Stem Cell Biology and Regenerative Medicine

Mariusz Z. Ratajczak *Editor*

Adult Stem Cell Therapies: Alternatives to Plasticity

Humana Press

Stem Cell Biology and Regenerative Medicine

Series Editor
Kursad Turksen, Ph.D.
kursadturksen@gmail.com

For further volumes:
http://www.springer.com/series/7896

Mariusz Z. Ratajczak
Editor

Adult Stem Cell Therapies: Alternatives to Plasticity

Humana Press

Editor
Mariusz Z. Ratajczak
Stem Cell Institute
James Graham Brown Cancer Center
University of Louisville
Louisville, KY, USA

ISSN 2196-8985 ISSN 2196-8993 (electronic)
ISBN 978-1-4939-1000-7 ISBN 978-1-4939-1001-4 (eBook)
DOI 10.1007/978-1-4939-1001-4
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2014947333

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

The field of regenerative medicine is searching for stem cells that can be safely and efficiently employed in the regeneration of damaged organs. The ideal stem cell type would be pluripotent stem cells, which, according to their definition, have a broad potential to differentiate into cells from all three germ layers (meso-, ecto-, and endoderm). However, true pluripotent/multipotent stem cells in adult tissues are extremely rare and are still waiting to be tested in the clinic. In the meantime, various types of stem cells isolated from adult tissues are being employed in several in vivo experiments and in clinical trials aimed at regeneration of damaged organs (e.g., myocardial infarction, liver damage, ischemic kidney failure, or stroke). There is also mounting evidence that stem cells secrete a variety of growth factors, cytokines, chemokines, and bioactive lipids that regulate their biology in an autocrine/paracrine-manner and orchestrate interactions with the surrounding microenvironment. In addition to soluble factors, cells also secrete microvesicles (MVs), which are small, spherical membrane fragments shed from the cell surface or secreted from the endosomal compartment that seem to play an important and underappreciated role in improving the function of damaged organs. Such cell-derived paracrine signals may explain why the final therapeutic benefits are similar when applying different types of stem cells.

This book is focused on stem cells isolated from adult tissues and the various potential mechanisms involved in their therapeutic effects. The first chapter provides a general overview of the various stem cells isolated from embryonic and adult tissues that are endowed with pluri/multipotential differentiation potential and their advantages and limitations in clinical applications. In chapter 2, we describe the potential mechanisms involved in the therapeutic effects of adult stem cells, with special emphasis on a population of very small embryonic-like stem cells. Human umbilical cord blood is a promising source of stem cells, and this topic will be reviewed in the third chapter. Dr. Ewa Zuba-Surma et al. discuss various types of stem cells that are present in this promising source of cells for therapeutic applications. It has been postulated for many years that hematopoietic stem/progenitor cells express the CD34 antigen. Dr. Sonoda et al. focus on a population of CD34-negative cells that possess broader hematopoietic potential, and, as his group

proposes, is a population of the most primitive hematopoietic stem cells. The full hematopoietic potential of these cells is revealed after intra-bone injection. For more than forty years, hematopoietic stem cells isolated from bone marrow and, more recently, mobilized peripheral blood or umbilical cord blood have been employed to treat patients suffering from several malignant and non-malignant hematological and metabolic disorders. In the footsteps of hematologists, cardiologists have also tried to employ various types of cells to treat patients suffering from heart infarct or chronic heart insufficiency. This important issue is discussed by Dr. Wojciech Wojakowski et al. in chapter 5.

In the following chapter, Dr. Ahmed Abdel-Latif and Dr. Yuri Klyachkin discuss important mechanisms involved in the trafficking of stem cells during heart infarct, with special emphasis on the role of bioactive lipids such as sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P) in the mobilization of stem cells from hematopoietic tissues and their homing to injured myocardium. Another important clinical area with a research focus on stem cells is neurology. Dr. Cesar V. Borlongan et al. presents an overview of the potential applications of various types of stem cells in neurology. Moreover, recent evidence indicates that changes in trafficking of stem cells may also be involved in some psychiatric disorders, and this novel and exciting field is presented by Dr. Jerzy Samochowiec and Dr. Jolanta Kucharska-Mazur. Another important clinical area for stem cell therapies is dermatology, particularly in patients that are suffering from extensive skin burns. Dr. Justyna Drukala and Dr. Julia Borowczyk explore the involvement of local epidermal stem cells and mobilized stem cells circulating in peripheral blood.

An important clinical area for potential stem cell therapies is prematurely born neonates. In these youngest of patients, a potential source of stem cells is their own umbilical cord blood. This novel application of stem cells in neonatology is presented by Dr. Bogusław Machaliński. In the past few years, it has been proposed that ovaries contain stem cells that are the precursors of oocytes. Dr. Deepa Bhartiya et al. examine the concept that ovarian surface epithelium contains a population of very small embryonic-like stem cells that are precursors of oocytes. Next, Dr. Dong-Myung Shin et al. addresses the molecular mechanisms that explain the quiescent state of very small embryonic-like stem cells. Reversal of this state is an important issue in broadly applying these cells in the clinic. Dr. Giovanni Camusi et al. and Dr. Peter Quesenberry et al. discuss the potential role of membrane-derived microvesicles in cell-to-cell communication and their potential involvement in organ regeneration. Besides soluble factors, cell membrane-derived vesicles are responsible for several beneficial effects of stem cell therapies. The final chapter presented by Dr. Jerzy Kawiak et al. is focused on changes in the profile of circulating stem cells during systemic sepsis. The number and profile of the phenotypes of various types of stem cells that are mobilized into peripheral blood in this stress situation as well as a profile of some factors that affect their trafficking may be of novel prognostic value.

