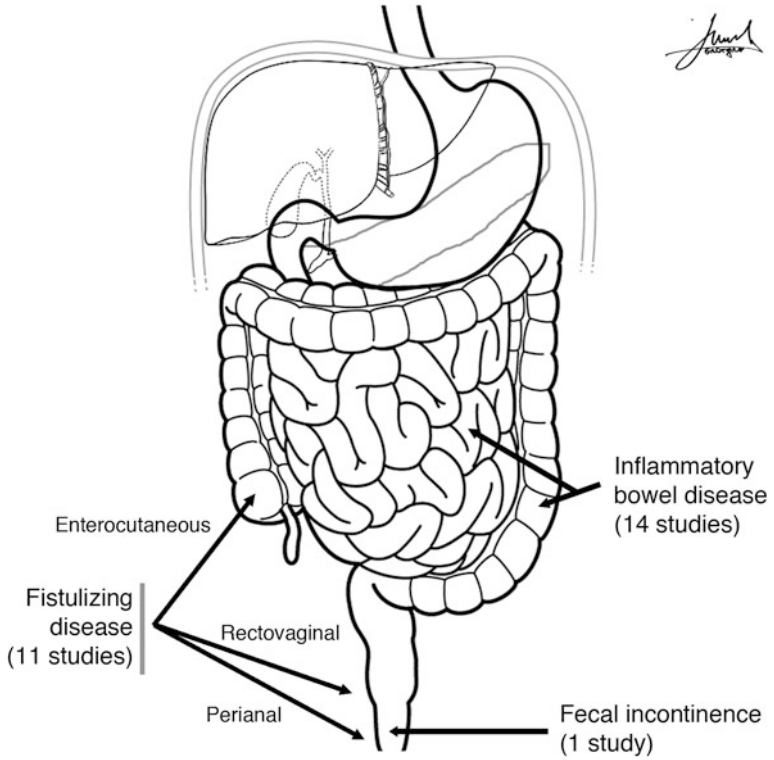

Fig. 12.2 Schematic view of gastrointestinal system diseases (excluded liver and endocrine pancreas) under investigation in ongoing stem cell therapy clinical trials registered at the principal official clinical trials registry, provided by the US NIH in the www.clinicaltrials.gov web page. Number of registered trials (cut-off June 2011) for each topic is indicated in brackets (Inflammatory Bowel Disease includes Crohn's Disease and Ulcerative Colitis). For more detailed information see Table 12.2

was 3–117 months, one had a moderate self-limiting recurrence and another died of infectious complications; all patients except two maintained immunosuppressive therapy.

Based on their results, clinical trials with *HSCs*as the primary treatment begin. Of note, the phase I study by Oyama et al. [79] in which autologous peripheral CD34+ HSCs were obtained, expanded and infused (with prior conditioning treatment) in 12 patients with refractory Crohn's. After 6 months, 11 had remission (Crohn's Disease Activity Index [CDAI] < 150) and after 18.5 months only one had experienced recurrence. Another trial successfully used peripheral HSCs unselected for CD34 in four patients (mean follow-up, 16.5 months) [80]. Oyama's group published their phase I/II long-term results (NCT00278577) for patients with CDAI greater than 250 and/or Crohn Severity Index greater than 16 despite antitumor necrosis factor therapy. The mean CD34+ cells infused was 6.35×10^6 cells/kg and 18 of 24 patients had at least 5 years of follow-up.

All patients reached remission (CDAI < 150) and it was sustained in more than 70 % during 5 years with 80 % patients steroid-free and 60 % medication free (this is the most effective treatment for this condition to the moment); no mortality related to treatment was reported [81].

Later, the interest in using *MSCs* grew. Nowadays, most MSCs trials are ongoing although some have started to report results. For example, one phase I applied expanded autologous BM-MSCs intravenously. Authors compared autologous Chron's BM-MSCs with healthy donor's BM-MSCs finding no differences. They selected ten refractory patients with a baseline median CDAI of 326 (224–378) and 9 completed the study. The protocol was feasible and safe, CDAI improved in five, clinical response (CDAI drop > 70) was seen in three at sixth week but remission (CDAI < 150) was not achieved in anyone and three required surgery due to worsening. Endoscopic improvement was found in two out of seven patients; lower CD4+ T cells account and higher CD4 + CD127+ regulatory T cells were observed in biopsies, and cytokine levels decreased in mucosa whereas increase in the serum [82]. Onken et al. carried out a phase II trial with expanded allogeneic MSCs (Prochymal™ [83], NCT00294112); final results have not yet been published but have been communicated in conferences. Ten patients with moderate–severe refractory Crohn's (CDAI > 220) received intravenous Prochymal (4–16 million cells per kilogram). A statistically significant decrease in mean CDAI was observed after 28 days (from 341 to 236) with a mean decrease of 62 in 7 days; all patients improved CDAI and clinical remission (IBDQ < 170) was achieved in three [84] without any noteworthy adverse events. In a subsequent 12-month follow-up study (fulfilled by eight patients), seven serious adverse events were found in four; they were classified as "unlikely causal relationship with the MSC procedure" and no tumors or ectopic tissues in CT-scan were found [85]. A Russian group published a study with 39 patients with ulcerative colitis and 11 with Crohn's compared with 30 and 10 controls, respectively. They applied allogeneic selected and expanded BM-MSCs intravenously after immunodepressant discontinuation and corticosteroids and aminosalicylate reduction. The follow-up was 4–8 months. Clinicomorphological remission in 31/39 ulcerative colitis and 9/11 Crohn's and a statistically significant improvement compared with controls were found. Corticosteroids could be discontinued in 34/50 patients and reduced to 5 mg/day in another seven [86].

12.8.1.2 Registered Clinical Trials

In June 2011, there were 20. As eight of them are related to fistulising disease (explained later), we are going to analyze 12 highlighting the most advanced ones. As it can be observed in Table 12.2, the vast majority are phase II (5) or III (4) and there are no phase IV trials. The SCs types mostly applied are HSCs (6) and MSCs (5) and the administration type are nearly equal (seven allogeneic and five autologous). Only one trial has published results (see Table 12.3).

Table 12.3 Summary of clinical trials related to gastrointestinal system (excluded liver and endocrine pancreas) listed on www.clinicaltrials.gov in June 2011

Disease	Recruitment status	Phase I	Phase I/ phase II	Phase II	Phase III	Phase IV	Subtotal	Total
Crohn's disease	Under recruitment	1	1	2	3	0	7	12
	Active but not recruitment	0	0	1	0	0	1	
	Completed, not published	0	0	1	1	0	2	
	Completed, published	0	1	0	0	0	1	
	Withdrawn	0	0	1	0	0	1	
Fistulizing disease	Under recruitment	0	3	3	1*	0	6/7*	11
	Active but not recruitment	0	0	0	0	0	0	
	Completed, not published	1	1	0	2	0	4	
	Completed, published	0	0	1	0	0	1	
	Withdrawn	0	0	0	0	0	0	
Ulcerative colitis	Under recruitment	0	0	1	0	0	1	2
	Active but not recruitment	0	1	0	0	0	1	
	Completed, not published	0	0	0	0	0	0	
	Completed, published	0	0	0	0	0	0	
	Withdrawn	0	0	0	0	0	0	
Fecal incontinence	Under recruitment	0	0	0	0	0	0	1
	Active but not recruitment	0	0	0	0	0	0	
	Completed, not published	1	0	0	0	0	0	
	Completed, published	0	0	0	0	0	0	
	Withdrawn	0	0	0	0	0	0	
								26

The disease, recruitment status and phase of development are indicated. Recruitment status is continuously changing information so it must be taken only as a reference. The symbol * represents the Crohńs phase III trial which has among secondary outcomes draining fistula reduction (explained in the text) so it is contemplated twice.

Using *HSCs* there are five autologous trials and one allogeneic. The phase I trial (NCT00692939) studies safety and efficacy of autologous CD34+ peripheral blood HSCs in the long term (5 years) in patients with non-located refractory Crohn's. The allogeneic trial (NCT01288053) is a Phase I/II applying matched sibling

allogeneic HSCs (with donor T cells removed to prevent graft versus host disease) without previous myeloablation in patients with refractory to all available Crohn's medications or with a relapse after autologous HSCs. The primary goals are survival and duration of remission in 5 years follow-up. The other phase I/II trial (NCT00278577, published) has been commented before. The only really ongoing phase II (NCT00271947) is comparative and randomized (non-myeloablative autologous HSCs vs standard therapy) and applied to severe refractory Crohn's patients (CDAI 225–400); the follow-up is 1 year. In the phase III trial (NCT00297193), with a follow-up period of 1 year, autologous HSCs are first mobilized, then myeloablation is done and HSCs are supplied at 4 or 59 weeks to determine whether the effects are due to mobilization agents or to HSCs.

Regarding *BM-MSCs*, all trials employ allogeneic cells. One phase II (NCT00294112) was commented before (see published ones) and the other (NCT01090817) is a single group safety and efficacy study during 6 weeks for refractory (included biological) Crohn's with surgical treatment not recommended or refused by the patient. The three phase III trials are being conducted by Custer et al. from Osiris Therapeutics Inc with Prochymal™; the first two (NCT00482092 and NCT00543374) are double-blinded, placebo controlled and multicenter efficacy trials. The first one seeks to assess relapses, clinical and quality of life improvements or reduction in draining fistulas number in the short term (28 days) in patients with moderate to severe Crohn (CDAI 250–450) refractory to at least one steroid and at least one immunosuppressant and at least one biologic drug. Cells (600 or 1200 million) were applied intravenously. The second is applied to subjects with at least 100 points reduction in CDAI at 28 day; it studies response duration (follow-up 6 months) and contemplates reinfusion and the ability to re-induce benefits. Both were stopped in March 2009 because an interim analysis showed an excessive response in placebo group, but not any safety concern, as reported by Osiris in a press release and as it was commented in Nature Biotechnology [87]. In another press release (May 2010), Osiris announced that enrollment had resumed according to the prespecified design [88]. Following those events the company registered the last trial (NCT01233960) to provide retreatment and study deeply efficacy and security for patients from the first trial receiving the treatment recently. Three infusions of 200 million cells are contemplated and the follow-up is 180 days.

There is one Phase II trial with the first *placental-derived stem cell (PDSCs)* product (PDA001). The main benefits observed in the preclinical studies are: powerful immunosuppressive properties, safety and unlimited source. It is a multicenter, comparative (with placebo) and randomized safety and efficacy study for patients with CDAI 220–450 and refractory to an agent (not all as other trials) during a 6 weeks follow-up.

12.8.1.3 Critical Analysis

Most of the published studies show some improvement in clinical scores and provide safety data. There are some methodological concerns such as the small number of patients in each study (maybe explained because they are phase I or II

trials), the variation in cell products, the lack of a control group (unless for the Russian study, but it is striking that no data about patients complexity are provided) and the limited information provided about additional therapies. It is worthy to highlight that the definitive results of Prochymal™ phase II trial, with a big company support, have not yet been published despite the authors are reporting results since 2006.

In studies concerning MSCs it is also important to consider whether authors fulfill the three minimal criteria established to define them [10]. Ongoing trials will probably resolve some of these doubts and will try to resolve some other questions as whether allogeneic or autologous HSCs is better, clarify if the response is owed to SCs or conditioning treatments, etc. It is also striking that two allogeneic phase III studies had to be stopped more than a year due to methodological concerns. Authors commented that there were no safety concerns but right after resuming enrollment, another trial to study reinfusions (with dose augmentation) and with much more safety and security evaluation issues was registered, so it seems better to wait all trials' results.

Special consideration should be given to the unresolved issue of SCs carcinogenesis. There are some reports of MSCs malignant transformation after prolonged in vitro culture [89, 90], but some authors have now attributed transformation to cross-contamination with tumor cells [91, 92] and other studies do not find that phenomenon [93]. Furthermore, this issue has not been observed in vivo and another study relates SCs fusion with "pre-malignant" cells with carcinogenesis [94]. More studies are needed to clearly define this potential causal relationship. Recent studies have investigated ASCs behavior in the presence of tumor cells. Some have found migration toward the tumor and tumor growth acceleration [95, 96] but also neoplastic cells apoptosis [97, 98]. The real clinical significance of these findings is unknown. Based on these, some authors have explored the benefit from this migratory capacity using SCs as a vehicle for cytotoxic drugs [99].

In conclusion we think that more animal data on safety, type of SCs to use, best dosage and therapeutic mechanisms involved are urgently required. Ongoing trials results and other large randomized controlled trials are necessary before firm conclusions can be drawn and SCs must only be applied under the strictly regulated clinical trial setting.

12.8.2 Fistulizing Disease

The SC mostly applied in this condition is ASCs.

12.8.2.1 Relevant Published Studies

In 2002, the first autologous **ASCs** application was done attempting to seal a recurrent and refractory rectovaginal fistula in a Crohn's disease patient [100]. The

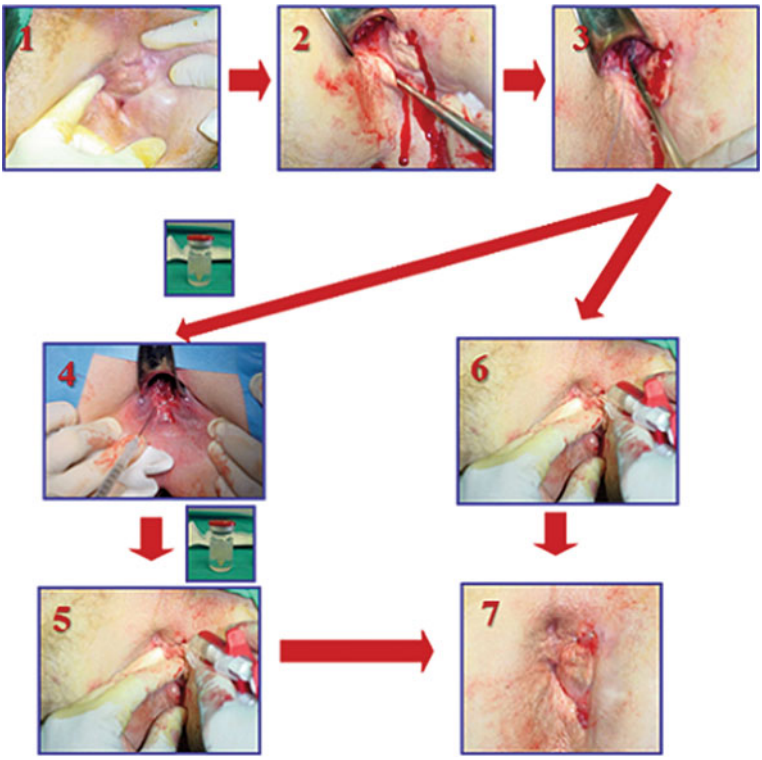

Fig. 12.3 Perianal complex fistula surgical protocol applied in the Phase II trial published by our group. *1* Tract and internal opening identification; *2* tract curettage; *3* internal opening closure with stitches (common to both groups). Treatment group: *4* ASCs injection (one half around internal opening and intersphincteric tract and another toward external opening) and *5* tract sealing with fibrin glue. Control group: *6* tract sealing with fibrin glue. *7* (common): perianal region post procedure

authors combined surgery (rectal opening closure and vaginal advancement flap) with intralesional ASCs achieving healing maintained nowadays.

"La Paz" University Hospital experience: A *phase I* study, prospective, single-center, uncontrolled and open-label applied ASCs for five Crohn's patients with eight refractory complex fistulas (enterocutaneous, rectovaginal or perianal) [101]. ASCs mixed with ringer lactate or fibrin glue (Tissucol® Duo; Baxter) were applied in internal openings or around and into fistula tract. After a follow-up of 12–30 months, treatment was successful in six fistulas and it was demonstrated that the procedure was **feasible and safe** (no adverse effects or tumors appeared) [102]. The *Phase II* study (NCT00115466) was an open-label, controlled (autologous ASCs vs fibrin glue), randomized and multicenter study with 50 patients with complex perianal fistulas (14 with and 35 without Crohn's disease). The procedure (see Fig. 12.3) included tract curettage, internal opening closure with stitches and tract sealing with fibrin glue (in the treatment group half of the ASCs was injected around internal opening and intersphincteric tract and the other half in tract wall

toward external opening prior to sealing). Fistula closure was observed in 17 out of 24 in ASC group (71, 46 % after the first and 25 % after the second dose) compared to 4 out of 25 (16, 8 and 8 %, respectively) in control group with the relative risk of cure being 4.43 (95 % CI 1.74–11.27; $p < 0.001$). These results were similar regardless of whether patients had Crohn or not. After 1 year, 17.6 % in ASCs group had experienced recurrence. No adverse events related to ASCs were observed. ASCs combined with fibrin glue were considered **effective and safe**and this procedure probably preserve better (or maybe improve) anal continence as the tract is not resected and sphincter is not damaged [103]. The results of five unscheduled biopsies (obtained by reasons not attributed to SCs therapy) from external openings have been reported. In the very early specimens, improved healing data are observed and no cellular changes suggesting carcinogenesis have been found for up to 2 years [104]. Another study compares intralesional stromal vascular fraction (SVF) cells with expanded ASCs in Crohn's enterocutaneous fistulas being this last option better in achieving cure (three out of four vs one out of four patients after 8 weeks) [105]. The first application of *allogeneic ASCs* has also been reported in a Crohn's rectovaginal fistula [106]. SVF with fibrin glue has been tested for a tracheo-mediastinic fistula; closure after 3 months, strong neovascularization (maintained at 1 year) and mediastinal cavity collapse without neoplastic cells on bronchoalveolar lavages or other treatment related adverse events were observed [107].

Other group experiences: a prospective study with autologous BM-MSCs in Chon's patients has been reported. 11 patients with actively draining complex perianal fistulas and one with multiple enterocutaneous fistulas were included, all unresponsive to or unsuitable for all previous medical treatment or unsuccessfully treated by surgery. Simultaneous biological therapy was not allowed. Fistula tracts were cleaned with saline solution and the solution with MSCs injected around and into the tract under local anesthesia several times (median: four) until clinical response. Sustained complete closure (inability to insert a cannula through external opening and no drainage despite finger compression) was found on seven and incomplete ($\geq$50 % reduction in draining fistulas number and/or no discharging upon finger compression) in three; a significant ($p < 0.01$) improvement in CDAI and PDAI was observed in all at 12 months with disease remission levels. These results were confirmed by MRI and endoscopic assessment. The percentage of mucosal and circulating regulatory T cells significantly increased during treatment and remained stable until follow-up ending ($p < 0.0001$ and $p < 0.01$, respectively). No adverse events were reported [108].

12.8.2.2 Registered Clinical Trials

In June 2011, there were 11 trials and the one commented in Crohn's section which had among secondary outcomes draining fistula reduction (Tables 12.2 and 12.3). The vast majority (9) is under phase I or II and there are no phase IV trials; the mostly used SC is largely ASCs (10 studies) and autologous application (8) is the most frequent. Two of them have published results.