There are many individuals that I wish to thank, without whose efforts this book would not have been possible. First and foremost, a special thanks goes to all the authors who wrote chapters and shared with readers their experience and novel

research. These individuals are recognized experts in the various areas of stem cell research. I would also like to thank Dr. Kursad Turksen and our acquisitions editor, Ms. Aleta Kalkstein, for their patience, encouragement, and valuable help. It was an intellectual pleasure to work with them on this book.

Contents

Contributors

Ahmed Abdel-Latif Department of Cardiology, University of Kentucky, Lexington, KY, USA

Sandra Acosta Center of Excellence for Aging & Brain Repair, Department of Neurosurgery and Brain Repair, Morsani College of Medicine, University of South Florida, Tampa, FL, USA

Marta Adamiak Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland

J. Aliotta Department of Medicine, Division of Hematology/Oncology, Rhode Island Hospital and Brown University, Providence, RI, USA

Sandhya Anand Stem Cell Biology Department, National Institute for Research in Reproductive Health, Indian Council of Medical Research, Mumbai, India

Deepa Bhartiya Stem Cell Biology Department, National Institute for Research in Reproductive Health, Indian Council of Medical Research, Mumbai, India

Cesar V. Borlongan Center of Excellence for Aging & Brain Repair, Department of Neurosurgery and Brain Repair, Morsani College of Medicine, University of South Florida, Tampa, FL, USA

Julia Borowczyk Laboratory of Cell & Tissue Engineering, Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland

Giovanni Camussi Department of Medical Sciences, University of Turin, Turin, Italy

D. Chatterjee Department of Medicine, Division of Hematology/Oncology, Rhode Island Hospital and Brown University, Providence, RI, USA

Agata Czekaj Third Division of Cardiology, Medical University of Silesia, Katowice, Poland

Travis Dailey Center of Excellence for Aging & Brain Repair, Department of Neurosurgery and Brain Repair, Morsani College of Medicine, University of South Florida, Tampa, FL, USA

M. Del Tatto Department of Medicine, Division of Hematology/Oncology, Rhode Island Hospital and Brown University, Providence, RI, USA

Maria Chiara Deregibus Department of Medical Sciences and Translational Center for Regenerative Medicine, University of Turin, Turin, Italy

M. Dooner Department of Medicine, Division of Hematology/Oncology, Rhode Island Hospital and Brown University, Providence, RI, USA

Justyna Drukała Laboratory of Cell & Tissue Engineering, Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland

L. Goldberg Department of Medicine, Division of Hematology/Oncology, Rhode Island Hospital and Brown University, Providence, RI, USA

Pranesh Gunjal Stem Cell Biology Department, National Institute for Research in Reproductive Health, Indian Council of Medical Research, Mumbai, India

Jinbeom Heo Department of Biomedical Sciences, Department of Physiology, University of Ulsan College of Medicine, Seoul, Korea

Grażyna Hoser Laboratory of Flow Cytometry, The Centre of Medical Postgraduate Education, Warsaw, Poland

Alessandra Iavello Department of Medical Sciences, University of Turin, Turin, Italy

Jaeho Jeong Department of Biomedical Sciences, Department of Physiology, University of Ulsan College of Medicine, Seoul, Korea

Yuji Kaneko Center of Excellence for Aging & Brain Repair, Department of Neurosurgery and Brain Repair, Morsani College of Medicine, University of South Florida, Tampa, FL, USA

Hyunsook Kang Department of Biomedical Sciences, Department of Physiology, University of Ulsan College of Medicine, Seoul, Korea

Jerzy Kawiak Laboratory of Flow Cytometry, The Centre of Medical Postgraduate Education, Warsaw, Poland

YongHwan Kim Department of Biomedical Sciences, Department of Physiology, University of Ulsan College of Medicine, Seoul, Korea

Yuri Klyachkin Department of Cardiology, University of Kentucky, Lexington, KY, USA

Jolanta Kucharska-Mazur Department of Psychiatry, Pomeranian Medical University, Szczecin, Poland

Magda Kucia Stem Cell Institute, James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA

Jisun Lim Department of Biomedical Sciences, Department of Physiology, University of Ulsan College of Medicine, Seoul, Korea

Bogusław Machaliński Department of General Pathology, Pomeranian Medical University, Szczecin, Poland

Zbigniew Madeja Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland

Mibel Pabon Center of Excellence for Aging & Brain Repair, Department of Neurosurgery and Brain Repair, Morsani College of Medicine, University of South Florida, Tampa, FL, USA

Seema Parte Stem Cell Biology Department, National Institute for Research in Reproductive Health, Indian Council of Medical Research, Mumbai, India

Hiren Patel Stem Cell Biology Department, National Institute for Research in Reproductive Health, Indian Council of Medical Research, Mumbai, India

M. Pereira Department of Medicine, Division of Hematology/Oncology, Rhode Island Hospital and Brown University, Providence, RI, USA

P. Quesenberry Department of Medicine, Division of Hematology/Oncology, Rhode Island Hospital and Brown University, Providence, RI, USA

B. Ramratnam Department of Medicine, Division of Hematology/Oncology, Rhode Island Hospital and Brown University, Providence, RI, USA

Janina Ratajczak Stem Cell Institute, James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA

Mariusz Z. Ratajczak Stem Cell Institute, James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA

Jerzy Samochowiec Department of Psychiatry, Pomeranian Medical University, Szczecin, Poland

Dong-Myung Shin Department of Biomedical Sciences, Department of Physiology, University of Ulsan College of Medicine, Seoul, Korea

Tomasz Skirecki Laboratory of Flow Cytometry, The Centre of Medical Postgraduate Education, Warsaw, Poland

Yoshiaki Sonoda Department of Stem Cell Biology and Regenerative Medicine, Graduate School of Medical Science, Kansai Medical University, Osaka, Japan

A. Sorokina Department of Medicine, Division of Hematology/Oncology, Rhode Island Hospital and Brown University, Providence, RI, USA

Kalpana Sriraman Stem Cell Biology Department, National Institute for Research in Reproductive Health, Indian Council of Medical Research, Mumbai, India

Meaghan Staples Center of Excellence for Aging & Brain Repair, Department of Neurosurgery and Brain Repair, Morsani College of Medicine, University of South Florida, Tampa, FL, USA

Malwina Suszynska Stem Cell Institute, James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA

Naoki Tajiri Center of Excellence for Aging & Brain Repair, Department of Neurosurgery and Brain Repair, Morsani College of Medicine, University of South Florida, Tampa, FL, USA

Michał Tendera Third Division of Cardiology, Medical University of Silesia, Katowice, Poland

Ciro Tetta Translational Center for Regenerative Medicine, University of Turin, Turin, Italy

S. Wen Department of Medicine, Division of Hematology/Oncology, Rhode Island Hospital and Brown University, Providence, RI, USA

Wojciech Wojakowski Third Division of Cardiology, Medical University of Silesia, Katowice, Poland

Ewa K. Zuba-Surma Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland

About the Editor

Mariusz Z. Ratajczak, M.D., Ph.D., D.Sci. ***d.h.c*** is the Henry M. and Stella M. Hoenig Endowed Chair in Cancer Biology and the Director of the Stem Cell Institute at the University of Louisville's James Graham Brown Cancer Center, Kentucky, USA. Dr. Ratajczak earned his M.D. at the Pomeranian Medical University in Szczecin, Poland, and his D.Sci. and Ph.D. in hematopoietic transplantation at the Center for Clinical and Experimental Medicine in Warsaw, Poland. He completed fellowships at the Polish Academy of Sciences in Warsaw and the University of Pennsylvania in Philadelphia, USA. Dr. Ratajczak is an internationally known specialist in the field of adult stem cell biology. He is section editor of *Leukemia* and *Blood Cancer Journal,* and he is a member of the editorial board for *Journal of Cellular and Molecular Medicine, Stem Cells,* and *Stem Cell Reviews and Reports.* He also has published numerous books and more than 400 peer-reviewed publications and is a frequent speaker at conferences worldwide.

Chapter 1
Regenerative Medicine and the Search for Pluripotent/Multipotent Stem Cells

Mariusz Z. Ratajczak

1.1 Introduction

By the beginning of the third millennium, mankind has acquired powerful technologies attributed, as some people believe, only to supreme beings. While the development of physics has enabled us to explore nuclear energy, the development of biology and genetics has begun to explain the mysteries of the creation of organisms and their regeneration, thus leading humanity into the fascinating world of stem cells [1, 2]. Various therapeutic strategies employing stem cells have been proposed as alternative therapies for a multitude of diseases that are difficult to treat using standard methods. It is believed that technologies leading to optimization of the clinical use of stem cells in the new developing clinical discipline of regenerative medicine will become the key to increased longevity [3].

The goal of regenerative medicine is the use of stem cells in therapies for injured organs and tissues. It is believed that in the future, transplantation of entire organs will be largely replaced by the transplantation of a suspension of stem cells directed to the given organ, which will perform the task of rebuilding the injured tissues. The rapidly evolving field of regenerative medicine offers hope that stem cells can be employed to treat injured organs such as myocardium after heart infarction, brain after stroke, spinal cord after mechanical injury, damaged liver, extensive skin burns, as well as diabetes and Parkinson's disease [4, 5].

For the purposes of regenerative medicine, the ideal stem cells would be pluripotent stem cells (PSCs), which, according to their definition, have a broad potential to differentiate into cells from all three germ layers (meso-, ecto-, and endoderm). Such cells can be isolated from embryonic tissues. The use of PSCs in clinical medicine has brought hope to the world for the development of new therapeutic methods. At the same time, it has stimulated a broad discussion of many religious and ethical concerns. The ethics of using these cells is controversial, as it touches on the

M. Z. Ratajczak (✉)
Stem Cell Institute, James Graham Brown Cancer Center, University of Louisville, 500 South Floyd Street, Rm. 107, 40202 Louisville, KY, USA
e-mail: mzrata01@louisville.edu

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,* Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_1,

definition of the beginning of human life, which differs between the major religions of the world [1, 6, 7]. The major technical problem with these cells, however, is that they may grow teratomas after transplantation into recipients.