Using **ASCs**, *our group* is participating in three *phase* III trials with perianal fistulas, two for patients without IBD (FATT I, NCT00475410 and the long term evaluation NCT01020825) and another one for Crohn's patients (FATT II, not registered). FATT I and FATT II are prospective, multicenter, randomized, active-

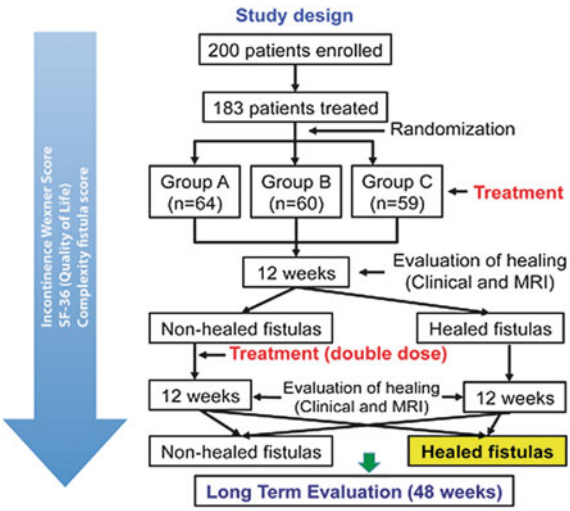

Fig. 12.4 Study protocol of the phase III trial applied for complex perianal fistula not associated with Crohn's Disease (FATT I, NCT00475410), the firstly registered phase III trial in fistulising disease

controlled, and single-blinded studies. FATT I compares application of autologous ASCs alone, ASCs and fibrin glue and fibrin glue alone. The surgical procedure was similar to that described for phase II publication. A second double-dose treatment administration was allowed if healing (clinical and MRI) was not achieved at 12th week (see Figs. 12.4 and 12.5). Although the data are not yet published, we can provide an advancement analysis: the rate of patients healed was 36.5 % when treated with fibrin glue, 54.3 % with ASCs plus fibrin glue and 53.1 % with ASCs alone (no statistical differences). In a further analysis on the fistula and treatment characteristics, patients treated at "La Paz" University Hospital had significantly higher healing rates: 18, 82, and 54 %, respectively ($p < 0.001$) and more patients with a suprasphincteric tract. No SCs related serious adverse events were found. We can conclude that treatment is safe and can cure more than 50 % of cryptoglandular complex perianal fistula without removing fistula tract [109]. A phase I–IIa study on feasibility and safety of allogeneic ASCs in rectovaginal fistulas (ALOREVA, NCT00999115) and another with perianal complex fistula (NCT01372969), both with Crohn's patients, are also running.

Other groups ASCs registered trials use them autologously. NCT00992485 is a phase I to test safety and efficacy at eighth week for Crohn's fistula and does not specify whether fistulas are simple or complex and not include previous treatment failure as inclusion criteria. NCT01157650 is a phase I–II trial for Crohn's enterocutaneous, rectovaginal or complex perianal fistula with 3 years follow-up; it is focused on safety but also efficacy and cellular product characteristics related to efficacy. NCT01011244 is a phase II continuing NCT00992485 (see above). The last two phase II trials are for complex perianal fistula (with a diameter less than 2 cm) in no-Crohn's patients applying 10 or 20 $\times$ 10^6 cells/ml (if healing was not achieved at sixth week a second double dose is accepted). One is the short term

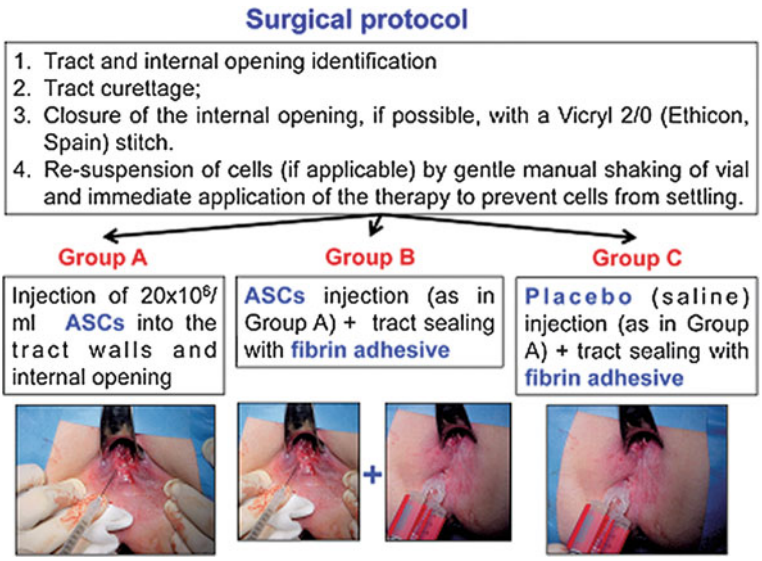

Fig. 12.5 Surgical procedure applied in Crohn's disease clinical trial (FATT I, NCT00475410). This is the firstly registered phase III trial in fistulising disease

(NCT01314092, follow-up 8 weeks) and the other the long term for healed patients (NCT01314079, follow-up 6 months).

Using BM-MSCs, we find one Prochymal™ phase III (which has among secondary outcomes draining fistula reduction) and NCT01144962. NCT01144962 is a phase I/II with allogeneic BM-MSCs to assess safety and efficacy in Crohn's perianal fistula refractory to drugs, with CDAI less than 250 and with medical treatment allowed unless biological therapy. The protocol includes curettage, internal opening closure and injection of 30, 60, or 90 million BM-MSCs. The follow-up is 24 weeks. Healing is assessed clinically and by MRI.

12.8.2.3 Critical Analysis

The most substantial limitations are similar to previously commented: the small number of patients in each assay, the lack of long-term follow-up (only some ongoing trials contemplate it) and that not all are randomized and controlled. In the case of fistula it is important to reflect that fistula type is not homogenous between trials (simple or complex) and the definition of complex varies among them. Maybe the condition of Crohn's Disease, such as previous surgical treatment, should be considered prior to analyze results and make subgroups.

It is important to take into account the cellular product applied and if it is clearly defined in the protocol; bone marrow mononucleated cells (BM-MNCs) or SVF therapies use cells without MSCs selection, and contains a heterogeneous

mixture of inflammatory cells and cytokines in addition to the modest population of SCs. In the case of SVF it also contains mature endothelial and HSCs, the latter representing up to 20 % [11].

It is also important to wait for the final results of ongoing trials before conclusions are made. Several MSCs phase III (for graft versus host disease, Crohn's and perianal fistula) have encountered some methodological difficulties (patients receiving control treatment performed better than anticipated, necessity of subgroup analysis, etc.) even though previous studies had shown beneficial effects. Maybe the most important conclusion by now is that the treatments have been safe, almost no serious adverse events related to therapy have been reported and that the protocol is minimally-invasive and the risk of affecting continence is insignificant.

12.8.3 Ulcerative Colitis

12.8.3.1 Relevant Published Studies

There are two from the same group. In the first one, allogeneic BM-MSCs were tested and remission duration increased and relapsing risk and hospital admissions were reduced compared with medication (5-aminosalicylic acid and glucocorticosteroid) [110]. The second one studies allogeneic intravenous BM-MSCs efficacy and safety during 2 years. Authors studied 40 patients who had not received biological drugs and 12 who underwent infliximab; 44 of them received BM-MSCs. A statistically significant (compared with conventional treatment) reduction in clinical and morphological inflammatory scores was found in 34 (72.7 %) patients whereas no therapeutic effect was reached on 12. MSCs allowed canceling corticosteroids in most patients with hormone-dependent and steroid resistant forms and in seven to reduce prednisolone to 5 mg/day. They also concluded than remission duration increased and recurrence risk and hospitalizations reduced [111].

12.8.3.2 Registered Clinical Trials

In June 2011, there were two (Tables 12.2 and 12.3).

NCT01221428 is a phase I/II single group safety and efficacy study about intravenous (2×10^7 SCs) and posteriorly mesenteric artery (1×10^7) administration of umbilical cord allogeneic MSCs for refractory disease. The follow-up is three months and authors study endoscopic, pathological, and clinical response.

NCT01240915 is a randomized, placebo controlled, and multicenter phase II study on safety and efficacy of Multistem® (PF-05285401) in moderate to severe refractory disease. Multistem® is obtained from bone marrow and other non-embryonic tissues and composed by healthy donors allogeneic MAPCs [112]. Placebo and different dosages and temporary administration protocols will be

compared. The follow-up is 8 weeks and the outcomes are adverse events and endoscopic and rectal bleeding scores.

12.8.3.3 Critical Analysis

Limitations are based on the reality that there are only two published studies from one group and no one is randomized (only one compares with the same cohort retrospectively). There are also a few registered trials so we have to wait for their results and for the registration of more studies to enable the drawing of conclusions.

12.8.4 Fecal Incontinence

An observational pilot study has been **published** in obstetric anal incontinence due to external sphincter disruption. Autologous myoblasts were injected into external sphincter in 10 women refractory to non-surgical therapy. Authors concluded that the injection was safe, well tolerated, and significantly improves symptoms [113].

Registered trials: A phase I trial (NCT01011686) focussing on safety studies the injection of autologous ASCs into the anal sphincter of patients without sphincteric lesions. The follow-up is 4 weeks and Wexner score, manometry, anorectal ultrasound and safety are assessed. The registry specifies than few subjects were enrolled.

Critical analysis: similar to ulcerative colitis about the limited existing scientific literature and registered trials.

12.8.5 Another Conditions

Celiac Disease: a published study tested high-dose chemotherapy followed by autologous HSCs in seven patients with a refractory disease subtype associated with high risk of enteropathy-associated T-cell lymphoma. A significant reduction in duodenal biopsies aberrant T cells, clinical and biochemical markers improvement without major nonhematologic toxicity or related mortality were found. So the treatment is feasible, safe and might result in long-term improvement [114].

In one study on MSCs application for acute severe and refractory *Graft Vs Host Disease (GVHD)*, authors observed that one patient who experienced perforated diverticulitis this was reversed twice by MSCs infusions. However, patient died from fungal infections and a Douglas pouch's abscess was found [115] so this publication has been criticized by others [116].

12.9 Future Perspectives

Over the last decade, SCs therapy for non-hematological diseases has grown exponentially. Experimental studies have generated sufficient knowledge to allow clinical research in the context of clinical trials and compassionate use.

SCs recognition as a drug and the legislation regulating their use will ensure that SCs will probably arrive to the market (provided the phase III studies confirm the promising results shown to date) in a safe and properly controlled way.

Allogeneic use (based mainly on the immune tolerance induced by some SCs) could extend the applications to emergency conditions and could allow SCs banks creation. The SCs obtaining process is very expensive so simplification of cell expansion (probably with closed systems under development) and the creation of international banks could make the access easier and cheaper for developing countries.

Nevertheless there are several main problems that hinder more extensive application and need to be resolved. More animal data on safety, data on the choice of SCs to use (one for all applications or disease-depending cell selection), the best dosage, the most efficient application route and therapeutic mechanisms involved are urgently required. SCs isolation must be standardized between different laboratories and properly defined to produce a 'consistent product' and compare results provided by different authors. Probably differentiation molecular pathways knowledge must augment in order to "direct" SCs better to the treatment objective. Regarding safety, the interaction between cancer and SCs, despite the numerous preclinical studies carried out, remains controversial. The possibility of stem cell transformation in vivo must be clarified; SCs migration toward neoplasms is feared to be able to induce cancer progression (although some therapeutic progress has been obtained using this feature for local delivery of chemotherapy reducing systemic toxicity) and several SCs are thought to be the origin of some cancers. Actually, neoplastic disease is considered a major contraindication in the context of most ongoing clinical trials. As soon as this issue is resolved, the number of possible applications would probably increase.

In the field of gastrointestinal system diseases (excluded liver and endocrine pancreas), several phase I–II trials have been reported, there are numerous ongoing phase II–III trials and probably some of them will report result soon revealing the real benefit (or not) of these therapies. Nevertheless large randomized controlled clinical trials are needed before firm conclusions can be drawn. Analyzing animal models, if current trends with clinical trials persist, the spectrum of treated diseases will increase.

Basic research must continue to obtain certain cells (for example mature enterocytes) and cells perfectly able to integrate into and function within the organ, thereby participating in the complex homeostasis of an organism. If this could be achieved, the jump from reparative (substitution of a tissue by another—mainly scar-) to regenerative (restitution of the original) therapy could be made. Today, this may seem like science fiction, but the results obtained today would also have seemed so a few years ago.

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References

1. Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105(3):369–377
2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284(5411):143–147
3. Joannides A, Gaughwin P, Schwiening C, Majed H, Sterling J, Compston A, Chandran S (2004) Efficient generation of neural precursors from adult human skin: astrocytes promote neurogenesis from skin-derived stem cells. Lancet 364(9429):172–178
4. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13(12):4279–4295
5. Liu D, Wang F, Zou Z, Dong S, Shi C, Wang J, Ran X, Su Y (2010) Long-term repopulation effects of donor BMDCs on intestinal epithelium. Dig Dis Sci 55(8):2182–2193
6. Rizvi AZ, Swain JR, Davies PS, Bailey AS, Decker AD, Willenbring H, Grompe M, Fleming WH, Wong MH (2006) Bone marrow-derived cells fuse with normal and transformed intestinal stem cells. Proc Nat Acad Sci USA 103(16):6321–6325
7. Houlihan DD, Newsome PN (2008) Critical review of clinical trials of bone marrow stem cells in liver disease. Gastroenterology 135(2):438–450
8. Yannaki E, Athanasiou E, Xagorari A, Constantinou V, Batsis I, Kaloyannidis P, Proya E, Anagnostopoulos A, Fassas A (2005) G-CSF-primed hematopoietic stem cells or G-CSF per se accelerate recovery and improve survival after liver injury, predominantly by promoting endogenous repair programs. Exp Hematol 33(1):108–119
9. Uccelli A, Moretta L, Pistoia V (2006) Immunoregulatory function of mesenchymal stem cells. Eur J Immunol 36(10):2566–2573
10. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. Cytotherapy 8(4):315–317
11. Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Penicaud L, Casteilla L (2004) Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 109(5):656–663
12. Francois S, Bensidhoum M, Mouiseddine M, Mazurier C, Allenet B, Semont A, Frick J, Sache A, Bouchet S, Thierry D, Gourmelon P, Gorin NC, Chapel A (2006) Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promotes their widespread engraftment to multiple organs: a study of their quantitative distribution after irradiation damage. Stem Cells 24(4):1020–1029
13. Mouiseddine M, Francois S, Semont A, Sache A, Allenet B, Mathieu N, Frick J, Thierry D, Chapel A (2007) Human mesenchymal stem cells home specifically to radiation-injured tissues in a non-obese diabetes/severe combined immunodeficiency mouse model. Br J Radiol 80(Spec No 1): S49–55

14. Verfaillie CM (2002) Adult stem cells: assessing the case for pluripotency. Trends Cell Biol 12(11):502–508
15. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7(2):211–228
16. Qiao XT, Ziel JW, McKimpson W, Madison BB, Todisco A, Merchant JL, Samuelson LC, Gumucio DL (2007) Prospective identification of a multilineage progenitor in murine stomach epithelium. Gastroenterology 133(6):1989–1998
17. Cheng H, Leblond CP (1974) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian theory of the origin of the four epithelial cell types. Am J Anat 141(4):537–561
18. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449(7165):1003–1007
19. Yan L, Cai C, Li J, Xu S, Chang Q, Li Y, Wu B (2009) Present status and perspectives of stem cell-based therapies for gastrointestinal diseases. Stem Cell Rev 5(3):278–282
20. Brittan M, Hunt T, Jeffery R, Poulsom R, Forbes SJ, Hodivala-Dilke K, Goldman J, Alison MR, Wright NA (2002) Bone marrow derivation of pericryptal myofibroblasts in the mouse and human small intestine and colon. Gut 50(6):752–757
21. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, Clevers H (2009) Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459(7244):262–265
22. Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, Sato T, Stange DE, Begthel H, van den Born M, Danenberg E, van den Brink S, Korving J, Abo A, Peters PJ, Wright N, Poulsom R, Clevers H (2010) Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. Cell Stem Cell 6(1):25–36
23. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
24. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872
25. Iwamuro M, Komaki T, Kubota Y, Seita M, Kawamoto H, Yuasa T, Shahid JM, Hassan RA, Hassan WA, Nakaji S, Nishikawa Y, Kondo E, Yamamoto K, Fox IJ, Kobayashi N (2010) Hepatic differentiation of mouse iPS cells in vitro. Cell Transplant 19(6):841–847
26. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, RingdÈn O (2008) Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 371(9624):1579–1586
27. Espinoza-Delgado I, Childs RW (2004) Nonmyeloablative transplantation for solid tumors: a new frontier for allogeneic immunotherapy. Expert Rev Anticancer Ther 4(5):865–875
28. Lundqvist A, Childs R (2005) Allogeneic hematopoietic cell transplantation as immunotherapy for solid tumors: current status and future directions. J Immunother 28(4):281–288
29. Nakagami H, Maeda K, Morishita R, Iguchi S, Nishikawa T, Takami Y, Kikuchi Y, Saito Y, Tamai K, Ogihara T, Kaneda Y (2005) Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. Arterioscler Thromb Vasc Biol 25(12):2542–2547
30. Joggerst SJ, Hatzopoulos AK (2009) Stem cell therapy for cardiac repair: benefits and barriers. Expert Rev Mol Med 11:e20
31. Chen L, Tredget EE, Wu PY, Wu Y (2008) Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS ONE 3(4):e1886
32. Taupin P (2006) Adult neural stem cells, neurogenic niches, and cellular therapy. Stem Cell Rev Rep 2(3):213–219

33. Zani A, Pierro A, Elvassore N, De Coppi P (2009) Tissue engineering: an option for esophageal replacement? Semin Pediatr Surg 18(1):57–62
34. Fascetti-Leon F, Malerba A, Boldrin L, Leone E, Betalli P, Pasut A, Zanon GF, Gamba PG, Vitiello L, De Coppi P (2007) Murine muscle precursor cells survived and integrated in a cryoinjured gastroesophageal junction. J Surg Res 143(2):253–259
35. Hutchinson L, Stenstrom B, Chen D, Piperdi B, Levey S, Lyle S, Wang T, Houghton J-M (2010) Human Barrett's adenocarcinoma of the esophagus, associated myofibroblasts and endothelium can arise from bone marrow derived cells after allogeneic stem cell transplant. Stem Cells Dev 20(1):11–17
36. Askarov M, Vostrikova O, Vorobjova N, Onishenko N (2008) Effects of autologous bone marrow cells on apoptosis and regeneration of non-healing autoimmune gastric ulcers. Bull Exp Biol Med 146(5):647–651
37. Hayashi Y, Tsuji S, Tsujii M, Nishida T, Ishii S, Iijima H, Nakamura T, Eguchi H, Miyoshi E, Hayashi N, Kawano S (2008) Topical transplantation of mesenchymal stem cells accelerates gastric ulcer healing in rats. Am J Physiol Gastrointest Liver Physiol 294(3):G778–G786
38. Komori M, Tsuji S, Tsujii M, Murata H, Iijima H, Yasumaru M, Nishida T, Irie T, Kawano S, Hori M (2005) Efficiency of bone marrow-derived cells in regeneration of the stomach after induction of ethanol-induced ulcers in rats. J Gastroenterol 40(6):591
39. Ismail A, Ramsis R, Sherif A, Thabet A, El-Ghor H, Selim A (2009) Use of human amniotic stem cells for common bile duct reconstruction: vascularized support of a free amnion graft. Med Sci Monit 15(9): BR243–BR247
40. Lin YX, Yan LN, Cheng NS (2009) Application of bone marrow cells: a novel therapy for bile leak? Med Hypotheses 73(3):374–376
41. Semont A, Francois S, Mouiseddine M, Francois A, Sache A, Frick J, Thierry D, Chapel A (2006) Mesenchymal stem cells increase self-renewal of small intestinal epithelium and accelerate structural recovery after radiation injury. Adv Exp Med Biol 585:19–30
42. Okamoto R, Matsumoto T, Watanabe M (2006) Regeneration of the intestinal epithelia: regulation of bone marrow-derived epithelial cell differentiation towards secretory lineage cells. Hum Cell 19(2):71–75
43. Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R (2003) Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood 101(8):2999–3001
44. Zhang J, Gong JF, Zhang W, Zhu WM, Li JS (2008) Effects of transplanted bone marrow mesenchymal stem cells on the irradiated intestine of mice. J Biomed Sci 15(5):585–594
45. Sala FG, Kunisaki SM, Ochoa ER, Vacanti J, Grikscheit TC (2009) Tissue-engineered small intestine and stomach form from autologous tissue in a preclinical large animal model. J Surg Res 156(2):205–212
46. Avansino JR, Chen DC, Hoagland VD, Woolman JD, Stelzner M (2006) Orthotopic transplantation of intestinal mucosal organoids in rodents. Surgery 140(3):423–434
47. Agopian V, Chen D, Avansino J, Stelzner M (2009) Intestinal stem cell organoid transplantation generates neomucosa in dogs. J Gastrointest Surg 13(5):971–982
48. Spence JR, Mayhew CN, Rankin SA, Kuhar MF, Vallance JE, Tolle K, Hoskins EE, Kalinichenko VV, Wells SI, Zorn AM, Shroyer NF, Wells JM (2011) Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature 470(7332):105–109
49. Hori Y, Nakamura T, Kimura D, Kaino K, Kurokawa Y, Satomi S, Shimizu Y (2002) Experimental study on tissue engineering of the small intestine by mesenchymal stem cell seeding. J Surg Res 102(2):156–160
50. Nakase Y, Hagiwara A, Nakamura T, Kin S, Nakashima S, Yoshikawa T, Fukuda K-I, Kuriu Y, Miyagawa K, Sakakura C, Otsuji E, Shimizu Y, Ikada Y, Yamagishi H (2006) Tissue engineering of small intestinal tissue using collagen sponge scaffolds seeded with smooth muscle cells. Tissue Eng 12(2):403–412
51. Sirbu-Boeti MP, Chivu M, Paslaru LL, Efrimescu C, Herlea V, Pecheanu C, Moldovan L, Dragomir L, Bleotu C, Ciucur E, Vidulescu C, Vasilescu M, Boicea A, Manoiu S, Ionescu

MI, Popescu I (2009) Transplantation of mesenchymal stem cells cultured on biomatrix support induces repairing of digestive tract defects, in animal model. Chirurgia (Bucur) 104(1):55–65
52. Metzger M, Caldwell C, Barlow AJ, Burns AJ, Thapar N (2009) Enteric nervous system stem cells derived from human gut mucosa for the treatment of aganglionic gut disorders. Gastroenterology 136(7): 2214–2225.e2213
53. Lindley RM, Hawcutt DB, Connell MG, Almond SN, Vannucchi MÄ, Faussone-Pellegrini MS, Edgar DH, Kenny SE (2008) Human and mouse enteric nervous system neurosphere transplants regulate the function of aganglionic embryonic distal colon. Gastroenterology 135(1): 205–216.e206
54. Tadauchi A, Narita Y, Kagami H, Niwa Y, Ueda M, Goto H (2009) Novel cell-based therapeutic strategy for ischemic colitis with use of bone marrow-derived mononuclear cells in rats. Dis Colon Rectum 52(8):1443–1451
55. Lorenzi B, Pessina F, Lorenzoni P, Urbani S, Vernillo R, Sgaragli G, Gerli R, Mazzanti B, Bosi A, Saccardi R, Lorenzi M (2008) Treatment of experimental injury of anal sphincters with primary surgical repair and injection of bone marrow-derived mesenchymal stem cells. Dis Colon Rectum 51(4):411–420
56. Kang SB, Lee HN, Lee JY, Park JS, Lee HS (2008) Sphincter contractility after muscle-derived stem cells autograft into the cryoinjured anal sphincters of rats. Dis Colon Rectum 51(9):1367–1373
57. Saihara R, Komuro H, Urita Y, Hagiwara K, Kaneko M (2009) Myoblast transplantation to defecation muscles in a rat model: a possible treatment strategy for fecal incontinence after the repair of imperforate anus. Pediatr Surg Int 25(11):981–986
58. Aghaee-Afshar M, Rezazadehkermani M, Asadi A, Malekpour-Afshar R, Shahesmaeili A, Nematollahi-mahani SN (2009) Potential of human umbilical cord matrix and rabbit bone marrow-derived mesenchymal stem cells in repair of surgically incised rabbit external anal sphincter. Dis Colon Rectum 52(10):1753–1761
59. Kajbafzadeh AM, Elmi A, Talab SS, Esfahani SA, Tourchi A (2010) Functional external anal sphincter reconstruction for treatment of anal incontinence using muscle progenitor cell auto grafting. Dis Colon Rectum 53(10):1415–1421
60. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M (2009) Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. Gastroenterology 136(3):978–989
61. Hayashi Y, Tsuji S, Tsujii M, Nishida T, Ishii S, Iijima H, Nakamura T, Eguchi H, Miyoshi E, Hayashi N, Kawano S (2008) Topical Implantation of mesenchymal stem cells has beneficial effects on healing of experimental colitis in rats. J Pharmacol Exp Ther 326(2):523–531
62. Wei Y, Nie Y, Lai J, Wan YJ, Li Y (2009) Comparison of the population capacity of hematopoietic and mesenchymal stem cells in experimental colitis rat model. Transplantation 88(1):42–48
63. Khalil PN, Weiler V, Nelson PJ, Khalil MN, Moosmann S, Mutschler WE, Siebeck M, Huss R (2007) Nonmyeloablative stem cell therapy enhances microcirculation and tissue regeneration in murine inflammatory bowel disease. Gastroenterology 132(3):944–954
64. Ando Y, Inaba M, Sakaguchi Y, Tsuda M, Quan GK, Omae M, Okazaki K, Ikehara S (2008) Subcutaneous adipose tissue-derived stem cells facilitate colonic mucosal recovery from 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats. Inflamm Bowel Dis 14(6):826–838
65. Dryden GW (2009) Overview of stem cell therapy for Crohn's disease. Expert Opin Biol Ther 9(7):841–847
66. Lanzoni G, Roda G, Belluzzi A, Roda E, Bagnara GP (2008) Inflammatory bowel disease: moving toward a stem cell-based therapy. World J Gastroenterol 14(29):4616–4626
67. Deng X, Szabo S, Chen L, Paunovic B, Khomenko T, Tolstanova G, Tarnawski AS, Jones MK, Sandor Z (2011) New cell therapy using bone marrow-derived stem cells/endothelial progenitor cells to accelerate neovascularization in healing of experimental ulcerative colitis. Curr Pharm Des 17(16):1643–1651

68. Jung KH, Song SU, Yi T, Jeon MS, Hong SW, Zheng HM, Lee HS, Choi MJ, Lee DH, Hong SS (2011) Human bone marrow-derived clonal mesenchymal stem cells inhibit inflammation and reduce acute pancreatitis in rats. Gastroenterology 140(3):998–1008
69. Schneider G, Saur D (2011) Mesenchymal stem cells: therapeutic potential for acute pancreatitis. Gastroenterology 140(3):779–782
70. Pascual I, Fernández de Miguel G, Gómez-Pinedo U, de Miguel F, García Arranz M, García-Olmo D (2008) Adipose-derived mesenchymal stem cells in biosutures do not improve healing of experimental colonic anastomoses. Br J Surg 95(9):1180–1184
71. Pascual I, Fernandez de Miguel G, Garcia Arranz M, Garcia-Olmo D (2010) Biosutures improve healing of experimental weak colonic anastomoses. Int J Colorectal Dis 25(12):1447–1451
72. Adas G, Arikan S, Karatepe O, Kemik O, Ayhan S, Karaoz E, Kamali G, Eryasar B, Ustek D (2011) Mesenchymal stem cells improve the healing of ischemic colonic anastomoses (experimental study). Langenbecks Arch Surg 396(1):115–126
73. Tyndall A, Pistoia V (2009) Mesenchymal stem cells combat sepsis. Nat Med 15(1):18–20
74. Gonzalez-Rey E, Anderson P, González MA, Rico L, Büscher D, Delgado M (2009) Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut 58(7): 929–939
75. Lucas P (2007) Stem cells for mesothelial repair: an understudied modality. Int J Artif Organs 30(6):550–556
76. Lucas PA, Warejcka DJ, Zhang L-M, Newman WH, Young HE (1996) Effect of rat mesenchymal stem cells on development of abdominal adhesions after surgery. J Surg Res 62(2):229–232
77. Lopez-Cubero SO, Sullivan KM, McDonald GB (1998) Course of Crohn's disease after allogeneic marrow transplantation. Gastroenterology 114(3):433–440
78. Ditschkowski M, Einsele H, Schwerdtfeger R, Bunjes D, Trenschel R, Beelen DW, Elmaagacli AH (2003) Improvement of inflammatory bowel disease after allogeneic stem-cell transplantation. Transplantation 75(10):1745–1747
79. Oyama Y, Craig RM, Traynor AE, Quigley K, Statkute L, Halverson A, Brush M, Verda L, Kowalska B, Krosnjar N, Kletzel M, Whitington PF, Burt RK (2005) Autologous hematopoietic stem cell transplantation in patients with refractory Crohn's disease. Gastroenterology 128(3):552–563
80. Cassinotti A, Annaloro C, Ardizzone S, Onida F, Della Volpe A, Clerici M, Usardi P, Greco S, Maconi G, Porro GB, Deliliers GL (2008) Autologous haematopoietic stem cell transplantation without CD34+ cell selection in refractory Crohn's disease. Gut 57(2):211–217
81. Burt RK, Craig RM, Milanetti F, Quigley K, Gozdziak P, Bucha J, Testori A, Halverson A, Verda L, de Villiers WJ, Jovanovic B, Oyama Y (2010) Autologous nonmyeloablative hematopoietic stem cell transplantation in patients with severe anti-TNF refractory Crohn disease: long-term follow-up. Blood 116(26):6123–6132
82. Duijvestein M, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidder HH, Verhaar AP, Fibbe WE, van den Brink GR, Hommes DW (2010) Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut 59(12):1662–1669
83. Taupin P (2006) OTI-010 osiris therapeutics/jcr pharmaceuticals. Curr Opin Investig Drugs 7(5):473–481
84. Onken JGD, Hanson J (2006) Successful outpatient treatment of refractory Crohn's disease using adult mesenchymal stem cells. ACG 2006 Final Program Book, 121
85. Onken JJT, Custer L (2008) Long-term safety of prochymal adult mesenchymal stem cells in Crohn's disease. Gastroenterology 134(4): A661
86. Lazebnik LB, Konopliannikov AG, Kniazev OV, Parfenov AI, Tsaregorodtseva TM, Ruchkina IN, Khomeriki SG, Rogozina VA, Konopliannikova OA (2010) Use of allogeneic mesenchymal stem cells in the treatment of intestinal inflammatory diseases. Ter Arkh 82(2):38–43
87. Allison M (2009) Genzyme backs osiris, despite prochymal flop. Nat Biotechnol 27(11):966–967

88. Prochymal: Press release about protocols 603 and 610. (2010)
89. Rubio D, Garcia-Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC, Bernad A (2005) Spontaneous human adult stem cell transformation. Cancer Res 65(8):3035–3039
90. Rosland GV, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, Mysliwietz J, Tonn JC, Goldbrunner R, Lonning PE, Bjerkvig R, Schichor C (2009) Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. Cancer Res 69(13):5331–5339
91. Garcia S, Bernad A, Martin MC, Cigudosa JC, Garcia-Castro J, de la Fuente R (2010) Pitfalls in spontaneous in vitro transformation of human mesenchymal stem cells. Exp Cell Res 316(9):1648–1650
92. Torsvik A, Rosland GV, Svendsen A, Molven A, Immervoll H, McCormack E, Lonning PE, Primon M, Sobala E, Tonn JC, Goldbrunner R, Schichor C, Mysliwietz J, Lah TT, Motaln H, Knappskog S, Bjerkvig R (2010) Spontaneous malignant transformation of human mesenchymal stem cells reflects cross-contamination: putting the research field on track—letter. Cancer Res 70(15):6393–6396
93. Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, Montagna D, Maccario R, Villa R, Daidone MG, Zuffardi O, Locatelli F (2007) Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. Cancer Res 67(19): 9142–9149
94. He X, Tsang TC, Pipes BL, Ablin RJ, Harris DT (2005) A stem cell fusion model of carcinogenesis. J Exp Ther Oncol 5(2):101–109
95. Rhodes L, Muir S, Elliott S, Guillot L, Antoon J, Penfornis P, Tilghman S, Salvo V, Fonseca J, Lacey M, Beckman B, McLachlan J, Rowan B, Pochampally R, Burow M (2009) Adult human mesenchymal stem cells enhance breast tumorigenesis and promote hormone independence. Breast Cancer Res Treat 121(2):293–300
96. Xu W-T, Bian Z-Y, Fan Q-M, Li G, Tang T-T (2009) Human mesenchymal stem cells (hMSCs) target osteosarcoma and promote its growth and pulmonary metastasis. Cancer Lett 281(1):32–41
97. Cousin B, Ravet E, Poglio S, De Toni F, Bertuzzi M, Lulka H, Touil I, André M, Grolleau J-L, Péron J-M, Chavoin J-P, Bourin P, Pénicaud L, Casteilla L, Buscail L, Cordelier P (2009) Adult stromal cells derived from human adipose tissue provoke pancreatic cancer cell death both in vitro and in vivo. PLoS ONE 4(7):e6278
98. Sun B, Roh K-H, Park J-R, Lee S-R, Park S-B, Jung J-W, Kang S-K, Lee Y-S, Kang K-S (2009) Therapeutic potential of mesenchymal stromal cells in a mouse breast cancer metastasis model. Cytotherapy 11(3):289–298
99. Lamfers M, Idema S, van Milligen F, Schouten T, van der Valk P, Vandertop P, Dirven C, Noske D (2009) Homing properties of adipose-derived stem cells to intracerebral glioma and the effects of adenovirus infection. Cancer Lett 274(1):78–87
100. Garcia-Olmo D, Garcia-Arranz M, Garcia LG, Cuellar ES, Blanco IF, Prianes LA, Montes JA, Pinto FL, Marcos DH, Garcia-Sancho L (2003) Autologous stem cell transplantation for treatment of rectovaginal fistula in perianal Crohn's disease: a new cell-based therapy. Int J Colorectal Dis 18(5):451–454
101. Garcia-Olmo D, Garcia-Arranz M, Herreros L (2005) A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. Dis Colon Rectum 48:1416–1423
102. Garcia-Olmo D, Garcia-Arranz M, Herreros D (2008) Expanded adipose-derived stem cells for the treatment of complex perianal fistula including Crohn's disease. Expert Opin Biol Ther 8(9):1417–1423
103. Garcia-Olmo DMD, Herreros DMD, Pascual IMD, Pascual JAMD, Del-Valle EMD, Zorrilla JMD, De-La-Quintana PPD, Garcia-Arranz MPD, Pascual MPD (2009) Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. Dis Colon Rectum 52(1):79–86

104. Garcia-Arranz M, Gomez-Pinedo U, Hardisson D, Herreros D, Guadalajara H, Garcia-Gomez I, Garcia-Verdugo JM, Garcia-Olmo D (2010) Histopathological analysis of human specimens removed from the injection area of expanded adipose-derived stem cells. Histopathology 56(7):979–982
105. Garcia-Olmo D, Herreros D, Pascual M, Pascual I, De-La-Quintana P, Trebol J, Garcia-Arranz M (2008) Treatment of enterocutaneous fistula in Crohn's disease with adipose-derived stem cells: a comparison of protocols with and without cell expansion. Int J Colorectal Dis 24(1):27–30
106. Garcia-Olmo D, Herreros D, De-La-Quintana P, Guadalajara H, Trebol J, Georgiev-Hristov T, Garcia-Arranz M (2010) Adipose-derived stem cells in Crohn's rectovaginal fistula. Case Report Med 2010:961758
107. Alvarez PD-A, Garcia-Arranz M, Georgiev-Hristov T, Garcia-Olmo D (2008) A new bronchoscopic treatment of tracheomediastinal fistula using autologous adipose-derived stem cells. Thorax 63(4):374–376
108. Ciccocioppo R, Bernardo ME, Sgarella A, Maccario R, Avanzini MA, Ubezio C, Minelli A, Alvisi C, Vanoli A, Calliada F, Dionigi P, Perotti C, Locatelli F, Corazza GR (2011) Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. Gut 60(6):788–798
109. Garcia-Olmo D, Herreros M, Guadalajara H, DeLaQuintana P, Trebol J, Georgiev-Hristov T, Garcia-Arranz M (2011) ASCRS annual meeting abstracts: expanded adipose derived autologous stem cells for the treatment of complex cryptoglandular fistulas. A phase III clinical trial (FATT1: fistula advanced therapy trial 1) and long term evaluation (LTE). Dis Colon Rectum 54(5):e68–e69
110. Lazebnik LB, Kniazev OV, Parfenov AI, Ruchkina IN, Rogozina VA, Konopliannikov AG (2010) Transplantation of allogeneic mesenchymal stem cells from the bone marrow increases duration of remission and reduces the risk of ulcerative colitis relapse. Eksp Klin Gastroenterol (3):5–10
111. Lazebnik LB, Kniazev OV, Konopliannikov AG, Parfenov AI, Ruchkina IN, Mikhailova ZF, Tsaregorodtseva TM, Khomeriki SG, Rogozina VA, Gudkova RB, Shcherbakov PL, Konopliannikova OA (2010) Allogeneic mesenchymal stromal cells in patients with ulcerative colitis: two years of observation. Eksp Klin Gastroenterol (11):3–15
112. Van Bokkelen G (2011) Company profile: athersys. Regen Med 6(1):39–43
113. Frudinger A, Kolle D, Schwaiger W, Pfeifer J, Paede J, Halligan S (2010) Muscle-derived cell injection to treat anal incontinence due to obstetric trauma: pilot study with 1 year follow-up. Gut 59(1):55–61
114. Al-toma A, Visser OJ, van Roessel HM, von Blomberg BM, Verbeek WH, Scholten PE, Ossenkoppele GJ, Huijgens PC, Mulder CJ (2007) Autologous hematopoietic stem cell transplantation in refractory celiac disease with aberrant T cells. Blood 109(5):2243–2249
115. Ringden O, Uzunel M, Sundberg B, Lonnies L, Nava S, Gustafsson J, Henningsohn L, Le Blanc K (2007) Tissue repair using allogeneic mesenchymal stem cells for hemorrhagic cystitis, pneumomediastinum and perforated colon. Leukemia 21(11):2271–2276
116. Ball L, Bredius R, Lankester A, Schweizer J, van den Heuvel-Eibrink M, Escher H, Fibbe W, Egeler M (2008) Third party mesenchymal stromal cell infusions fail to induce tissue repair despite successful control of severe grade IV acute graft-versus-host disease in a child with juvenile myelo-monocytic leukemia. Leukemia 22(6):1256–1257

Chapter 13
Germ Line Stem Cells: A Promising Alternative Source for Stem-Cell-Based Therapies in Regenerative Medicine

Ellen Goossens and Herman Tournaye

Abstract Spermatogonial stem cells (SSCs) are exceptional cells with potential clinical applications in fertility preservation and regenerative medicine. Like other tissue-specific stem cells, SSCs are defined by their ability to balance between self-renewal and differentiation. This balance regulates the stem cell pool and guarantees the daily production of gametes. The preservation and transplantation of SSCs may become a promising strategy to treat reproductive stem cell loss disorders. As a consequence, the banking of testicular tissue from prepubertal patients is being introduced in a clinical setting. Moreover, with the increasing reports about the plasticity of mouse and human SSCs, their potential application in regenerative medicine is highly interesting. In contrast to males, females are born with a finite population of primordial follicles. Although the existence of ovarian stem cells (OSCs) has been suggested recently, it is still a matter of controversy. This review provides an update on the role of germ line stem cells in fertility restoration after germ cell loss, and gives an overview of (future) clinical applications making use of these fascinating cells.