Therefore, an alternative possibility is being explored to search for stem cells isolated from postnatal tissues that can be efficiently employed in regenerative medicine. A few years ago, the concept was proposed that adult stem cells (e.g., hematopoietic stem cells) are plastic and may extensively transdifferentiate into cells from different germ layers, but this possibility lacks solid experimental support. As a result, the concept of stem cell plasticity or transdifferentiation has been challenged, and some positive effects of stem cell therapies have been explained by alternative mechanisms such as cell fusion and paracrine effects of stem cells employed in therapy due to released growth factors, cytokines, chemokines, and microvesicles. Alternatively, it has been proposed that stem cells employed for therapy derived from bone marrow (BM), mobilized peripheral blood (mPB), or umbilical cord blood (UCB), may, from the beginning, contain heterogeneous populations of stem cells, including some very rare multipotent or pluripotent stem cells. A great deal of effort has been made to unleash the power of these cells, and several preclinical studies in experimental models employing these cells are ongoing.

1.2 Definition of Stem Cell, Hierarchy, and Heterogeneity of the Stem Cell Compartment

A stem cell has been described as a cell that is able to renew itself and to differentiate into daughter cells [2]. However, this definition is too simple, because many types of stem cells differ between themselves according to their proliferative potential and ability to differentiate. The stem cell pool balances the number of somatic cells throughout the organism and is thus responsible for the renewal of somatic cells that are depleted over time as well as regeneration of damaged organs and tissues [3].

Stem cells are very heterogeneous, and it is difficult to describe them by one common definition. There is a large degree of hierarchy and heterogeneity within the pool of stem cells, ranging from the most developmentally primitive to those that are more-or-less organ/tissue-specific (Table 1.1). An adult mammal develops from the earliest stem cell, the fertilized ovum (zygote), which is the totipotent stem cell that, according to the definition, gives rise to cells of both placenta and embryo. After the first division during embryonic development, the totipotent stem cell differentiates immediately into PSCs, which are located in the morula (the embryonic stage consisting of approximately 30 cells) and subsequently in the embryonic node of the blastocyst called the inner cell mass (which consists of approximately 250 cells) [2]. PSCs cannot regenerate the placenta, but they give rise to all three germ layers (ecto-, meso-, and endoderm), and they may differentiate into multipotent stem cells (which can give rise to cells of two germ layers) and finally into so-called monopotent or tissue-specific stem cells (TSSC), which are able to differentiate into only one type of tissue. It has been calculated that, starting from the zygote, after

Table 1.1 Developmental hierarchy in the stem cell (SC) compartment

Totipotent SCs	Give rise to both embryo and placenta. The physiological totipotent stem cell is a fertilized oocyte (zygote) or first blastomere. Its artificial counterpart is a clonote, obtained by nuclear transfer into an enucleated oocyte
Pluripotent SCs	Give rise to all three germ layers of the embryo after injection into the developing blastocyst. Pluripotent stem cells from the inner cell mass (ICM) of the blastocyst are known as embryonic stem cells (ESCs)
Multipotent SCs	Give rise to cells from one or two of the germ cell layers (ecto-, meso-, or endoderm) only
Monopotent SCs	Tissue-committed stem cells that give rise to cells of one lineage, e.g., hematopoietic stem cells, epidermal stem cells, intestinal epithelium stem cells, neural stem cells, liver stem cells, or skeletal muscle stem cells

approximately 47 divisions a total of 10^6 cells are created, which belong to 200 different cell types composing the tissues and organs of the human body. All of these cell types are usually derived from specific monopotent TSSCs.

TSSCs "work" hard throughout the entire lifetime of an individual. It is known that the intestinal epithelium is replaced every 48 h, the epidermis every 14 days, granulocytes every week, and erythrocytes have a physical half-life of 100–150 days [8, 9]. The replacement of depleted cells is slower in other organs and tissues; nevertheless, it has been proven that even such organs as heart or brain exhibit slow biological regeneration. It is hard to imagine that a single cell could live in an organ for 80 years.

Taking into consideration the enormous potential of stem cells and the important role they play in everyday regeneration of several types of tissues, stem cells have become an object of extensive interest for clinicians and are considered key potential targets for modern pharmacology to improve quality of life and extend life span.

1.3 The Potential Sources of Stem Cells to Regenerate Damaged Tissues and Organs

From a historical point of view, hematopoietic stem cells (HSCs) were the first stem cells to be employed in the clinic and have been used successfully for more than 40 years. HSCs are an example of TSSCs that play a role in hematopoiesis [10, 11]. Such cells are relatively easily isolated from BM, mPB, or UCB. For purposes of regenerative medicine, non-hematopoietic stem cells may also be isolated from adipose tissue or expanded ex vivo from biopsies of epidermis, skeletal muscle, or even myocardium [8, 12, 13]. Conversely, due to obvious ethical and technical considerations, it is much more difficult to obtain stem cells from liver, pancreatic islets, or the central nervous system of healthy donors.