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13.1 Spermatogonial Stem Cells

13.1.1 From Primordial Germ Cell to Spermatogonial Stem Cell

The most important function of the mammalian testis is the production of sperm. Spermatogenesis occurs in the seminiferous tubules over a long reproductive period. This continuous process is supported by a stem cell population, the spermatogonial stem cells (SSC).

Germ cell development starts with the specification of the primordial germ cells (PGCs) early in human fetal life. The extraembryonic ectoderm expresses bone morphogenetic protein 4 and bone morphogenetic protein 8b, two growth factors indispensable for the creation of PGCs [1]. PGCs arise from the proximal epiblast and will translocate to the extraembryonic mesoderm during the 4th and 5th week of embryonic development. During the formation of the allantois, by the end of the 5th week to early 6th week, the PGCs leave the embryo and start migrating via the dorsal mesenterium of the gut to the gonadal ridge. The migration, cell proliferation, and survival of PGCs depend on the Kit-SCF system. PGCs that express the c-kit receptor, are attracted by cells which produce soluble stem cell factor (SCF), all along the migratory path [2]. During their migration PGCs proliferate but once they have reached the gonadal ridge, they enter into a mitotic arrest while differentiating into gonocytes. These gonocytes become enclosed in testicular cords formed by Sertoli cell precursors and peritubular myoid cells. Initially, gonocytes are located in the center of the testicular cords, away from the basal membrane. Shortly after birth, these gonocytes move to the basal membrane of the seminiferous tubules where they resume proliferation giving rise to A_s-spermatogonia or SSCs [3, 4]. In the adult testis only 0.03 % of all germ cells are SSCs. They have the potential to self-renew and to differentiate in order to produce spermatozoa [5].

13.1.2 Spermatogonial Stem Cell Phenotype

Spermatogonia are small single cells with an ovoid nucleus and the nucleoli close to the nuclear membrane. Electron microscopy shows a small Golgi-apparatus, few mitochondria, and many free ribosomes. The SSC population is a very small subpopulation of the spermatogonia, located on the basement membrane of the seminiferous tubules.

Different markers can be used to characterize mouse SSCs: α_6-integrin (CD49f) [6], β_1-integrin (CD29) [6], glial cell-line derived neurotrophic factor (GDNF) family receptor alpha 1 (GFRα1) [7], stimulated by retinoic acid gene 8 (Stra8) [8], thymocytes differentiation antigen 1 (Thy1 or CD90) [9], CD9 [10], rearranged during transfection proto-oncogene (RET) [11] and Nanog [12] for positive selection, and c-kit (CD117), CD45, α_6-integrin and H-2 K^b (major histocompatibility complex 1, MCH1 in human) for negative selection [9, 13, 14]. Although it

is possible to highly enrich cell populations for SSCs (100–200-fold) using combinations of positive and negative markers, so far, a pure SSC suspension could not be produced.

Human spermatogonia express some markers of rodent spermatogonia (α_6-integrin, GFRα1, Thy1). However, other markers are not shared. For example, testis specific protein Y-encoded (TSPY) [15, 16], melanoma-associated antigen 4 (MAGE-A4) [17], and stage-specific embryonic antigen 4 (SSEA4) [12, 18] are not expressed in mouse spermatogonia. Conversely, β_1-integrin, which is found on mouse SSCs, is not appropriate for human SSCs 139. A combination of $SSEA4^+CD49^+$ G-protein coupled receptor 125 $(GFR125)^+C\text{-}kit^{-/low}$ was proposed to select for human SSCs, but the value of these markers still needs to be validated [12].

To prove the presence of true SSCs, functional assays are required. The only two functional assays available for SSCs are the re-establishment of spermatogenesis in infertile recipients by spermatogonial stem cell transplantation (SSCT) [19] and the clonal proliferation in culture [20] .

Using SSCT, it was shown that cells expressing OCT4 showed higher stem cell activity compared with the $OCT4^-$ cells [21]. The expression of promyelocytic leukemia zinc-finger (PLZF), SOX3, neurogenin3, and NANOS2 was also determined in cells displaying stem cell activity [22–25].

Although, during the last 10 years, a lot of progress has been made in characterizing SSCs, a specific SSC marker could not be identified yet. Since there is growing evidence that the SSC population might be heterogeneous, the identification of such a marker might be very difficult, if not impossible [25–29]. Very recently, a new marker for SSCs was proposed in rodents: Inhibitor of DNA binding 4 (ID4), the marker with the most restricted expression pattern observed to date. However, not all single spermatogonia expressed this marker [30]. Sal-like protein 4 (SALL4), for example, is specifically expressed in undifferentiated spermatogonia. But, whereas SALL4 expression mostly overlapped with PLZF, its co-expression with GFRα1 revealed heterogeneity. Populations of $SALL4^+$/$GFR\alpha1^-$ and $SALL4^+/GFR\alpha1^+$ cells could be detected, with GFRα1 expression being more restricted (clones of 1–4 cells) than the expression of PLZF and SALL4 (also clones of 8 and 16 cells) [31].

It is still far from clear whether stem cell activity is a privilege for the population of single spermatogonia or also for the populations of paired and aligned spermatogonia. In reality, in damaged testes, not only single spermatogonia have the capacity to self-renew. Here, paired and aligned spermatogonia are able to revert to the single state and start spermatogenesis [26].

13.1.3 Spermatogonial Stem Cell Niche

The most important role of SSCs is the unlimited production of sperm. In mammals, spermatogenesis is a strictly regulated and continuous process. Every day, millions of spermatozoa are produced in the normal adult testes. This

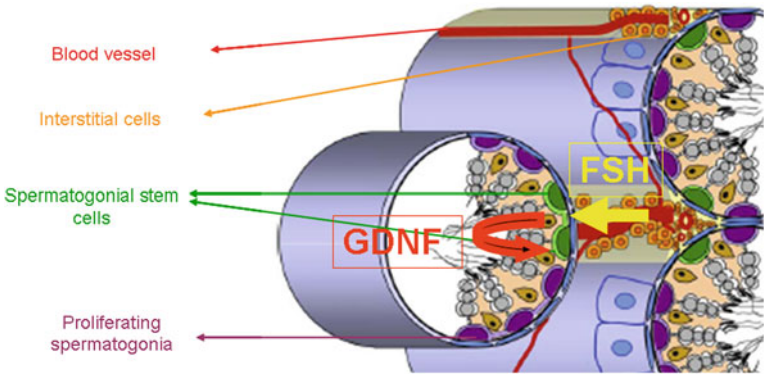

Fig. 13.1 Spermatogonial stem cell niche. Under the stimulation of FSH, Sertoli cells will secrete GDNF. GDNF binds to SSCs inducing self-renewal. Sertoli cells located close to blood vessels will receive more FSH than Sertoli cells positioned next to a neighboring tubule. Thus SSCs are more likely to self-renew in those parts of the tubule adjacent to the interstitial tissue

multiplication of germ cells is not regulated by the stem cells themselves but by the micro-environment surrounding the stem cells, e.g. the stem cell niche. The discovery of the stem cell niche in *Drosophila* in 2000 has accelerated the elucidation of this regulatory system in mammals [32] (Fig. 13.1).

Niches are specialized micro-environments regulating tissue homeostasis by controlling stem cell self-renewal and differentiation. The SSC niche in the mammalian testis is located at the basal membrane of the seminiferous tubules and consists of Sertoli cells, peritubular myoid cells, Leydig cells, and other interstitial cells [33]. Each niche houses one stem cell, which is connected to the basement membrane through (α_1- and) α_6-integrins. The fate of the stem cells is regulated by paracrine factors secreted by the niche.

The main regulators of stem cell fate are the Sertoli cells. These cells are polarized columnar epithelial cells dividing the seminiferous tubule into two compartments: the basal and the adluminal compartment. The barrier separating the compartments is built up by tight junctions between Sertoli cells and is called blood-testis barrier (BTB). Whereas the basal compartment houses mainly spermatogonia, the adluminal compartment comprises the differentiating germ cells. This compartmentalization indicates that, during germ cell differentiation, germ cells have to cross the BTB. The opening of the BTB regulates germ cell development by permitting the passage of preleptotene and leptotene spermatocytes. As such, the differentiation process from leptotene spermatocytes up to mature spermatozoa is disconnected from the systemic circulation, but Sertoli cells provide developing germ cells with essential nutrients while establishing an immune-privileged environment for haploid germ cells [34].

Next to Sertoli cells, also interstitial cells have been suggested to regulate germ cell fate. While colony-stimulating factor 1 was expressed in Leydig and peritubular myoid cells, its receptor was detected on $THY1^+$ cells [35].

As earlier mentioned, spermatogonia are localized in the basal compartment. However, not every spermatogonium is a SSC. So there must be an additional factor determining stem cell self-renewal. In 2007, it was reported that stem cell niches are not distributed randomly along the tubule but are localized in areas nearby the vasculature [36]. The mechanism behind SSC renewal has been described as follows. Under the stimulation of follicle stimulating hormone (FSH), Sertoli cells produce and secrete GDNF, which is an important self-renewal factor for SSCs [7]. GDNF binds to the RET and the GFRα1 co-receptor mediating intracellular protein kinase signaling through the Sarcoma family kinase (SFK) and 3-kinase/serine-threonine kinase AKT family (PI3 K/AKT) pathways. The latter regulate the expression of specific genes, such as Etv5 and Bcl6b, which are involved in SSC self-renewal. Other genes, not regulated by GDNF, e.g. Zbtb16, Taf4b, and Lin28, are controlled by different signals, and might play a role in blocking SSC differentiation [37].

When the seminiferous epithelium is depleted by cytotoxic agents or irradiation, restoration of spermatogenesis must occur from stem cells. Although SSCs are less sensitive than differentiating spermatogonia, they still can be lost. It was observed that, in this event, the percentage of paired spermatogonia was lower than in the normal testis, indicating that, after damage, stem cells prefer self-renewal to differentiation [38].

13.1.4 Spermatogonial Stem Cell Differentiation

SSC proliferation is slightly different for non-primate and primate mammals. For non-primates, the prevailing model is the A_s-model [39, 40]. The A_s or single undifferentiated type A-spermatogonia is considered to be the most primitive cell or true spermatogonial stem cell. When single spermatogonia divide to form new stem cells, they usually migrate individually. If they are destined for differentiation, they remain connected by cytoplasmic bridges and become paired spermatogonia. The division of paired spermatogonia results in four aligned spermatogonia, which are also connected one to another. Aligned spermatogonia divide a few times more leading to chains of 8, 16, and occasionally 32 cells. Most of the aligned spermatogonia will undergo morphological changes and transform into differentiating spermatogonia.

Using the transplantation assay, it was possible to demonstrate that stem cell activity was limited to the single, paired, and aligned spermatogonia [41]. Interconnected spermatogonia gradually lose their potential to self-renew, although they can revert back to single spermatogonia after testicular damage. Differentiating spermatogonia possess a weaker potential to self-renew [42].

In primates, two morphologically different classes of stem cells are observed: the dark or 'reserve' stem cells and the pale or 'renewing' stem cells [43]. The self-renewal of pale spermatogonia is analogous to that in the non-primate model, because most of the pale spermatogonia appear in clones of two, four, and eight cells, but single cells also may exist. Furthermore, pale and dark cells can transform into each other. Dark spermatogonia are often found in clusters. Such a cluster could be the result of a transformation of pale spermatogonia into dark spermatogonia at low renewal frequency. Conversely, after cytotoxic injury, dark spermatogonia may transform into pale spermatogonia and start proliferating [44]. Pale spermatogonia are responsible for the maintenance of the original size of the stem cell population. Because these cells, which are found in clones of two or four cells, cycle continuously, the true stem cells are probably the rarely dividing single spermatogonia [45].

13.1.5 Spermatogenesis In vitro

A primary step in the establishment of a system for in vitro spermatogenesis is the long-term survival and proliferation of SSCs. Initially, rodents SSCs were cultured for 4 months on embryonic fibroblasts in a medium containing fetal bovine serum [46]. The efficacy of SSC culture could be improved by enriching the cell suspension for SSCs or by using pup testicular cells [47]. In addition, suppressing the differentiation of SSCs improved their in vitro maintenance [48]. SSCs could be kept in culture for more than 5 months, when GDNF, epithelial derived growth factor, basic fibroblast growth factor 2 (FGF2), and leukemia inhibitory factor were added to the culture medium [49]. SSCs could be expanded 10^{85}-fold in 2 years of culture [50]. Afterwards, feeder layers and serum could be omitted [51, 52], which is an advantage for any potential clinical application (Fig. 13.2).

A few years ago, the culture of bovine SSCs has been reported [53], but it was only until very recently that SSC culture was realized using frozen-thawed adult and prepubertal human SSCs [54, 55].

The second–and most difficult–step in the establishment of in vitro spermatogenesis is the differentiation process to spermatozoa. Culture and differentiation of male germ cells has been performed for various purposes in the past, but none of these attempts has resulted in a sufficient number of mature gametes. Morphologically normal spermatozoa could be obtained from immature mouse germ cells through 3D-culture [56] and bovine spermatogonia could be differentiated into spermatocytes and spermatids in a minimal essential medium containing fetal calf serum [57]. Further research is required to reveal the applicability of this culture technique for human germ cells.

In vitro tissue explants represent an alternative strategy to culture testicular cells. Bovine testicular tissue pieces could be maintained in culture for 2 weeks resulting in an increase in colony numbers [58]. Lately, functional spermatids and

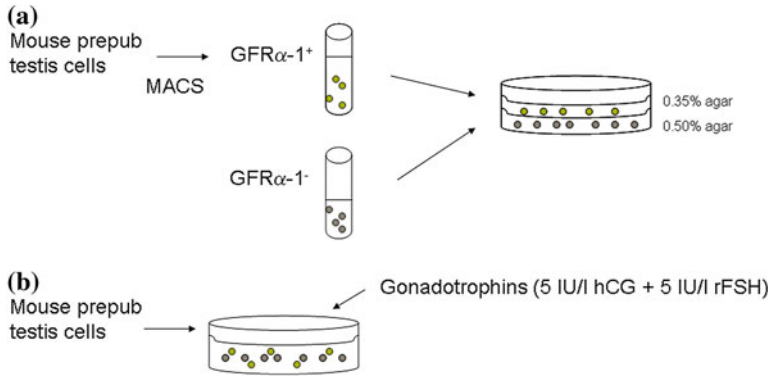

Fig. 13.2 Spermatogenesis in vitro. **a** In a first part of their study, Stukenborg and colleagues separated mouse prepubertal germ cells from interstitial cells by MACS using antibodies against GFRα1. The undifferentiated spermatogonia (GFRα1$^+$ cells) were cultured in an agar matrix. Germ cells expanded but the efficiency of proliferation increased and differentiation was observed when the GFRα1$^-$ fraction was cultured in a matrix on top of the agar containing the germ cells. **b** In a second part of the study, both GFRα1$^+$ and GFRα1$^-$ fractions were cultured in the same matrix. Aggregates of cells were formed and post-meiotic germ cells appeared. Moreover, the addition of gonadotrophins led to the production of mature spermatozoa

sperm could be produced from cryopreserved neonatal mouse testicular tissue in vitro in serum-free culture media [59].

13.1.6 Regeneration of Spermatogenesis in Infertile Patients

A depletion of SSCs is a major cause of male infertility. Fertility problems often occur after chemo- or radiotherapy, used as conditioning treatment for cancer or bone marrow transplantation [60]. Loss of SSCs is also found in association with certain genetic disorders, such as the 47, XXY Klinefelter syndrome [61]. It is out of question that prevention of sterility needs special attention in both oncology and reproductive medicine. The inability to father his genetically own children could have a high impact on the psychological well-being of the patient in adulthood. Cryopreservation of a sperm sample is the standard fertility preservation strategy in adult males. For prepubertal boys, there is currently no validated technique for fertility restoration. Autologous transplantation of cryopreserved SSCs or testicular tissue has been suggested, but this is still in a preclinical phase [62]. On the other hand, autologous transplantation is not always suitable. Testicular biopsies from cancer patients may contain malignant cells. Obviously, it is unacceptable to reintroduce these cells into a cured patient. Autologous transplantation is also not possible in Klinefelter patients given their testis pathology. For these cases, in vitro spermatogenesis could be the solution to avoid sterility.

13.1.7 Cryopreservation of Stem Cells

Because the presence of both the extracellular matrix and supporting cells is critical for germ cell survival and function, the cryopreservation of testicular tissue is to be preferred over cryopreservation of testicular cell suspensions or isolated SSCs. Controlled slow freezing with dimethylsulfoxide is routinely used to cryopreserve immature testicular tissue [63, 64]. In rodents, the transplantation of frozen-thawed prepubertal testicular tissue has already led to the birth of healthy offspring [65]. Controlled slow freezing protocols have also been used for human testicular tissue [66, 67]. Nevertheless, this type of freezing requires expensive computerized equipment and is time-consuming. A more economical and time-saving alternative is uncontrolled slow freezing. As controlled freezing, uncontrolled freezing of prepubertal testicular tissue has been successfully used in different animal species, and has been fully validated in mice as a means to preserve the reproductive potential [68–70]. Because, next to uncontrolled freezing, vitrification is also inexpensive, convenient, and fast executable, this technique was tested with piglets and mouse testicular tissue [71–73]. Since vitrification was shown to yield similar results compared to slow freezing, this method might be considered for human testicular tissue too [74].

13.1.8 Spermatogonial Stem Cell Transplantation

Almost 20 years ago, Brinster and colleagues introduced the transplantation of SSCs in fundamental research. A germ cell suspension obtained from fertile donors was injected into the seminiferous tubules of infertile recipient mice. During the first month after transplantation, the transplanted spermatogonia were able to traverse the BTB and to relocate onto the basement membrane. From that moment on, SSCs colonized the tubules, started to proliferate and initiated spermatogenesis. The recipient mice were able to reproduce in vivo and produce transgenic offspring [75] even after transplanting cryopreserved cell suspensions [76]. Shortly after, SSCT was performed in other mammalian species, including primates [77–79]. As long as the phylogenetic distance is not too large, the transplantation between different species can be successful too [80, 81]. These results, especially those from primate studies, are promising enough to advise the banking and transplantation of human SSCs as a fertility preservation strategy in patients who are at risk of SSC loss.

However, before introducing this application in the clinic, it is desirable to evaluate the efficiency and the safety of the procedure. Why this is so important is illustrated by the fact that, in the mouse, it was shown that litter sizes were smaller after transplantation compared to fertile controls [82]. This seemed to be associated to a lower sperm concentration and reduced sperm motility after transplantation [83]. On the other hand, when donors and recipients were genetically

related, the offspring did not show irregularities on the karyotype or on the epigenetic level for most of the investigated modifications [84, 85]. Only the acetylation of H4K5 and H4K8, which is important for the histone-to-protamine exchange during spermiation, was impaired in spermatogonia and spermatocytes. The function of H4K5ac and H4K8ac in these cell types is still unknown, but might have an impact on DNA repair.

13.1.9 Malignant Cells can Contaminate the Testicular Tissue

Many pediatric malignancies can metastasize through the blood or can be present in the testis, causing a potential risk of contamination of the collected testicular tissue. In the human, the threshold number of malignant cells able to cause malignant relapse when transplanted to the testis is unknown, but the transplantation of as few as 20 leukemic cells could cause malignant recurrence in rats [86]. It should thus be evident that the detection of even the slightest contamination of the testicular tissue is extremely important.

In case contaminating cells are present, the isolation of SSCs from these malignant cells prior to transplantation is required. The use of magnetic activated cell sorting and/or FACS has been studied in the depletion of cancer cells from mouse and human testicular cell suspensions. However, the reported decontamination strategies seemed not fully efficient, maybe because not enough markers were used or the markers were not specific enough [13, 87, 88]. The isolation of SSCs by selective matrix adhesion was also insufficient to deplete all contaminating cells from the cultures [89].

13.1.10 Testicular Tissue Grafting

In case there is no risk for malignant cell contamination (e.g. blood-borne diseases), testicular tissue grafting can be suggested as an alternative to SSCT. Testis tissue can be grafted ectopically (under the back skin, in the scrotum) or homotopically (in the testis). Mature spermatozoa could be retrieved from ectopic grafts and used to create progeny by ICSI [90, 91]. Although full spermatogenesis could be achieved in grafts using immature testis tissue [92–94], the ectopic grafting of adult testicular tissue showed only limited spermatogonial survival, with most of the tubules being completely regressed [95, 96]. Therefore, researchers explored other grafting sites in order to improve the establishment of human spermatogenesis after xenografting. Transplantation of immature human testicular tissue to the mouse peritoneal bursa inside the scrotum resulted in long-term survival of spermatogonia and differentiation up to pachytene spermatocytes [97]. Only very recently, it was shown that differentiation up to secondary spermatocytes was possible if human grafts were placed inside the mouse testis [98].

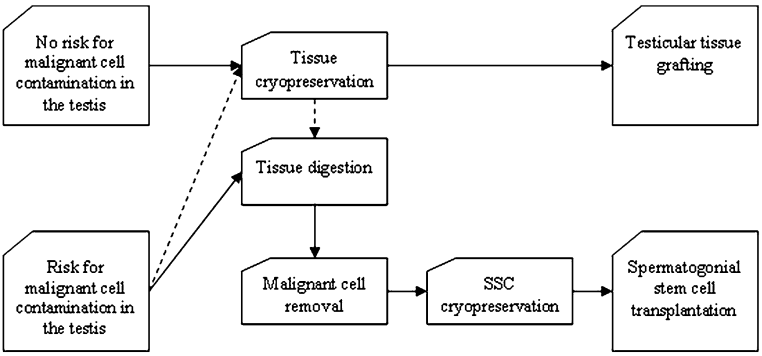

Fig. 13.3 Fertility preservation. Preservation and restoration strategies depend on the malignancy of the disease. When there is no risk for contaminating malignant cells in the testis, tissue preservation and transplantation is the method of choice. In case the risk for contaminating cells in the testis is substantial, the tissue should be digested and decontaminated before (*solid arrow*) or after (*dashed arrow*) cryopreservation

In the mouse model, intratesticular grafting showed a better re-establishment of spermatogenesis compared to SSCT. Cryopreservation of the grafts did not adversely affect the colonization efficiency and restoration of spermatogenesis [99]. Moreover, whereas some epigenetic modifications were altered after SSCT, this was not the case after grafting [100]. Therefore, we recommend transplanting tissue in patients with non-malignant diseases or non-metastasizing tumors. Only for patients who are at risk of having malignant cells in their testes at the time of biopsy, SSCT would be the method of choice (Figs. 13.3, 13.4).

13.1.11 Are Spermatogonial Stem Cells Pluripotent?

At this moment, there is no straightforward answer to this question. Several research groups have reported that both neonatal and adult mouse and human SSCs are capable to generate pluripotent cells in vitro and to differentiate into derivatives of the three embryonic germ cell layers [101–103]. These pluripotent cells displayed phenotypical similarities to embryonic stem cells (ESC), they showed the potential to differentiate into various types of somatic cells in vitro and they induced teratoma formation after injection into nude mice. Furthermore, germ line chimeras could be produced after injecting ESC-like cells into blastocysts.

Similar phenomena were reported for human SSCs. ESC-like cell lines could be successfully established from adult human SSCs. The cells displayed cellular and molecular characteristics of human ESCs and differentiated into various cell types of the three germ layers [104, 105] .

However, since it has been suggested that some of the testis-derived cells shared more characteristics with epiblast stem cells than with ESCs, the

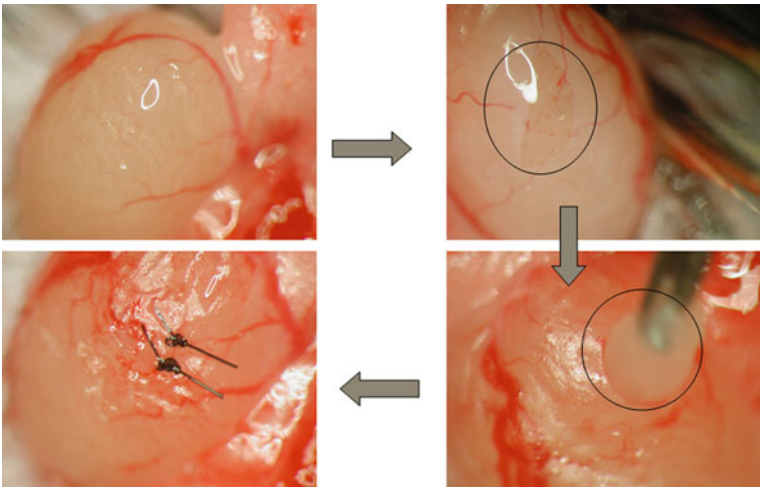

Fig. 13.4 Intratesticular tissue grafting. After making an incision in the tunica albuginea, a couple of tubules are removed from the testis to make space for the graft. A small fragment of testicular tissue is placed under the tunica and the tunica is closed by two stitches

pluripotency of SSCs has been put into question [101, 106]. Therefore, a study was designed to compare human adult germ line stem cells (GSCs) with human ESCs and human testicular fibroblasts [107]. The "pluripotent" adult GSCs had a similar gene expression profile to human testicular fibroblasts, but differed from ESCs.

This dissimilarity in SSC potential has been suggested to originate from differences in the derivation protocol [108], but the debate is still ongoing. Further characterization of testis-derived cells is required before we may have a thoroughly understanding of their pluripotent potential.

13.1.12 Cell Therapy

The derivation of autologous pluripotent cells has become a main goal in translational stem cell research. The use of ESCs is ethically controversial, leading to searches for other sources of pluripotent stem cells. Compared with ESCs and induced pluripotent stem cells, SSC-derived pluripotent cells would have clear advantages. There would be less ethical concerns (no need to destroy embryos or to create embryos for treatment) and it would be possible to derive patient-specific cells. Some centers started banking human SSCs for the preservation of male fertility in boys. These banked SSCs could also be used for advanced treatments in regenerative medicine. Since SSCs display similar molecular characteristics as ESCs, exceptional opportunities could be envisaged.

A few research teams tried to derive somatic cells from mouse SSCs. Under in vitro conditions, mouse SSCs were able to generate tissues of ectodermal,

mesodermal, and endodermal origin (functional neurons, glia, cardiomyocytes, and other somatic cell types) without first reverting to a less differentiated state [109–112]. Our research group demonstrated the differentiation of SSCs into hematopoietic cells in vivo. Donor cells that had been transplanted to the bone marrow of recipient mice presented molecular and functional characteristics of hematopoietic cells in vitro as well as in vivo [113] (Fig. 13.5).

The mechanism responsible for this cell conversion is still subject for discussion. Some scientists believe in cell reprogramming [114, 115], while others consider SSCs to become pluripotent before differentiating into another cell type [116]. It is also possible that only a certain subpopulation of SSCs is able to transdifferentiate when exposed to a suitable microenvironment. The SSCs expressing Pou5f1 and C-kit^{+} might be pluripotent while the Pou5f1^{+}/C-kit^{-} SSCs are committed to the germ line [103].

Next to autologous therapy, SSCs could be an attractive target cell population for transgenerational therapy as well since these cells are the only stem cells in the human that can transmit parental genetic information to the next generation. It is now possible to transfect both adult and immature mouse stem cells by retroviral-mediated gene delivery in vitro [47]. Also the co-injection of retroviral particles and germ cells into recipient testes resulted in the incorporation of the reporter gene [117].

13.2 Ovarian Stem Cells

13.2.1 From Primordial Germ Cells to Oocytes

The origin of male and female germ cells shows a lot of similarities. Like SSCs, oogonia evolve from PGCs. Before gastrulation, somatic lineages already start to develop in the epiblast cells. However, about six cells, located in the posterior region of the epiblast, receive a higher amount of BMP4. These cells are suppressed to become somatic cells and will develop into PGCs [118]. PGCs migrate from the extraembryonic mesoderm of the wall of the yolk sac toward the gonadal ridges. Following the entrance of the PGCs in the indifferent gonad, the ovary will form and allow the PGCs to differentiate into oogonia and—later—oocytes, which are arrested during meiosis. It has long been believed that, in this stage of fetal life, PGCs and oogonia have disappeared. The arrested oocytes become progressively enclosed by pre-granulosa cells and form the primordial follicles [119]. At birth ovaries are filled with primordial follicles each composed by a meiotically arrested oocyte surrounded by a single layer of flat granulosa cells. After puberty these resting oocytes can be recruited into the pool of growing follicles, of which only one will be ovulated monthly [120].

13.2.2 Ovarian Stem Cells in Postnatal Life?

Until less than 10 years ago, it was accepted as true that the adult mammalian ovary did not contain self-renewing stem cells. According to this long-held dogma in reproductive biology, females are born with a finite population of non-growing follicles that declines with age. In 2004, Tilly's group provided evidence to challenge this doctrine. They showed that, in adult mouse ovaries, neo-oogenesis actually takes place from ovarian stem cells (OSCs) located in the surface epithelium of the ovary (OSE) [121]. Furthermore, the same group claimed the formation of immature oocytes after bone marrow transplantation, suggesting an extraovarian source of OSCs [122, 123]. However, later, it was stated that bone marrow cells were not involved in oocyte formation, but that they improve the function of the ovary after chemotherapy-induced damage [124, 125]. Another report hypothesizes that a small number of PGCs remain in the postnatal ovary, which are able to resume mitosis, enter meiosis, and give rise to oocytes [126] (Fig. 13.6).

It has been reported that, in human, the OSE shows characteristics of both mesenchymal and epithelial cells. The mesenchymal cells in the OSE were found to be capable of developing into OSCs by a mesenchymal-epithelial transition, giving rise to oocytes and de novo folliculogenesis [127, 128]. Even when OSE cells were put in culture, some of the cells were able to form oocyte-like cells [129]. Recently, VASA-positive cells could be purified from neonatal and adult mouse ovaries and maintained in culture for months. The transplantation of these cultured cells in chemotherapy-sterilized recipients led to the development of oocytes and the production of viable offspring [130].

While the presence of PGC-derived stem cells in the ovary is scrutinized [131], neo-oogenesis in the adult ovary remains a matter of big controversy.

13.2.3 Fertility Preservation and Restoration in Girls

In prepubertal girls ovarian tissue cryopreservation is available. The technique involves surgical removal of the cortex containing the immature follicles. After being dissected, the cortex is cut into thin strips and cryopreserved by slow freezing or vitrification. Potential future use of the tissue includes autotransplantation onto the remaining ovary or to a heterotopic site. The former strategy has already led to the first pregnancies and life births [132]. In the latter case, the administration of gonadotrophins is required to stimulate follicular development. The oocytes are harvested and used for in vitro fertilization [133, 134].

In analogy with testis tissue transplantation in male cancer survivors, the risk of reintroducing malignant cells should be taken into consideration. An alternative strategy, currently under development, is in vitro maturation of primordial follicles [135]. Mouse ovarian strips and isolated follicles cultured in a 3D matrix generated mature oocytes, which could be fertilized and yielded healthy life offspring [136, 137].

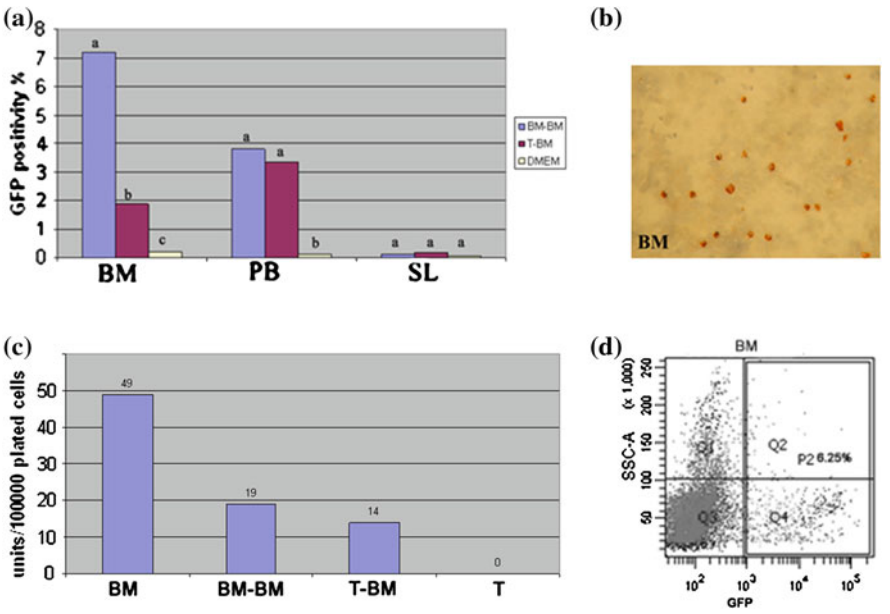

Fig. 13.5 Transdifferentiation of SSCs into bone marrow cells. **a** GFP^+ mouse SSCs were transplanted to the bone marrow (BM) of chemotherapy-treated GFP^- recipient mice (T-BM). Four months after transplantation, FACS analyses showed GFP^+ donor cells in the bone marrow, the peripheral blood (PB), and the spleen of the recipients. As a positive control GFP^+ bone marrow cells were transplanted to the bone marrow of GFP^- recipients. DMEM injected recipients were used as negative controls. **b** Immunostaining confirmed the presence of GFP^+ cells (brown) in the bone marrow of transplanted recipients. **c** A colony forming units assay was employed to test the function of the transdifferentiated cells in vitro. Donor-derived bone marrow cells were isolated from the bone marrow of T-BM recipients and put in culture. The number of colonies that grew up in the dishes was comparable to the BM–BM transplanted controls. **d** To test the in vivo function of the donor-derived bone marrow cells, a second bone marrow transplantation was performed. Twelve weeks after the second transplantation, GFP^+ cells could be observed in the bone marrow of the recipients

Although these techniques are very promising, they should still be considered experimental for humans.

The existence of OSCs would probably not change the strategy for fertility restoration, but can have potential uses in transgenerational gene therapy. So far, no studies have been conducted addressing this topic.

On the other hand, the possible contribution of circulating OSCs or the potential of blood-borne cells to populate the adult ovary requires serious consideration. Especially for those women that have conceived after bone marrow transplantation, genetic analysis of the patients and their children should be implemented [138].

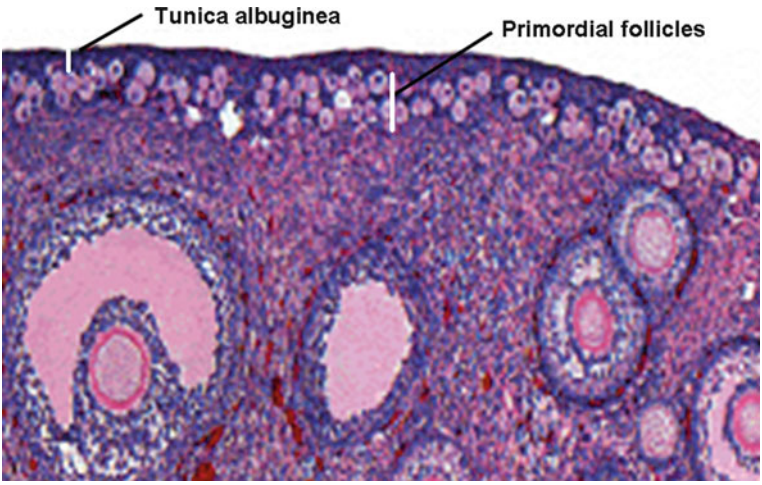

Fig. 13.6 The ovarian cortex. The germ cell epithelium is located in the tunica albuginea of the ovary. The layer underneath the tunica contains primary follicles of which some develop into secondary follicles. Each month, one dominant follicle will enlarge to form a Graafian follicle. The banking and transplantation of the tunica albuginea is a promising technique for fertility preservation in young girls

13.2.4 Conclusive Thought

Banking and transplantation of SSCs may become a promising method to preserve the fertility of prepubertal patients. According to recent discoveries, the potential of SSCs to become pluripotent or to transdifferentiate into other cell types is interesting and may create an additional role for SSCs as a source for stem cell therapy. Nevertheless, the methods established in mice still need to be tested and adapted to human applications.

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References

1. Ying Y, Qi X, Zhao GQ (2001) Induction of primordial germ cells from murine epiblasts by synergistic action of BMP4 and BMP8B signaling pathways. Proc Natl Acad Sci U S A 98(14):7858–7862
2. Fleischman RA (1993) From white spots to stem cells: the role of the Kit receptor in mammalian development. Trends Genet 9(8):285–290
3. Vergouwen RP, Jacobs SG, Huiskamp R, Davids JA, de Rooij DG (1991) Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. J Reprod Fertil 93(1):233–243

4. Ohta H, Wakayama T, Nishimune Y (2004) Commitment of fetal male germ cells to spermatogonial stem cells during mouse embryonic development. Biol Reprod 70(5):1286–1291
5. Kubota H, Brinster RL (2006) Technology insight: In vitro culture of spermatogonial stem cells and their potential therapeutic uses. Nat Clin Pract Endocrinol Metab 2(2):99–108
6. Shinohara T, Avarbock MR, Brinster RL (1999) beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 96(10):5504–5509
7. Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287(5457):1489–1493
8. Giuili G, Tomljenovic A, Labrecque N, Oulad-Abdelghani M, Rassoulzadegan M, Cuzin F (2002) Murine spermatogonial stem cells: targeted transgene expression and purification in an active state. EMBO Rep 3(8):753–759
9. Kubota H, Avarbock MR, Brinster RL (2003) Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci U S A 100(11):6487–6492
10. Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2004) CD9 is a surface marker on mouse and rat male germline stem cells. Biol Reprod 70(1):70–75
11. Naughton CK, Jain S, Strickland AM, Gupta A, Milbrandt J (2006) Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol Reprod 74(2):314–321
12. Izadyar F, Wong J, Maki C, Pacchiarotti J, Ramos T, Howerton K, Yuen C, Greilach S, Zhao HH, Chow M, Chow YC, Rao J, Barritt J, Bar-Chama N, Copperman A (2011) Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. Hum Reprod 26(6):1296–1306
13. Fujita K, Ohta H, Tsujimura A, Takao T, Miyagawa Y, Takada S, Matsumiya K, Wakayama T, Okuyama A (2005) Transplantation of spermatogonial stem cells isolated from leukemic mice restores fertility without inducing leukemia. J Clin Invest 115(7):1855–1861
14. Yoshinaga K, Nishikawa S, Ogawa M, Hayashi S, Kunisada T, Fujimoto T (1991) Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. Development 113(2):689–699
15. Schnieders F, Dork T, Arnemann J, Vogel T, Werner M, Schmidtke J (1996) Testis-specific protein, Y-encoded (TSPY) expression in testicular tissues. Hum Mol Genet 5(11):1801–1807
16. Kido T, Lau YF (2006) The rat Tspy is preferentially expressed in elongated spermatids and interacts with the core histones. Biochem Biophys Res Commun 350(1):56–67
17. Aubry F, Satie AP, Rioux-Leclercq N, Rajpert-De Meyts E, Spagnoli GC, Chomez P, De Backer O, Jegou B, Samson M (2001) MAGE-A4, a germ cell specific marker, is expressed differentially in testicular tumors. Cancer 92(11):2778–2785
18. Maki CB, Pacchiarotti J, Ramos T, Pascual M, Pham J, Kinjo J, Anorve S, Izadyar F (2009) Phenotypic and molecular characterization of spermatogonial stem cells in adult primate testes. Hum Reprod 24(6):1480–1491
19. Nagano M, Brinster RL (1998) Spermatogonial transplantation and reconstitution of donor cell spermatogenesis in recipient mice. APMIS 106(1):47–55 (discussion 56–47)
20. Yeh JR, Zhang X, Nagano MC (2007) Establishment of a short-term in vitro assay for mouse spermatogonial stem cells. Biol Reprod 77(5):897–904
21. Ohmura M, Yoshida S, Ide Y, Nagamatsu G, Suda T, Ohbo K (2004) Spatial analysis of germ stem cell development in Oct-4/EGFP transgenic mice. Arch Histol Cytol 67(4):285–296
22. Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP (2004) Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 36(6):653–659
23. Yoshida S, Takakura A, Ohbo K, Abe K, Wakabayashi J, Yamamoto M, Suda T, Nabeshima Y (2004) Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. Dev Biol 269(2):447–458