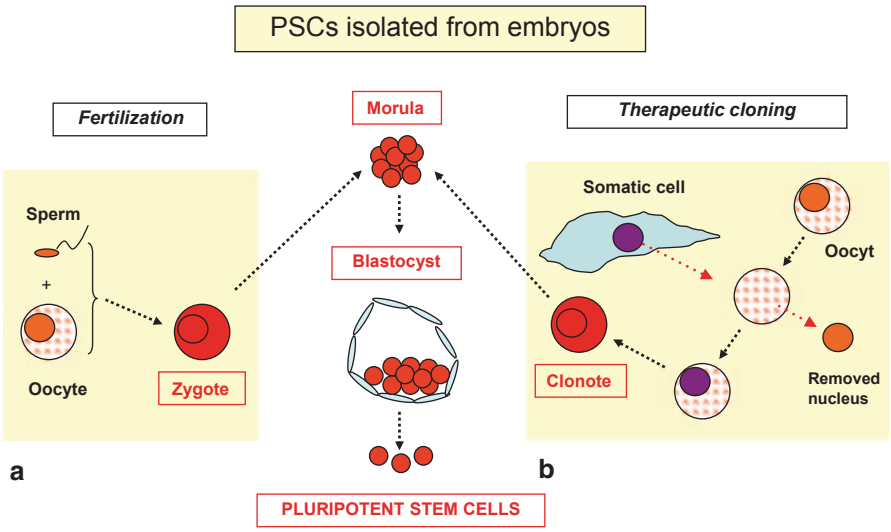

Fig. 1.1 Pluripotent stem cells (*PSCs*) obtained from embryos. **a** *PSCs* isolated from zygote-derived blastocysts derived in the normal process of fertilization. **b** *PSCs* can also be obtained by means of so-called therapeutic cloning as the result of transfer of the nucleus from adult somatic cells into an enucleated oocyte. A totipotent stem cell generated by this strategy is called a clonote, which, like a zygote, will give rise to a blastocyst that is a potential source of *PSCs*

Nevertheless, based on encouraging data in experimental animals, several types of stem cells isolated from embryonic and adult tissues hold promise for treating patients. Thus, we will discuss embryonic and non-embryonic sources of stem cells.

1.4 Embryonic Sources of PSCs and Induced Pluripotent Stem Cells (iPSCs)

There are two potential sources of PSCs isolated from embryos (Fig. 1.1), namely those isolated from surplus embryos stored in in vitro fertilization clinics or PSCs obtained by employing nuclear transfer to oocytes in the process of so-called therapeutic cloning. Both strategies require oocyte donors. An additional interesting strategy for obtaining PSCs is generation of induced pluripotent stem cells (iPSCs) by employing genetic modification of adult somatic cells. Since the latter strategy creates cells that may complete blastocyst development, it will be discussed together with stem cells isolated from embryos.

Pluripotent stem cells isolated from embryos As mentioned above, embryonic tissues are a potential source of PSCs, and such cells can be obtained from the developing morula or blastocyst (Fig. 1.1) using, for example, frozen surplus embryos that are stored in in vitro fertility clinics. By this means, several human embryonic

stem cell lines have been established [13, 14]. However, these cell lines have been demonstrated to change their properties over time. Obtaining PSCs from stored frozen human embryos for therapeutic purposes is also controversial, and it is commonly known that there will be tissue histocompatibility differences between stem cells derived from such embryos and potential recipients. Specifically, established embryonic cell lines will differentiate into cells with different sets of histocompatibility antigens than the potential recipient, and will be recognized by the immune system of the recipient as allogeneic, because the embryo has come from a human leukocyte antigen (HLA)-incompatible donor [15]. In addition, given ethical and technical considerations, it is hard to imagine that it would be possible to obtain such embryos on demand for a specific patient from biological parents. Finally, studies in experimental animals have demonstrated that administration of established embryonic cell lines may lead to development of teratomas [16, 17]. Nevertheless, the difficult dilemma remains of what to do with frozen embryos in embryo banks worldwide: whether to keep them indefinitely in a hibernation state, thaw and destroy them, or use them in basic science research.

Pluripotent stem cells obtained as a result of therapeutic cloning Taking into consideration the ethical aspects as well as technical problems in obtaining normal human embryos and with the awareness that PSCs received from such embryos will differentiate into tissues that are not histocompatible with the recipient's tissues, a strategy has been developed for obtaining PSCs from early embryos created artificially in the lab as a result of so-called therapeutic cloning. The strategy of therapeutic cloning consists in creating a cell in vitro called a clonote (Fig. 1.1), which is equivalent to a zygote in developmental potential [18, 19]. During the creation of clonotes, the nucleus is removed from the donated oocyte and the cytoplasm of the ovum is used as a biochemical incubator to dedifferentiate the injected patient-derived somatic cell nucleus (from a fibroblast or lymphocyte). After transfer (microinjection) of the somatic cell nucleus into the cytoplasm of the ovum, the microinjected chromosomes undergo dedifferentiation. Generally speaking, this process consists of demethylation of deoxyribonucleic acid (DNA) and rearrangement and establishment of proper methylation patterns and acetylation of histone proteins that are present in embryonic stem cells. All of this leads to the loosening of chromatin structure and to the return of developmentally differentiated DNA derived from the donor's somatic cell to the state it had in the fertilized ovum. As described above, these clonotes generated by nuclear transfer are, like the fertilized ovum, totipotent, and artificially created embryos derived from such cells could be a potential source of PSCs when the embryo grows to the level of morula or blastula. What is most important, such cells will be histocompatible with the donor of the nucleus employed in therapeutic cloning [20, 21]. Thus, this is a potential strategy for generating custom-made iPSCs for therapy. Nevertheless, besides some ethical concerns, the main obstacles to broader usage of therapeutic cloning are the necessity of obtaining human oocytes and observations that PSCs derived from animal clonotes, like animal embryonic cells, create teratomas (Table 1.2) [22, 23].