24. Raverot G, Weiss J, Park SY, Hurley L, Jameson JL (2005) Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. Dev Biol 283(1):215–225
25. Sada A, Suzuki A, Suzuki H, Saga Y (2009) The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. Science 325(5946):1394–1398
26. Nakagawa T, Sharma M, Nabeshima Y, Braun RE, Yoshida S (2011) Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science 328(5974):62–67
27. Zheng K, Wu X, Kaestner KH, Wang PJ (2009) The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol 9:38
28. Grisanti L, Falciatori I, Grasso M, Dovere L, Fera S, Muciaccia B, Fuso A, Berno V, Boitani C, Stefanini M, Vicini E (2009) Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. Stem Cells 27(12):3043–3052
29. Suzuki H, Sada A, Yoshida S, Saga Y (2009) The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins NANOS2 and NANOS3. Dev Biol 336(2):222–231
30. Oatley MJ, Kaucher AV, Racicot KE, Oatley JM (2011) Inhibitor of DNA binding 4 is expressed selectively by single spermatogonia in the male germline and regulates the self-renewal of spermatogonial stem cells in mice. Biol Reprod 85(2):347–356
31. Phillips BT, Gassei K, Orwig KE (2011) Spermatogonial stem cell regulation and spermatogenesis. Philos Trans R Soc Lond B Biol Sci 365(1546):1663–1678
32. Tran J, Brenner TJ, DiNardo S (2000) Somatic control over the germline stem cell lineage during *Drosophila* spermatogenesis. Nature 407(6805):754–757
33. Ogawa T, Ohmura M, Ohbo K (2005) The niche for spermatogonial stem cells in the mammalian testis. Int J Hematol 82(5):381–388
34. Cheng CY, Mruk DD (2002) Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. Physiol Rev 82(4):825–874
35. Oatley JM, Oatley MJ, Avarbock MR, Tobias JW, Brinster RL (2009) Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development 136(7):1191–1199
36. Yoshida S, Sukeno M, Nabeshima Y (2007) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317(5845):1722–1726
37. Wu X, Schmidt JA, Avarbock MR, Tobias JW, Carlson CA, Kolon TF, Ginsberg JP, Brinster RL (2009) Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of germline stem cell regulatory molecules. Proc Natl Acad Sci U S A 106(51):21672–21677
38. van Beek ME, Meistrich ML, de Rooij DG (1990) Probability of self-renewing divisions of spermatogonial stem cells in colonies, formed after fission neutron irradiation. Cell Tissue Kinet 23(1):1–16
39. Oakberg EF (1971) Spermatogonial stem-cell renewal in the mouse. Anat Rec 169(3):515–531
40. Huckins C (1971) The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat Rec 169(3):533–557
41. Ohbo K, Yoshida S, Ohmura M, Ohneda O, Ogawa T, Tsuchiya H, Kuwana T, Kehler J, Abe K, Scholer HR, Suda T (2003) Identification and characterization of stem cells in prepubertal spermatogenesis in mice small star, filled. Dev Biol 258(1):209–225
42. Barroca V, Lassalle B, Coureuil M, Louis JP, Le Page F, Testart J, Allemand I, Riou L, Fouchet P (2009) Mouse differentiating spermatogonia can generate germinal stem cells in vivo. Nat Cell Biol 11(2):190–196
43. Clermont Y (1966) Renewal of spermatogonia in man. Am J Anat 118(2):509–524
44. van Alphen MM, van de Kant HJ, de Rooij DG (1988) Depletion of the spermatogonia from the seminiferous epithelium of the rhesus monkey after X irradiation. Radiat Res 113(3):473–486
45. Ehmcke J, Simorangkir DR, Schlatt S (2005) Identification of the starting point for spermatogenesis and characterization of the testicular stem cell in adult male rhesus monkeys. Hum Reprod 20(5):1185–1193

46. Nagano M, Avarbock MR, Leonida EB, Brinster CJ, Brinster RL (1998) Culture of mouse spermatogonial stem cells. Tissue Cell 30(4):389–397
47. Nagano M, Shinohara T, Avarbock MR, Brinster RL (2000) Retrovirus-mediated gene delivery into male germ line stem cells. FEBS Lett 475(1):7–10
48. Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL (2003) Maintenance of mouse male germ line stem cells in vitro. Biol Reprod 68(6):2207–2214
49. Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, Shinohara T (2003) Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod 69(2):612–616
50. Kanatsu-Shinohara M, Shinohara T (2007) Culture and genetic modification of mouse germline stem cells. Ann N Y Acad Sci 1120:59–71
51. Kanatsu-Shinohara M, Ogonuki N, Iwano T, Lee J, Kazuki Y, Inoue K, Miki H, Takehashi M, Toyokuni S, Shinkai Y, Oshimura M, Ishino F, Ogura A, Shinohara T (2005) Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. Development 132(18):4155–4163
52. Kanatsu-Shinohara M, Inoue K, Ogonuki N, Morimoto H, Ogura A, Shinohara T (2011) Serum- and feeder-free culture of mouse germline stem cells. Biol Reprod 84(1):97–105
53. Aponte PM, Soda T, Teerds KJ, Mizrak SC, van de Kant HJ, de Rooij DG (2008) Propagation of bovine spermatogonial stem cells in vitro. Reproduction 136(5):543–557
54. Sadri-Ardekani H, Akhondi MA, van der Veen F, Repping S, van Pelt AM (2011) In vitro propagation of human prepubertal spermatogonial stem cells. JAMA 305(23):2416–2418
55. Sadri-Ardekani H, Mizrak SC, van Daalen SK, Korver CM, Roepers-Gajadien HL, Koruji M, Hovingh S, de Reijke TM, de la Rosette JJ, van der Veen F, de Rooij DG, Repping S, van Pelt AM (2009) Propagation of human spermatogonial stem cells in vitro. JAMA 302(19):2127–2134
56. Stukenborg JB, Schlatt S, Simoni M, Yeung CH, Elhija MA, Luetjens CM, Huleihel M, Wistuba J (2009) New horizons for in vitro spermatogenesis? An update on novel three-dimensional culture systems as tools for meiotic and post-meiotic differentiation of testicular germ cells. Mol Hum Reprod 15(9):521–529
57. Izadyar F, Spierenberg GT, Creemers LB, den Ouden K, de Rooij DG (2002) Isolation and purification of type A spermatogonia from the bovine testis. Reproduction 124(1):85–94
58. Oatley JM, de Avila DM, Reeves JJ, McLean DJ (2004) Testis tissue explant culture supports survival and proliferation of bovine spermatogonial stem cells. Biol Reprod 70(3):625–631
59. Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, Kubota Y, Ogawa T (2011) In vitro production of functional sperm in cultured neonatal mouse testes. Nature 471(7339):504–507
60. Howell SJ, Shalet SM (2005) Spermatogenesis after cancer treatment: damage and recovery. J Natl Cancer Inst Monogr 34:12–17
61. Aksglaede L, Wikstrom AM, Rajpert-De Meyts E, Dunkel L, Skakkebaek NE, Juul A (2006) Natural history of seminiferous tubule degeneration in Klinefelter syndrome. Hum Reprod Update 12(1):39–48
62. Tournaye H, Goossens E, Verheyen G, Frederickx V, De Block G, Devroey P, Van Steirteghem A (2004) Preserving the reproductive potential of men and boys with cancer: current concepts and future prospects. Hum Reprod Update 10(6):525–532
63. Schlatt S (2002) Spermatogonial stem cell preservation and transplantation. Mol Cell Endocrinol 187(1–2):107–111
64. Milazzo JP, Vaudreuil L, Cauliez B, Gruel E, Masse L, Mousset-Simeon N, Mace B, Rives N (2008) Comparison of conditions for cryopreservation of testicular tissue from immature mice. Hum Reprod 23(1):17–28
65. Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, Nakata K, Kurome M, Nagashima H, Toyokuni S, Kogishi K, Honjo T, Ogura A (2002) Birth of offspring following transplantation of cryopreserved immature testicular pieces and in vitro micro insemination. Hum Reprod 17(12):3039–3045

66. Kvist K, Thorup J, Byskov AG, Hoyer PE, Mollgard K, Yding Andersen C (2006) Cryopreservation of intact testicular tissue from boys with cryptorchidism. Hum Reprod 21(2):484–491
67. Keros V, Hultenby K, Borgstrom B, Fridstrom M, Jahnukainen K, Hovatta O (2007) Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. Hum Reprod 22(5):1384–1395
68. Jahnukainen K, Ehmcke J, Hergenrother SD, Schlatt S (2007) Effect of cold storage and cryopreservation of immature non-human primate testicular tissue on spermatogonial stem cell potential in xenografts. Hum Reprod 22(4):1060–1067
69. Ohta H, Sakaide Y, Wakayama T (2008) The birth of mice from testicular spermatozoa retrieved from frozen testicular sections. Biol Reprod 78(5):807–811
70. Honaramooz A, Megee SO, Dobrinski I (2002) Germ cell transplantation in pigs. Biol Reprod 66(1):21–28
71. Zeng W, Snedaker AK, Megee S, Rathi R, Chen F, Honaramooz A, Dobrinski I (2009) Preservation and transplantation of porcine testis tissue. Reprod Fertil Dev 21(3):489–497
72. Abrishami M, Anzar M, Yang Y, Honaramooz A (2010) Cryopreservation of immature porcine testis tissue to maintain its developmental potential after xenografting into recipient mice. Theriogenology 73(1):86–96
73. Curaba M, Verleysen M, Amorim CA, Dolmans MM, Van Langendonckt A, Hovatta O, Wyns C, Donnez J (2011) Cryopreservation of prepubertal mouse testicular tissue by vitrification. Fertil Steril 95(4):1229–1234 e1221
74. Curaba M, Poels J, van Langendonckt A, Donnez J, Wyns C (2011) Can prepubertal human testicular tissue be cryopreserved by vitrification? Fertil Steril **95**(6):2123 e2129–2112
75. Brinster RL, Avarbock MR (1994) Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A 91(24):11303–11307
76. Avarbock MR, Brinster CJ, Brinster RL (1996) Reconstitution of spermatogenesis from frozen spermatogonial stem cells. Nat Med 2(6):693–696
77. Honaramooz A, Behboodi E, Blash S, Megee SO, Dobrinski I (2003) Germ cell transplantation in goats. Mol Reprod Dev 64(4):422–428
78. Kim Y, Turner D, Nelson J, Dobrinski I, McEntee M, Travis AJ (2008) Production of donor-derived sperm after spermatogonial stem cell transplantation in the dog. Reproduction 136(6):823–831
79. Schlatt S, Rosiepen G, Weinbauer GF, Rolf C, Brook PF, Nieschlag E (1999) Germ cell transfer into rat, bovine, monkey and human testes. Hum Reprod 14(1):144–150
80. Dobrinski I, Avarbock MR, Brinster RL (1999) Transplantation of germ cells from rabbits and dogs into mouse testes. Biol Reprod 61(5):1331–1339
81. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL (1999) Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes. Biol Reprod 60(2):515–521
82. Goossens E, Frederickx V, De Block G, Van Steirteghem AC, Tournaye H (2003) Reproductive capacity of sperm obtained after germ cell transplantation in a mouse model. Hum Reprod 18(9):1874–1880
83. Goossens E, De Block G, Tournaye H (2008) Computer-assisted motility analysis of spermatozoa obtained after spermatogonial stem cell transplantation in the mouse. Fertil Steril 90(4 Suppl):1411–1416
84. Goossens E, De Rycke M, Haentjens P, Tournaye H (2009) DNA methylation patterns of spermatozoa and two generations of offspring obtained after murine spermatogonial stem cell transplantation. Hum Reprod 24(9):2255–2263
85. Goossens E, de Vos P, Tournaye H (2010) Array comparative genomic hybridization analysis does not show genetic alterations in spermatozoa and offspring generated after spermatogonial stem cell transplantation in the mouse. Hum Reprod 25(7):1836–1842
86. Jahnukainen K, Hou M, Petersen C, Setchell B, Soder O (2001) Intratesticular transplantation of testicular cells from leukemic rats causes transmission of leukemia. Cancer Res 61(2):706–710
87. Geens M, Van de Velde H, De Block G, Goossens E, Van Steirteghem A, Tournaye H (2007) The efficiency of magnetic-activated cell sorting and fluorescence-activated cell

sorting in the decontamination of testicular cell suspensions in cancer patients. Hum Reprod 22(3):733–742
88. Fujita K, Tsujimura A, Miyagawa Y, Kiuchi H, Matsuoka Y, Takao T, Takada S, Nonomura N, Okuyama A (2006) Isolation of germ cells from leukemia and lymphoma cells in a human in vitro model: potential clinical application for restoring human fertility after anticancer therapy. Cancer Res 66(23):11166–11171
89. Geens M, Goossens E, Tournaye H (2011) Cell selection by selective matrix adhesion is not sufficiently efficient for complete malignant cell depletion from contaminated human testicular cell suspensions. Fertil Steril 95(2):787–791
90. Honaramooz A, Snedaker A, Boiani M, Scholer H, Dobrinski I, Schlatt S (2002) Sperm from neonatal mammalian testes grafted in mice. Nature 418(6899):778–781
91. Schlatt S, Honaramooz A, Boiani M, Scholer HR, Dobrinski I (2003) Progeny from sperm obtained after ectopic grafting of neonatal mouse testes. Biol Reprod 68(6):2331–2335
92. Schlatt S, Kim SS, Gosden R (2002) Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and heterotopic grafting to castrated hosts. Reproduction 124(3):339–346
93. Honaramooz A, Li MW, Penedo MC, Meyers S, Dobrinski I (2004) Accelerated maturation of primate testis by xenografting into mice. Biol Reprod 70(5):1500–1503
94. Oatley JM, de Avila DM, Reeves JJ, McLean DJ (2004) Spermatogenesis and germ cell transgene expression in xenografted bovine testicular tissue. Biol Reprod 71(2):494–501
95. Schlatt S, Honaramooz A, Ehmcke J, Goebell PJ, Rubben H, Dhir R, Dobrinski I, Patrizio P (2006) Limited survival of adult human testicular tissue as ectopic xenograft. Hum Reprod 21(2):384–389
96. Geens M, De Block G, Goossens E, Frederickx V, Van Steirteghem A, Tournaye H (2006) Spermatogonial survival after grafting human testicular tissue to immunodeficient mice. Hum Reprod 21(2):390–396
97. Wyns C, Van Langendonckt A, Wese FX, Donnez J, Curaba M (2008) Long-term spermatogonial survival in cryopreserved and xenografted immature human testicular tissue. Hum Reprod 23(11):2402–2414
98. Van Saen D, Goossens E, Bourgain C, Ferster A, Tournaye H (2011) Meiotic activity in orthotopic xenografts derived from human postpubertal testicular tissue. Hum Reprod 26(2):282–293
99. Van Saen D, Goossens E, De Block G, Tournaye H (2009) Regeneration of spermatogenesis by grafting testicular tissue or injecting testicular cells into the testes of sterile mice: a comparative study. Fertil Steril 91(5 Suppl):2264–2272
100. Goossens E, Bilgec T, Van Saen D, Tournaye H (2011) Mouse germ cells go through typical epigenetic modifications after intratesticular tissue grafting. Hum Reprod (2011)
101. Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, Baba S, Kato T, Kazuki Y, Toyokuni S, Toyoshima M, Niwa O, Oshimura M, Heike T, Nakahata T, Ishino F, Ogura A, Shinohara T (2004) Generation of pluripotent stem cells from neonatal mouse testis. Cell 119(7):1001–1012
102. Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G (2006) Pluripotency of spermatogonial stem cells from adult mouse testis. Nature 440(7088):1199–1203
103. Izadyar F, Pau F, Marh J, Slepko N, Wang T, Gonzalez R, Ramos T, Howerton K, Sayre C, Silva F (2008) Generation of multipotent cell lines from a distinct population of male germ line stem cells. Reproduction 135(6):771–784
104. Conrad S, Renninger M, Hennenlotter J, Wiesner T, Just L, Bonin M, Aicher W, Buhring HJ, Mattheus U, Mack A, Wagner HJ, Minger S, Matzkies M, Reppel M, Hescheler J, Sievert KD, Stenzl A, Skutella T (2008) Generation of pluripotent stem cells from adult human testis. Nature 456(7220):344–349
105. Kossack N, Meneses J, Shefi S, Nguyen HN, Chavez S, Nicholas C, Gromoll J, Turek PJ, Reijo-Pera RA (2009) Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells 27(1):138–149

106. Seandel M, James D, Shmelkov SV, Falciatori I, Kim J, Chavala S, Scherr DS, Zhang F, Torres R, Gale NW, Yancopoulos GD, Murphy A, Valenzuela DM, Hobbs RM, Pandolfi PP, Rafii S (2007) Generation of functional multipotent adult stem cells from GPR125 + germline progenitors. Nature 449(7160):346–350
107. Ko K, Arauzo-Bravo MJ, Tapia N, Kim J, Lin Q, Bernemann C, Han DW, Gentile L, Reinhardt P, Greber B, Schneider RK, Kliesch S, Zenke M, Scholer HR (2010) Human adult germline stem cells in question. Nature 465(7301): E1 (discussion E3)
108. Geijsen N, Hochedlinger K (2009) gPS navigates germ cells to pluripotency. Cell Stem Cell 5(1):3–4
109. Glaser T, Opitz T, Kischlat T, Konang R, Sasse P, Fleischmann BK, Engel W, Nayernia K, Brustle O (2008) Adult germ line stem cells as a source of functional neurons and glia. Stem Cells 26(9):2434–2443
110. Streckfuss-Bomeke K, Vlasov A, Hulsmann S, Yin D, Nayernia K, Engel W, Hasenfuss G, Guan K (2009) Generation of functional neurons and glia from multipotent adult mouse germ-line stem cells. Stem Cell Res 2(2):139–154
111. Guan K, Wagner S, Unsold B, Maier LS, Kaiser D, Hemmerlein B, Nayernia K, Engel W, Hasenfuss G (2007) Generation of functional cardiomyocytes from adult mouse spermatogonial stem cells. Circ Res 100(11):1615–1625
112. Simon L, Ekman GC, Kostereva N, Zhang Z, Hess RA, Hofmann MC, Cooke PS (2009) Direct trans differentiation of stem/progenitor spermatogonia into reproductive and nonreproductive tissues of all germ layers. Stem Cells 27(7):1666–1675
113. Ning L, Goossens E, Geens M, Van Saen D, Van Riet I, He D, Tournaye H (2010) Mouse spermatogonial stem cells obtain morphologic and functional characteristics of hematopoietic cells in vivo. Hum Reprod 25(12):3101–3109
114. Baeyens L, Bouwens L (2008) Can beta-cells be derived from exocrine pancreas? Diabetes Obes Metab 10(Suppl 4):170–178
115. Eberhard D, Tosh D (2008) Trans differentiation and metaplasia as a paradigm for understanding development and disease. Cell Mol Life Sci 65(1):33–40
116. Kanatsu-Shinohara M, Shinohara T (2006) The germ of pluripotency. Nat Biotechnol 24(6):663–664
117. Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2004) Transgenic mice produced by retroviral transduction of male germ line stem cells in vivo. Biol Reprod 71(4):1202–1207
118. de Sousa Lopes SM, Hayashi K, Surani MA (2007) Proximal visceral endoderm and extraembryonic ectoderm regulate the formation of primordial germ cell precursors. BMC Dev Biol **7**:140
119. Eppig JJ (2001) Oocyte control of ovarian follicular development and function in mammals. Reproduction 122(6):829–838
120. Edson MA, Nagaraja AK, Matzuk MM (2009) The mammalian ovary from genesis to revelation. Endocr Rev 30(6):624–712
121. Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL (2004) Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 428(6979):145–150
122. Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT, Tilly JL (2005) Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. Cell 122(2):303–315
123. Lee HJ, Selesniemi K, Niikura Y, Niikura T, Klein R, Dombkowski DM, Tilly JL (2007) Bone marrow transplantation generates immature oocytes and rescues long-term fertility in a preclinical mouse model of chemotherapy-induced premature ovarian failure. J Clin Oncol 25(22):3198–3204
124. Eggan K, Jurga S, Gosden R, Min IM, Wagers AJ (2006) Ovulated oocytes in adult mice derive from non-circulating germ cells. Nature 441(7097):1109–1114
125. Fu X, He Y, Xie C, Liu W (2008) Bone marrow mesenchymal stem cell transplantation improves ovarian function and structure in rats with chemotherapy-induced ovarian damage. Cytotherapy 10(4):353–363