Table 1.2 Various potential sources of pluripotent stem cells

	PSCs isolated from embryos obtained by fertilization and stored in embryo banks	PSCs isolated from embryos obtained by therapeutic cloning	PSCs obtained as a result of transformation of somatic cells (induced PSCs)
Risk of developing teratomas	Yes	Yes	Yes
Histocompatibility problems	Yes	No	No/yes[a]
Requires ovum donor	Yes	Yes	No
Ethical considerations	Yes	Yes/no[b]	No

PSCs Pluripotent stem cells, *iPSCs* Induced pluripotent stem cells

[a] It has recently been reported that iPSCs may be immunogenic. This possibility, however, needs further study

[b] This problem is considered differently by the various major religions of the world. A number of religions potentially accept therapeutic cloning (for example, Judaism, Islam, and Buddhism), but unquestionably a majority reject reproductive cloning

On the other hand, if a clonote is placed in the uterus, it can, like a zygote, give rise to a new individual. This is the basis for so-called reproductive cloning, which, at least for human application, is highly dangerous and considered unethical [23].

Induced pluripotent stem cells (iPSC) These non-controversial, from an ethical point of view, stem cells are derived by genetic modification of postnatal somatic cells (Fig. 1.2). They are obtained as a result of transformation in vitro of mature somatic cells using genes encoding key transcription factors for development of embryonic stem cells (Oct-4, Nanog, Klf4, c-myc). These genes are introduced into somatic cells (for example, fibroblasts) using retroviral vectors [24]. As a result of this strategy, a transformed cell can be obtained that has a number of PSC properties (among others, it differentiates into cells derived from all three germ layers). Such transformation is, however, relatively rare as, on average, one cell in several thousand undergoing the aforementioned genetic manipulation yields to transformation (is induced to the embryonic stage) and begins to proliferate, creating a clone consisting of iPSCs [24, 25]. Recently, some modifications to this strategy have been described that employ more limited numbers of genes in the transduction process or the use of micro-ribonucleic acid (miRNA) or even small molecules [26]. Surprisingly, similar effects have been recently obtained by stressing somatic cells with a mildly acidic bath or even vigorous trituration to generate so-called stimulus-triggered acquisition of pluripotency cells (STAPs). If this strategy can be reproduced in other laboratories, and if it is also possible to obtain human STAPs, this method could completely transform the field of regenerative medicine [27, 28].

This process for generating iPSCs, however, is difficult to control, and cells obtained as a result of the aforementioned strategy, like embryonic cells obtained from expanding ICM cells from embryos, also create teratomas in experimental animal models [24, 26]. This problem must be solved prior to the clinical use of such cells. Furthermore, transduction of genes into somatic cells as a step in generating iPSCs additionally disturbs the structure and organization of the DNA, which in turn may

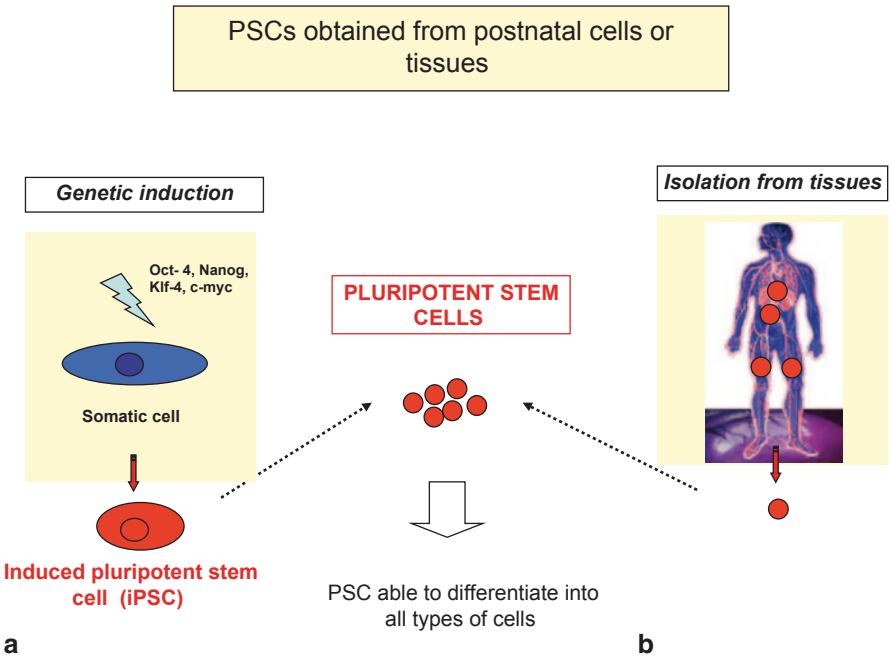

Fig. 1.2 Pluripotent stem cells (*PSCs*) obtained from postnatal tissues. **a** *PSCs* can also be obtained by transforming somatic cells (for example, fibroblasts) using genes that encode embryonic transcription factors (e.g., Oct-4, Nanog, Klf-4, c-myc). **b** *PSCs* or multipotent stem cells can also be obtained from tissues of mature individuals. Examples of such cells are listed in Tables 1.4, 1.5, and 1.6

potentially lead to induction of mutations and the creation of neoplastic cells [29]. There was also a report that iPSCs are potentially immunogenic and rejected by the recipient immune system.

iPSCs have been proposed as an ethically acceptable source of PSCs that are an alternative to embryonic stem cells isolated from embryos. The creation of iPSCs is not burdened with the problem of obtaining human ova, and, most importantly, cells generated from iPSCs, like those obtained by therapeutic cloning, will have the same histocompatibility genes as the potential recipient of such cells (Table 1.2). Interestingly, in a recent report, iPSCs and STAPs were reported to have totipotent character. This may again raise issues concerning the ethical application of such cells.