126. De Felici M (2010) Germ stem cells in the mammalian adult ovary: considerations by a fan of the primordial germ cells. Mol Hum Reprod 16(9):632–636
127. Okamoto S, Okamoto A, Nikaido T, Saito M, Takao M, Yanaihara N, Takakura S, Ochiai K, Tanaka T (2009) Mesenchymal to epithelial transition in the human ovarian surface epithelium focusing on inclusion cysts. Oncol Rep 21(5):1209–1214
128. Bukovsky A, Caudle MR, Svetlikova M, Upadhyaya NB (2004) Origin of germ cells and formation of new primary follicles in adult human ovaries. Reprod Biol Endocrinol 2:20
129. Bukovsky A, Svetlikova M, Caudle MR (2005) Oogenesis in cultures derived from adult human ovaries. Reprod Biol Endocrinol 3:17
130. Zou K, Yuan Z, Yang Z, Luo H, Sun K, Zhou L, Xiang J, Shi L, Yu Q, Zhang Y, Hou R, Wu J (2009) Production of offspring from a germline stem cell line derived from neonatal ovaries. Nat Cell Biol 11(5):631–636
131. Begum S, Papaioannou VE, Gosden RG (2008) The oocyte population is not renewed in transplanted or irradiated adult ovaries. Hum Reprod 23(10):2326–2330
132. Donnez J, Silber S, Andersen CY, Demeestere I, Piver P, Meirow D, Pellicer A, Dolmans MM (2011) Children born after autotransplantation of cryopreserved ovarian tissue. a review of 13 live births. Ann Med 43(6):437–450
133. Kim SS, Lee WS, Chung MK, Lee HC, Lee HH, Hill D (2009) Long-term ovarian function and fertility after heterotopic autotransplantation of cryobanked human ovarian tissue: 8 year experience in cancer patients. Fertil Steril 91(6):2349–2354
134. Oktay K, Buyuk E, Veeck L, Zaninovic N, Xu K, Takeuchi T, Opsahl M, Rosenwaks Z (2004) Embryo development after heterotopic transplantation of cryopreserved ovarian tissue. Lancet 363(9412):837–840
135. Smitz J, Dolmans MM, Donnez J, Fortune JE, Hovatta O, Jewgenow K, Picton HM, Plancha C, Shea LD, Stouffer RL, Telfer EE, Woodruff TK, Zelinski MB (2010) Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation. Hum Reprod Update 16(4):395–414
136. Abir R, Nitke S, Ben-Haroush A, Fisch B (2006) In vitro maturation of human primordial ovarian follicles: clinical significance, progress in mammals, and methods for growth evaluation. Histol Histopathol 21(8):887–898
137. Xu M, Kreeger PK, Shea LD, Woodruff TK (2006) Tissue-engineered follicles produce live, fertile offspring. Tissue Eng 12(10):2739–2746
138. Hutt KJ, Albertini DF (2006) Clinical applications and limitations of current ovarian stem cell research: a review. J Exp Clin Assist Reprod 3:6
139 Dym M, Kokkinaki M, He Z (2009) Spermatogonial stem cells: mouse and human comparisons. Birth Defects Res C Embryo Today 87(1), 27-34

Chapter 14
Cord Blood Stem Cells and Regenerative Medicine

David T. Harris

Abstract Umbilical cord blood (CB) can provide a virtually unlimited source of diverse stem cells that can be used for hematologic transplants as well as in a variety of regenerative medicine applications. Cord blood units can be frozen and preserved in liquid nitrogen dewars for prolonged periods of time, having been frozen in time at the peak of their biological activity. Multipotent stem cells are available in large numbers in both umbilical cord blood (CB) and cord tissue (CT). CB stem cells are capable of giving rise to hematopoietic, epithelial, endothelial, and neural tissues both in vitro and in vivo. CT stem cells are capable of giving rise to various mesenchymal lineages, including bone, cartilage, and fat. Thus, CB and CT stem cells are candidates to develop stem-cell-based therapies for a wide variety of diseases, including cardiovascular, ophthalmic, orthopedic, neurological, and endocrine diseases. CB and CT have now been used in several regenerative medicine clinical studies, examples of which include cerebral palsy and non-healing bone fractures. CB and CT represent an invaluable source of stem cells that have both immediate and future applications for the individual donor.

14.1 Introduction

The difficulty of finding suitable HLA-matched bone marrow donors for patients needing stem cell transplants, particularly patients from ethnic minorities [1, 2], as well as complications such as graft-versus-host disease (GVHD) which can occur

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more than 50 % of the time in unrelated bone marrow transplants (BMT) [3] led researchers to investigate alternate sources of stem cells for treatment of blood, immune, and metabolic disorders. Research in the early 1980s revealed that the blood in the umbilical cord and placenta after the birth of a child was comparable to bone marrow in stem cell transplantation [4–11], and offered a number of advantages over bone marrow, including a lower incidence of GVHD and less strict HLA-matching requirements [12, 13]. In the past 20 years, more than 25,000 transplants have been performed worldwide [14].

Stem cell transplantation for hematological malignancies and genetic disorders is an uncommon occurrence. However, research performed by several independent laboratories [15–20] demonstrated that cord blood also contains a mixture of pluripotent stem cells capable of giving rise to cells derived from the endodermal, mesodermal, and ectodermal lineages. In addition, mesenchymal stem cells can be isolated from CT and preserved for later use. Thus, CB and CT are readily available for use in tissue engineering and regenerative medicine applications, which are hypothesized to be more frequent events. It is estimated that almost 1 in 3 individuals in the United States, or 128 million people, could benefit over their lifetime from regenerative medicine, including therapies for cardiovascular, neurological, and orthopedic diseases [21]. Diseases such as myocardial infarction, stroke, and spinal cord injury might possibly be treated with greater efficacy using cell-therapy based approaches than current treatment options. Translation of these potential therapies from the laboratory to the clinic requires that the stem cells are medically and economically available. Political and ethical controversy surrounds the use of embryonic stem cells, and significant biological and regulatory concerns limit their clinical use. However, cord blood is non-controversial and if preserved at birth, will provide a source of autologous stem cells for an individual's use in regenerative medicine. Furthermore, cord tissue also exhibits the potential to replace embryonic stem cell sources in the regenerative medicine arena.

In vitro and in vivo research has shown that CB stem cells have the ability to regenerate numerous tissue types, and when transplanted into animals and humans, have produced measurable functional improvements [19, 22]. Clinical trials have begun using cord blood stem cells to treat cerebral palsy and peripheral vascular disease among others [22, 23]. In addition, recent efforts have focused on the isolation, characterization, and utilization of mesenchymal stem cells (MSC) isolated from CT. In fact, CT stem cells have also now made their way into clinical trials. In this paper we will review the latest developments in the use of cord blood and cord tissue stem cells for regenerative medicine.

In terms of clinically available stem cell sources, CB and CT represent an almost limitless supply of stem cells for various clinical uses. In the US there are more than 4 million babies born annually from whom such stem cells may be collected. Stem cells are present in large numbers that may be collected and processed economically, for less than $2,000 and can either be used immediately or banked for future applications [24]. The vast majority of CB collections are red blood cell (RBC)-depleted prior to cryopreservation. Several methods are in use to accomplish this goal including Hespan sedimentation to obtain a modified

buffy coat [12], density gradient centrifugation to obtain enriched mononuclear cells (MNC) [25], and two automated processes (Sepax and the AutoXpress Platform© (AXP) [26, 27], that result in a buffy coat product. The Hespan, Sepax, and AXP processing methods result in cord blood products containing all nucleated cell populations found in the original collection (MNC, neutrophils, some normal and nucleated RBC), while the Ficoll method enriches for the stem cell-containing MNC subpopulation, which is generally greater than 85 % MNC with a few neutrophils and nucleated RBC. The Ficoll and AXP methods routinely recover more than 95 % of the original mononuclear cell population in the sample, as well as more than 95 % of the CD34 + cells and the CFU-GM progenitors, which are both surrogate measures for stem cell recovery. Most of the cord blood stem cells are CD34+ and/or CD133+. These antigens are both useful markers for further purification and either can be utilized with antibody coupled to ferromagnetic beads. As a single cord blood collection can contain 100–500 $\times$ 10^6 MNC or more, one can expect to recover stem cells in the range of 0.5–1.5 % of the starting mononuclear cell fraction. Purity of the stem cells (CD34 + and/or CD133 + cells) can range from 50–95 %. The desired regenerative medicine end use will determine what cell dose and what level of stem cell purity are needed, but generally many applications require some processing and stem cell enrichment.

14.2 Cord Blood and Regenerative Medicine

Stem cell use in regenerative medicine is different from typical stem cell use for hematopoietic transplantation. These applications do not require the aggressive preconditioning regimes that often lead to a myriad of side-effects that may increase morbidity and mortality of the recipient. However, this lack of preconditioning does generally require the use of autologous stem cells for the therapy to be successful. Otherwise, immune rejection will most probably occur [28]. The growth of family cord blood banks over the past 20 years, with an estimated 1,500,000 total samples in storage [29], has now provided significant numbers of patients into these types of clinical trials. A review of several of these applications is shown below.

14.3 Neurological Applications

Cerebrovascular diseases are the third leading cause of death in the United States, not including the multitudes of individuals who survive only to suffer debilitating lifelong injuries and decreased quality of life. Approximately 700,000 people in the United States are affected by stroke annually; and 1 in 16 Americans who suffer a stroke will die from it [30]. The brain is extremely sensitive to hypoxia and some degree of tissue death is likely from stroke. At a relatively young age the

brain loses most of its plasticity so any significant tissue death can be profoundly devastating. The use of stem cells to rescue or replace damaged nervous tissue could provide a much needed therapy for this relatively common event. As early as 2001, it was demonstrated that the infusion of CB stem cells into an animal model of stroke could reverse many of the physical and behavioral deficits associated with this disease [31]. Studies demonstrated that direct injection of the stem cells into the brain was not required [32], and theorized stem cells initiated endogenous repair via the release of growth and repair factors triggered by the anoxia [33, 34]. Significantly, unlike current pharmacological interventions that require treatment within the first few hours after stroke, CB stem cell therapies were effective up to 48 h after the event [35]. In fact, administration of CB stem cells immediately after the ischemic event may not be optimal in that the inflammatory milieu may be toxic to the administered stem cells. Neuroprotective effects [35–40] as well as functional/behavioral improvements [35, 39, 41] from CB therapies have been widely reported. These observations implied that CB therapy mediated both direct restorative effects to the brain as well as trophic neuroprotection. For additional information one is referred to the 2007 review on cell therapies for stroke published in Stroke [42].

Cerebral palsy (CP) is a devastating brain disorder that affects many children worldwide. CB stem cells have shown promise in the treatment of CP in early human trials. Recently, considerable excitement has been generated by anecdotal reports of improvement after CB stem cell infusions in children treated in a clinical study at Duke University. Although not a randomized trial, this treatment has been used to treat more than 50 children with cerebral palsy. Although results of the study have not been published, preliminary observations have been encouraging, and many additional patients are actively enrolled. A placebo-controlled, FDA-registered clinical trial is also underway at the Georgia Health Sciences University [43]. Similar results for children with cerebral palsy have been reported recently by investigators treating children in Europe and Asia (personal communication, Novussanguis Foundation, Paris, France, May 2008). It appears that the younger the patient the more significant the benefits that have been observed. However, the optimal therapeutic regime and the mechanism(s) behind any beneficial effects have yet to be determined.

Additional neural applications for CB stem cells are being investigated at The University of Texas at Houston in an FDA-registered clinical trial to treat children with traumatic brain injury utilizing autologous cord blood stem cell infusions [44]. This study is based on successful results obtained with a similar autologous bone marrow stem cell study and numerous animal studies demonstrating the efficacy of stem cell treatments in models of traumatic brain injury [45].

Finally, observations that CB stem cells can become different types of nervous cells in vitro [19, 22, 23] have led to investigations as to its use in spinal cord injury. Spinal cord-injured rats infused with CB stem cells have shown significant improvements 5 days post-treatment compared to untreated animals. The CB stem cells were observed at the site of injury but not at uninjured regions of the spinal cord [31]. This finding is supported by another study demonstrating that CB stem

cells transplanted into spinal cord injured animals differentiated into various neural cells, improving axonal regeneration and motor function [46]. Significantly, in a recently reported clinical application of CB stem cells to treat a patient with a spinal cord injury, it was stated that transplantation of CB cells improved her sensory perception and mobility in the hip and thigh regions [47]. Both CT and MRI studies revealed regeneration of the spinal cord at the injury site. Neither additional patients nor additional studies in this area have yet been reported. Evaluating the safety and potential efficacy of CB stem cells for spinal cord injury in a clinical trial setting is a logical extension of the pre-clinical work and early case-study report.

14.4 Orthopedic Applications

The potential of CB stem cells to generate bone and cartilage has been recently examined. It is estimated that more than 1 million individuals in the USA annually suffer from articular joint injuries involving cartilage, ligaments, and/or tendons, as well as difficult to heal bone fractures [48]. CB contains both ES-like and MSC capable of differentiating into both bone and cartilage [49]. In fact, when CB stem cells were placed into animals with fractured femurs there was significant bone healing. Work from the laboratories of Szivek [50] and Harris (unpublished data) have also examined the ability of cord blood stem cells to become cartilage in comparison to tissues derived from bone marrow MSC and adipose stem cells, with early encouraging results. As discussed below, CT may be a better source of stem cells for these applications than CB.

14.5 Hearing Loss

A recent animal study demonstrated the CB stem cells may have clinical utility to repair inner ear damage and restore hearing [51]. Human CB stem cells, when intravenously injected into immunodeficient mice made deaf by exposure to kanamycin, high intensity noise, or a combination of these insults, migrated and engrafted into the cochlea of the deaf mice and the levels of engraftment correlated with both the severity of damage and the treatment dose. Analysis at 60 days post-treatment showed that the mice in the CB treatment group had well-repaired cochlea with dramatic hair cell regrowth, while control mice showed no sign of repair or hair cell regeneration. This study has led to a clinical trial to investigate autologous CB infusions for childhood hearing damage that is now being conducted at Memorial Hermann Hospital, Houston TX [52].

14.6 Cord Tissue and Regenerative Medicine

Cord tissue is an additional source of stem cell derived from the birthing process. CT is a rich source of MSC [53] that are able to differentiate into cells of mesenchymal origin, including bone, cartilage, fat, and connective tissue [54]. MSCs were originally isolated from bone marrow [55] but are known to be present in many other tissues, including adipose (fat) tissue [56], placenta [57], and whole umbilical cord tissue [57]. Young tissue-derived MSCs have some unique characteristics that set them apart from older adult-derived MSCs, the most notable of which is their higher proliferation rate and differentiation potential [58–61]. Therefore there may be significant benefits to collection, cryopreservation, and use of these younger MSC in regenerative medicine applications. MSCs also have a number of characteristics that suggest a broad range of potential therapeutic applications including high expansion potential and immunomodulatory ability [62]. Based on promising in vitro and in vivo results for a wide range of conditions [63–70], MSCs, primarily from bone marrow, are currently being used in more than 150 planned, ongoing, or recently-completed clinical trials [71] for conditions, including ischemic injury (heart attack, stroke, critical limb ischemia), autoimmune diseases (type I diabetes, MS, SLE), inflammatory conditions (COPD, Crohn's disease), orthopedic applications (bone fractures, cartilage injuries, osteoarthritis, osteogenesis imperfecta), and transplantation (both stem cells and organs).

As might be expected, when it is possible to harvest and bank MSCs from various tissues at different times throughout the lifespan of the donor, the question is necessarily posed as to why should someone use younger as opposed to older MSC? Numerous studies have indicated that MSC isolated from older donors, as well as from patients with longstanding (chronic) disease conditions are neither as prevalent [72–74] nor as potent [75–79] as those isolated from younger and healthier donors. MSCs collected from older donors and/or donors with chronic diseases (e.g., coronary disease, COPD, etc.) seem to be less able to differentiate into the different cell types needed for tissue engineering [72, 75–79], less able to proliferate and expand to achieve cell concentrations that would allow for multiple treatments [75–79], and are more prone to die off during culture and use [76]. Thus, younger CT stem cells are likely to be more useful for regenerative medicine applications than older bone marrow MSCs. Finally, there is anecdotal evidence that increased MSC donor age and disease status negatively impacts clinical utility and successful clinical outcomes. Data on the success rates of treatment of patients with myocardial infarction and chronic heart disease is mixed, but seems to be negatively correlated with patient age and chronic disease [80].

It is only now that CT stem cells are making their way into the clinic; and it is only now that clinical comparisons to bone marrow MSCs can be made. A recent report from Xue et al. examined the use of CT MSCs in patients with non-healing bone fractures [81]. This study reported significant clinical benefit from intravenously infused MSC. CT MSCs are also showing positive results in treating

GVHD following hematopoietic stem cell transplantation. Two pediatric patients with severe steroid-resistant GVHD were infused with CT MSCs. The GVHD improved dramatically in both patients following infusion of CT MSCs, although one patient received multiple infusions of MSCs over the course of treatment was complete [82]. CT MSCs have also been evaluated for potential therapeutic benefits in autoimmune diseases. Liang et al. reported that CT MSCs stabilized the disease course of a patient with progressive multiple sclerosis that was not responsive to conventional treatment [83]. A subsequent study from the same group reported dramatic improvements in a patient with systemic lupus erythematosus following intravenous infusion of CT MSCs [84]. Importantly none of the case reports indicated adverse effects associated with infusion of CT MSCs.

14.7 Conclusions

Although many individuals have elected to collect and bank cord blood for its potential use in the treatment of hematopoietic cancers and genetic blood, more and more, individuals are now banking both cord blood and cord tissue for uses that are only now being realized or have yet to be discovered. Primarily, these stem cell applications are in tissue engineering and regenerative medicine. Regenerative medicine has the ability to treat many of the above discussed conditions by replacing or repairing malfunctioning tissues. Because regenerative medicine focuses on functional restoration of damaged tissues, not just the abatement or moderation of symptoms, this field has the potential to cut healthcare costs significantly. In the United States cardiovascular disease is the leading cause of death, and the costs associated with major cardiovascular diseases are estimated to be almost $291 billion annually [85]. In the U.S. 1.7 million people sustain a traumatic brain injury annually, and the total costs, both direct medical and indirect costs, associated with traumatic brain injury in 2000 were estimated to be $60 billion [86]. The CDC estimates that 3.3 in 1,000 children in the U.S have cerebral palsy and that the lifetime cost of caring for an individual with cerebral palsy is close to $1 million [87]. However, in order for the promise of regenerative medicine to be realized, it is necessary to identify optimal stem cell sources for particular disease states, and make efforts to inform the lay and medical communities as to their options.

Already the therapeutic potential of cord blood stem cells to treat children born with neurological defects and cerebral palsy, post-natal strokes and type 1 diabetes is being evaluated in clinical trials,. Other trials will surely rapidly follow, including therapies for the eye, joints, wound healing, and spinal cord injuries. In the next decade there will likely be additional uses that are not yet anticipated. The key to these advances lies in the pluripotency of CB and CT stem cells and their ability to be used in many cases under the practice of medicine, since it appears in many instances that it is possible to merely infuse the stem cells directly without timely and costly in vitro culture and differentiation. In my opinion cord blood and

cord tissue stem cells are likely to become the preferred source of stem cells for many regenerative medicine applications, now and in the future.

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References

1. Hows JM (1987) Histocompatible unrelated donors for bone marrow transplantation. Bone Marrow Transpl 1:259–263
2. Bradley BA, Gilks WR, Gore SM, Klouda PT (1987) How many HLA typed volunteer donors for bone marrow transplantation (BMT) are needed to provide an effective service? Bone Marrow Transpl 2(Suppl 1):79
3. Sullivan KM, Weiden PL, Storb R, Witherspoon RP, Fefer A, Fisher L, Buckner CD, Anasetti C, Appelbaum FR, Badger C et al (1989) Influence of acute and chronic graft-versus-host disease on relapse and survival after bone marrow transplantation from HLA-identical siblings as treatment of acute and chronic leukemia. Blood 73(6):1720–1728
4. Broxmeyer HE, Gluckman E, Auerbach A, Douglas GW, Friedman H, Cooper S, Hangoc G, Kurtzberg J, Bard J, Boyse EA (1990) Human umbilical cord blood: a clinically useful source of transplantable hematopoietic stem/progenitor cells. Int J Cell Cloning 8(Suppl 1): 76–89, discussion 89–91
5. Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, Esperou H, Thierry D, Socie G, Lehn P et al (1989) Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. N Engl J Med 321(17):1174–1178
6. Gluckman E (1990) Stem cell harvesting from cord blood: a new perspective. In: Wunder Ha (ed) Peripheral blood stem cell autographs. Springer, Berlin
7. Broxmeyer HA, Kurtzberg J, Gluckman E, Auerbach A, Douglas G, Cooper S, Falkenburg JH, Bard J, Boyse EA (1991) Umbilical cord blood hematopoietic stem and repopulating cells in human clinical transplantation: an expanded role for cord blood transplantation. Blood Cells 17(2):330–337
8. Broxmeyer HE, Kurtzberg J, Gluckman E, Auerbach AD, Douglas G, Cooper S, Falkenburg JH, Bard J, Boyse EA (1991) Umbilical cord blood hematopoietic stem and repopulating cells in human clinical transplantation. Blood Cells 17(2):313–329
9. Broxmeyer HE, Douglas GW, Hangoc G, Cooper S, Bard J, English D, Arny M, Thomas L, Boyse EA (1989) Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. Proc Natl Acad Sci U S A 86(10):3828–3832
10. Vilmer E, Sterkers G, Rahimy C, Denamur E, Elion J, Broyart A, Lescoeur B, Tiercy JM, Gerota J, Blot P (1992) HLA-mismatched cord-blood transplantation in a patient with advanced leukemia. Transplantation 53(5):1155–1157
11. Wagner JE, Kernan NA, Steinbuch M et al (1995) Allogeneic sibling umbilical cord blood transplantation in children with malignant and nonmalignant disease. Lancet 346:214–219
12. Rubinstein P, Rosenfield RE, Adamson JW, Stevens CE (1993) Stored placental blood for unrelated bone marrow reconstitution. Blood 81:1679–1690
13. Gluckman E, Rocha V, Boyer-Chammard A (1997) Outcome of cord-blood transplantation from related and unrelated donors. N Eng J Med 337(6):373–381

14. Butler MG, Menitove JE (2011) Umbilical cord blood banking: an update. J Assist Reprod Genet 28(8):669–676
15. McGuckin C, Forraz N, Baradez MO et al (2005) Production of stem cells with embryonic characteristics from human umbilical cord blood. Cell Prolif 38:245–255
16. McGuckin CP, Forraz N, Allouard Q, Pettengell R (2004) Umbilical cord blood stem cells can expand hematopoietic and neuroglial progenitors in vitro. Exp Cell Res 295:350–359
17. Rogers I, Yamanaka N, Bielecki R, Wong CJ, Chua S, Yuen S, Casper RF (2007) Identification and analysis of in vitro cultured CD45-positive cells capable of multi-lineage differentiation. Exp Cell Res 313:1839–1852
19. Harris DT, He X, Badowski M, Nichols JC (2008) Regenerative medicine of the eye: a short review. In: Levicar N, Habib NA, Dimarakis I, Gordon MY (eds) Stem cell repair and regeneration, vol 3. Imperial College Press, London, p 211–225
20. Sunkomat JNE, Goldman S, Harris DT (2007) Cord blood-derived MNCs delivered intracoronary contribute differently to vascularization compared to CD34 + cells in the rat model of acute ischemia. J Mol Cell Cardiol 42(6) Suppl 1: S97
21. http://www.hhs.gov/asl/testify/2008/05/t20080508c.html
22. Harris DT, Rogers I (2007) Umbilical cord blood: a unique source of pluripotent stem cells for regenerative medicine. Curr Stem Cell Res Ther 2:301–309
23. Harris DT, Badowski M, Ahmad N, Gaballa M (2007) The potential of cord blood stem cells for use in regenerative medicine. Expert Opin Biol Ther 7(9):1311–1322
24. Badowski, MS and Harris, DT (2012) Collection, processing and banking of umbilical cord blood stem cells for transplantation and regenerative medicine. In: Singh SR (Ed) Somatic stem cells: Methods and protocols, methods in molecular biology, vol 879. Humana Press +Springer Science, pp 279–290
25. Harris DT, Schumacher MJ, Rychlik S et al (1994) Collection, separation and cryopreservation of umbilical cord blood for use in transplantation. Bone Marrow Transpl 13:135–143
26. Papassavas AC, Goika V, Chatzistamatiou T et al (2008) A strategy of splitting individual high volume cord blood units into two half subunits prior to processing increases the recovery of cells and facilitates ex vivo expansion of the infused hematopoietic progenitor cells in adults. Intl J Lab Hemat 30(2):124–132
27. Harris DT, McGaffey AP, Schwarz RH et al (2007) Comparing the mononuclear cell (MNC) recovery of AXP and Hespan. Obstet Gynecol 109(4):93S
28. Preynat-Seauve O, Krause K-H (2011) Stem cell sources for regenerative medicine: the immunological point of view. Semin. Immunopathol 33(6):519–524 DOI 10.1007/s00281-011-0271y
29. http://www.cirm.ca.gov/meetings/pdf/2006/06/061306_item_3a.pdf website
30. Furfaro MEK, Gaballa MA (2007) Do adult stem cells ameliorate the damaged myocardium? Is human cord blood a potential source of stem cells? Curr Vasc Pharmacol 5:27–44
31. Chen J, Sanberg PR, Li Y, Wang L, Lu M, Willing AE, Sanchez-Ramos J, Chopp M (2001) Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. Stroke 32:2682–2688
32. Willing AE, Lixian J, Milliken M, Poulos S, Zigova T, Song S, Hart C, Sanchez-Ramos J, Sanberg PR (2003) Intravenous versus intrastriatal cord blood administration in a rodent model of stroke. J Neurosci Res 73(3):296–307
33. Borlongan CV, Hadman M, Sanberg CD, Sanberg PR (2004) Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke. Stroke 35:2385–2389
34. Newman MB, Willing AE, Manressa JJ, Sanberg CD, Sanberg PR (2006) Cytokines produced by cultured human umbilical cord blood (HUCB) cells: implications for brain repair. Exp Neurol 199(1):201–208
35. Newcomb JD, Ajrno CT, Sanberg CD, Sanberg PR, Pennypacker KR, Willing AE (2006) Timing of cord blood treatment after experimental stroke determines therapeutic efficacy. Cell Transpl 15:213–223

36. Xiao J, Nan Z, Motooka Y, Low WC (2005) Transplantation of a novel cell line population of umbilical cord blood stem cells ameliorates neurological deficits associated with ischemic brain injury. Stem Cells Dev 14:722–733
37. Vendrame M, Gemma C, de Mesquita D, Collier L, Bickford PC, Sanberg CD, Sanberg PR, Pennypacker KR, Willing AE (2005) Anti-inflammatory effects of human cord blood cells in a rat model of stroke. Stem Cells Dev 14(5):595–604
38. Vendrame M, Gemma C, Pennypacker KR, Bickford PC, Davis Sanberg C, Sanberg PR, Willing AE (2006) Cord blood rescues stroke-induced changes in splenocyte phenotype and function. Exp Neurology 199(1):191–200
39. Nan Z, Grande A, Sanberg CD, Sanberg PR, Low WC (2005) Infusion of human umbilical cord blood ameliorates neurologic deficits in rats with hemorrhagic brain injury. Ann N Y Acad Sci 1049:84–96
40. Mäkinen S, Kekarainen T, Nystedt J, Liimatainen T, Huhtala T, Närvänen A, Laine J, Jolkkonen J (2006) Human umbilical cord blood cells do not improve sensorimotor or cognitive outcome following transient middle cerebral artery occlusion in rats. Brain Res 1123(1):207–215
41. Nystedt J, Mäkinen S, Laine J, Jolkkonen J (2006) Human cord blood CD34 + cells and behavioral recovery following focal cerebral ischemia in rats. Acta Neurobiologiae Experimentalis (Wars) 66(4):293–300
42. Bliss T, Guzman R, Daadi M, Steinberg GK (2007) Cell transplantation therapy for stroke. Stroke 38:817–826
43. http://www.clinicaltrials.gov/ct2/show/NCT01072370. Accessed 1 Dec 2011
44. http://clinicaltrials.gov/ct2/show/NCT01251003. Accessed 1 Dec 2011
45. Harting MT, Baumgartner JE, Worth LL, Ewing-Cobbs L, Gee AP, Day MC, Cox CS (2008) Cell therapies for traumatic brain injury. Neurosurg Focus 24(34):E18
46. Kuh SU, Cho YE, Yoon DH, Kim KN, Ha Y (2005) Functional recovery after human umbilical cord blood cells transplantation with brain derived-neurotropic factor into the spinal cord injured rats. Acta Neurochirurgica (Wein) 14: 985–992
47. Kang K-S, Kim SW, Oh YH, Yu JW, Kim K-Y, Park HK, Song C-H, Han HA (2005) Thirty-seven-year old spinal cord-injured female patient, transplanted of multipotent stem cells from human UC blood with improved sensory perception and mobility, both functionally and morphologically: a case study. Cytotherapy 7: 368–373
48. www.arthritis.org. Accessed 1 Dec 2011
49. Wang FS, Yang KD, Wang CJ (2004) Shockwave stimulates oxygen radical-mediated osteogenesis of the mesenchymal cells from human umbilical cord blood. J Bone Miner Res 19:973–982
50. Szivek JA, Wiley D, Cox L, Harris DT, Margolis DS, Grana WA (2006) Stem cells grown in dynamic culture on micropatterned surfaces can be used to engineer cartilage like tissue. Abstract presented at the Orthopaedic Research Society Meeting, San Diego, CA
51. Revoltella RP, Papini S, Rosellini A, Michelini M, Franceschini V, Ciorba A, Bertolaso L, Magosso S, Hatzopoulos S, Lorito G, Giordano P, Simoni E, Ognio E, Cilli M, Saccardi R, Urbani S, Jeffery R, Poulsom R, Martini A (2008) Cochlear repair by transplantation of human cord blood CD133 + cells to nod-scid mice made deaf with kanamycin and noise. Cell Transpl 17:665–678
52. http://www.clinicaltrials.gov/ct2/show/NCT01343394 Accessed 21 Dec 2011
53. Abdallah BM, Kassem M (2008) Human mesenchymal stem cells: from basic biology to clinical applications. Gene Ther 15(2):109–116
54. Uccelli A, Moretta L, Pistoia V (2008) Mesenchymal stem cells in health and disease. Nat Rev Immunol 8:726–736
55. Friedenstein AJ, Chailakhjan RK, Lalykina KS (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3:393–403

56. Bieback K, Kern S, Kocaomer A, Ferlik K, Bugert P (2008) Comparing mesenchymal stromal cells from different human tissues: bone marrow, adipose tissue and umbilical cord blood. Biomed Mater Eng 18(1 Suppl):S71–S76
57. Prather WR, Toren A, Meiron M, Ofir R, Tschope C, Horwitz EM (2009) The role of placental-derived adherent stromal cell (PLX-PAD) in the treatment of critical limb ischemia. Cytotherapy 11(4):427–434
58. Jo CH, Kim OS, Park EY et al (2008) Fetal mesenchymal stem cells derived from human umbilical cord sustain primitive characteristics during extensive expansion. Cell Tissue Res 3:423–433
59. Troyer DL, Weiss ML (2008) Wharton's jelly-derived cells are a primitive stromal cell population. Stem Cells 26(3):591–599
60. Pappa KI, Anagnou NP (2009) Novel sources of fetal stem cells: where do they fit on the developmental continuum? Regen Med 4(3):423–433
61. Can A, Karahuseyinoglu S (2007) Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. Stem Cells 25(11):2886–2895
62. Bobis S, Jarocha D, Majka M (2006) Mesenchymal stem cells: characteristics and clinical applications. Folia Histochem Cytobiol 44(4):215–230
63. Yang CC, Shih YH, Ko MH, Hsu SY, Cheng H, Fu YS (2008) Transplantation of human umbilical mesenchymal stem cells from Wharton's jelly after complete transection of the rat spinal cord. PLoS ONE 3(10):e3336
64. Liao W, Xie J, Zhong J et al (2009) Therapeutic effect of human umbilical cord multipotent mesenchymal stromal cells in a rat model of stroke. Transplantation 87(3):350–359
65. Liao W, Zhong J, Yu J et al (2009) Therapeutic benefit of human umbilical cord derived mesenchymal stromal cells in intracerebral hemorrhage rat: implications of anti-inflammation and angiogenesis. Cell Physiol Biochem 24(3–4):307–316
66. Koh SH, Kim KS, Choi MR et al (2008) Implantation of human umbilical cord-derived mesenchymal stem cells as a neuroprotective therapy for ischemic stroke in rats. Brain Res 1229:233–248
67. Weiss ML, Medicetty S, Bledsoe AR et al (2006) Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. Stem Cells 24(3):781–792
68. Ishikane S, Ohnishi S, Yamahara K et al (2008) Allogeneic injection of fetal membrane-derived mesenchymal stem cells induces therapeutic angiogenesis in a rat model of hind limb ischemia. Stem Cells 26(10):2625–2633
69. Bailey MM, Wang L, Bode CJ, Mitchell KE, Detamore MS (2007) A comparison of human umbilical cord matrix stem cells and temporomandibular joint condylar chondrocytes for tissue engineering temporomandibular joint condylar cartilage. Tissue Eng 13(8):2003–2010
70. Wu LF, Wang NN, Liu YS, Wei X (2009) Differentiation of Wharton's jelly primitive stromal cells into insulin-producing cells in comparison with bone marrow mesenchymal stem cells. Tissue Eng Part A 15(10):2865–2873
71. ClinicalTrials.gov | Mesenchymal Stem Cell. http://www.clinicaltrials.gov/ct2/results?intr=mesenchymal+stem+cell. Accessed 1 Dec 2011
72. D'Ippolito G, Schiller PC, Ricordi C, Roos BA, Howard GA (1999) Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. J Bone Miner Res 14(7):1115–1122
73. Tokalov SV, Gruner S, Schindler S, Wolf G, Baumann M, Abolmaali N (2007) Age-related changes in the frequency of mesenchymal stem cells in the bone marrow of rats. Stem Cells Dev 16:439–446
74. Stolzing A, Jones E, McGonagle D, Scutt A (2008) Age-related changes in human bone marrow-derived mesenchymal stem cells: Consequences for cell therapies. Mech Aging Dev 129:163–173
75. Bonyadi M, Waldman SD, Liu D, Aubin JE, Grynpas MD, Stanford SL (2003) Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice. Proc Natl Acad Sci U S A 100(10):5840–5845

76. Stolzing A, Scutt A (2006) Age-related impairment of mesenchymal progenitor cell function. Aging Cell 5(3):213–224
77. Murphy JM, Dixon K, Beck S, Fabian D, Feldman A, Barry F (2002) Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. Arthritis Rheum 46(3):704–713
78. Zhang H, Fazel S, Tian H, Mickle DA, Weisel RD, Fujii T, Li RK (2005) Increasing donor age adversely impacts beneficial effects of bone marrow but no smooth muscle myocardial cell therapy. Am J Physiol Heart Circ Physiol 289(5): H2089–H2096
79. Kretlow JD, Jin YQ, Liu W, Zhang WJ, Hong TH, Zhou G, Baggett LS, Mikos AG, Cao Y (2008) Donor age and cell passage affects differentiation potential of murine bone marrow-derived stem cells. BMC Cell Biology http://www.biomedcentral.com/1471-2121/9/60
80. http://ClinicalTrials.gov website
81. Xue G, He M, Zhao J, Chen Y, Tian Y, Zhao B (2011) Intravenous umbilical cord mesenchymal stem cell infusion for the treatment of combined malnutrition nonunion of the humerus and radial nerve injury. Regenerative Med 6:733–741
82. Wu KH, Chan CK, Tsai C, Chang YH, Sieber M, Chiu TH, Ho M, Peng CT, Wu HP, Huang JL (2011) Effective treatment of severe steroid-resistant acute graft-versus-host disease with umbilical cord-derived mesenchymal stem cells. Transplantation 91(12):1412–1416
83. Liang J, Zhang H, Hua B, Wang H, Wang J, Han Z, Sun L (2009) Allogeneic mesenchymal stem cells transplantation in treatment of multiple sclerosis. Mult Scler 15(5):644–646
84. Liang J, Gu F, Wang H, Hua B, Hou Y, Shi S, Lu L, Sun L (2010) Mesenchymal stem cell transplantation for diffuse alveolar hemorrhage in SLE. Nat Rev Rheumatol 6(8):486–489
85. http://www.cdc.gov/chronicdisease/resources/publications/AAG/dhdsp.htm. Accessed 30 Nov 2011
86. http://www.cdc.gov/traumaticbraininjury/statistics.html. Accessed 30 Nov 2011
87. http://www.cdc.gov/ncbddd/cp/data.html. Accessed 30 Nov 30 2011

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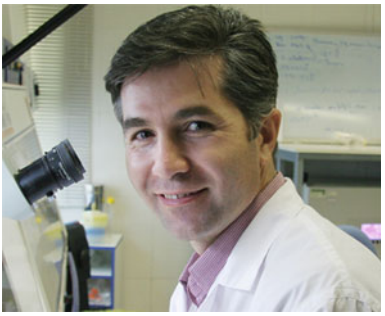

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