1.5 Stem Cells Obtained from Mature Postnatal Tissues

It should be pointed out that stem cells isolated from the adult tissues are the only stem cells currently employed in the clinic. Of course, the most important question regarding their use is their potential for multiple tissue differentiation.

Table 1.3 Alternative explanations for the phenomenon of trans-dedifferentiation or plasticity of adult TSSCs

Cell fusion	The relatively rare phenomenon by which infused HSCs may fuse with cells in damaged tissues and form heterokaryons. Cells created in this way express markers of both donor and recipient cells (pseudochimerism)
Paracrine stimulation	HSCs are a source of different trophic and angiopoietic factors that may promote tissue/organ repair
Microvesicle-dependent transfer of molecules	Some of the plasticity data can be explained by a transient modification of cell phenotype by the transfer of receptors, proteins, and mRNA between HSCs and damaged cells by means of membrane-derived microvesicles
Epigenetic changes	Factors present in the environment of damaged organs induce epigenetic changes in genes that regulate the pluripotency of HSCs (involvement of changes in DNA methylation and acetylation of histones). More evidence is needed that is robust and reproducible
Heterologous population of stem cells in BM	In addition to HSCs, BM contains other stem cell populations. Regeneration could be explained by the presence of endothelial progenitors that promote neo-vasculogenesis and also by the presence of other stem cells, including PSCs. The existence of these other populations could also explain the loss of contribution of BM cells to organ regeneration with the use of highly purified populations of HSCs

HSCs Hematopoietic stem cells, *mRNA* Mitochondrial ribonucleic acid, *DNA* Deoxyribonucleic acid, *BM* Bone marrow

In parallel with the first reports that it is possible to obtain human embryonic cell lines, a search for alternative sources of PSCs was initiated, and several years ago a theory was developed of so-called stem cell plasticity or the ability of TSSCs to transdifferentiate into other types of TSSCs. According to this theory, TSSCs such as HSCs obtained, for example, from BM would be able to dedifferentiate into stem cells typical of other organs such as myocardium, the central nervous system, or liver [30, 31]. Based on this theory, there were great expectations associated with the potential use of HSCs as a source of plastic stem cells. However, despite initially promising results [30, 32–38], the direct role of these cells in the regeneration of injured organs by reversing their phenotype has not been proven. Specifically, a series of studies with the use of phenotypically defined and purified subpopulations of HSCs have been disappointing, revealing negative results in models of regeneration of myocardium [39] and brain [40]. Several alternative explanations have been proposed (Table 1.3) to explain these negative results.

First, it is possible that some of the stem cell plasticity data can be explained by the phenomenon of cell fusion [41–44]. Specifically, transplanted HSCs might undergo fusion (melting) with the cells of injured organs. If so, cells in the injured organs treated with transplanted HSCs would be heterokaryons, created as a result of fusion of transplanted HSCs with cells belonging to the injured organ. However, cell fusion is an extremely rare event that can't fully account for the extensive positive trans-dedifferentiation/plasticity data claimed in several reports.

Alternatively, the positive effects observed following cell therapies might be explained by the involvement of stem cell-derived paracrine effects. Stem cells employed in therapy are a rich source of growth factors, cytokines, chemokines, and bioactive lipids, which may inhibit apoptosis and promote neovascularization in the damaged tissues. The function and phenotype of cells in the damaged tissues may also be modified by transfer of cell receptors, cytoplasmic proteins, and mitochondrial RNA (mRNA) from surrounding cells by microvesicles (MVs), which are spherical structures in which a part of the cell cytoplasm enriched for mRNA, miRNA, and functional proteins is encapsulated by cell membrane [45–46]. MVs released from the surface of cells employed to regenerate damaged organs may deliver these cargo molecules to damaged tissues. Evidence has accumulated that MV cargo has positive effects on cell survival and angiogenesis. Thus, paracrine effects associated with MVs most likely make the major contribution to the positive results reported in clinical trials employing adult stem cells.

We also cannot exclude the possibility that some factors present in the environment of damaged organs induce epigenetic changes in genes that regulate pluripotency of adult cells (involving changes in DNA methylation or acetylation of histones). This mechanism is obviously involved, for example, in generation of the recently reported STAPs [27, 28].

Finally, cells employed for therapy that are derived, for example, from hematopoietic tissues, may from the beginning contain heterogeneous populations of stem cells, including some rare multipotent or pluripotent stem cells that possess a broader differentiation potential. The presence of such cells in adult tissues will be discussed below [5, 47–54].

1.6 Do Pluripotent/Multipotent Stem Cells Reside in Postnatal Tissues?

Cumulative evidence from several laboratories shows that small cells that express some early-development embryonic markers may reside in adult tissues (Table 1.4) [49, 50, 55, 57–65], and some of these cells may even possess germline potential [66–73], indicating their close relationship to early stages of embryonic development (Table 1.5).

Morover, in support of the presence of early-development stem cells in postnatal life, several types of putative pluripotent/multipotent stem cells have been described and isolated, primarily from hematopoietic tissues, which are able to give rise to cells from more than one germ layer [51, 57,74–79]. These cells were isolated by employing various strategies, such as ex vivo expansion of partially purified immunomagnetic- or fluorescence-activated cell sorting (FACS)-sorted cells [51, 57, 74, 77, 78, 80–82]. However, in most of the expansion cultures, the rare cells that were able to initiate expansions and cross germ layer commitment were not characterized at the single-cell level [37, 57, 75, 76, 78], and in most of these cases, the phenotype of the putative pluri/multipotent cells with stem cell-like properties

Table 1.4 Examples of selected reports from other independent groups on potential pluripotent/multipotent stem cells in adult tissues

Cell name as originally described in the literature	References
Egg-laying hormone (ELH) cells: Very small cells ~5 μm in diameter isolated byelutriation and FACS sorting or by elutriation (E), lineage depletion (L), and recovered after homing (H) to BM. Give rise to long-term reconstituting hematopoietic stem cells (LT-HSCs) and epithelial cells	[74, 94, 95]
Small non-hematopoietic Sca-1$^+$Lin$^-$ CD45$^-$ cells: Isolated by FACS from murine BM, giving rise to type II pneumocytes and producing surfactant in lung alveolar epithelium. Recently, these cells have been confirmed to be very small embryonic-like stem cells (VSELs)	[75, 96]
Pluripotent CD45$^-$Sca-1$^+$c-kit$^-$ cells: Isolated by FACS from murine BM, muscles, and intestinal epithelium and able to differentiate into cells from all three germ layers	[51]
Spore-like stem cells: Very small cells, ~5 μm in diameter, isolated from various murine tissues, resistant to freeze/thawing, expressing Oct-4, and showing broad differentiation potential. The isolation procedure for these small cells was not revealed in the original paper	[86]
Rat embryonic-like stem cells (ELSCs): Very small cells, ~5 μm in diameter, isolated by FACS from rat bone marrow as an SSEA-1$^+$Lin$^-$ CD45$^-$ population. As reported, these cells express Oct-4 and are endowed with in vitro and in vivo cardiomyogenic and endothelial potential	[76]
Ovarian and testicular VSELs: Small Oct-4$^+$SSEA$^+$cells isolated by FACS from ovarian surface epithelium (OSE) from mice (SSEA-1$^+$) and humans (SSEA-4$^+$) and are precursors of female gametes. Human OSE-derived VSELs were characterized extensively for mRNA expression by gene arrays. Small Oct-4$^+$SSEA$^+$cells were also identified in murine and human testes as precursors of male gametes	[97–101]
Embryonic-like stem cells from UCB: Small CD45$^-$, CD33$^-$, CD7$^-$, CD235a$^-$ pluripotent stem cells (~ 3 μm in diameter) co-expressing embryonic stem cell markers, including Oct4 and Sox2, and able to differentiate into neuronal cells	[49, 50]
Human UCB-derived VSELs: Small Oct-4$^+$, SSEA-4$^+$, Nanog$^+$, Sox-2$^+$, Rex-1$^+$, and Tert$^+$cells	[102, 103]
Human peripheral blood (PB)-derived VSELs: Oct-4$^+$very small cells isolated by FACS. In one report, SSEA-4$^+$CD133$^+$CXCR4$^+$Lin$^-$ and CD45$^-$ VSELs from human PB formed vascularized bone fragments in immunodeficient mice	[104, 105]
Omnicytes: Small Oct-4$^+$stem cells identified in UCB and able to establish fetal–maternal chimerism.	[106]
UCB-deived nonhematopoietic CD34– Oct3/4$^+$, Sox2$^+$, Rex1$^+$cells: These cells are able to differentiate into the neural lineage (neurons, astrocytes, and oligodendrocytes)	[107]
Very Small Embryonic-Like Stem Cells (VSELs) CD45$^-$: Isolated from murine tissues, including mouse and human BM, UCB, and mPB, as a population of *Oct-4$^+$SSEA-1$^+$Sca-1$^+$Lin$^-$CD45$^-$* mouse cells and as a population of *Oct-4$^+$SSEA-4$^+$CD133$^+$Lin$^-$CD45$^-$* human cells	[48, 88, 89, 108]

FACS Fluorescence-activated cell sorting, *BM* Bone marrow, *UCB* Umbilical cord blood

Table 1.5 Selected reports from other groups on stem cells in adult non-gonadal tissues that possess germline potential and/or express embryonic stem cell markers (e.g., Oct-4, SSEA, MvH)

Cell name as originally described in the literature	References
Stem cells with germline potential from newborn mouse skin: Oct-4$^+$cells isolated by FACS from Oct-4–green fluorescence protein (GFP) mice that are able to give rise in vitro and in vivo to early oocytes	[66]
Porcine multipotent stem/stromal cells: Oct-3/4$^+$, Nanog$^+$, Sox-2$^+$cells isolated from porcine skin and adipose tissue able to differentiate into oocyte-like cells	[67]
SSEA-1$^+$ murine BM cells: Isolated from murine BM by anti-SSEA-1 immunomagnetic beads. In the presence of bone morphogenic factor-4 (BMP4) these cells differentiate into Oct-4$^+$Stella$^+$Mvh$^+$gamete precursors	[68]
BM-derived putative germ cells: Oct-4$^+$Mvh$^+$Dazl$^+$Stella$^+$cells present in BM, which may affect recurrence of oogenesis in mice sterilized by chemotherapy	[69, 70]

FACS Fluorescence-activated cell sorting, *BM* Bone marrow

Table 1.6 Examples of non-hematopoietic stem cells isolated from adult BM

Stem cells	Phenotype
Mesenchymal stem cells (MSCs)[a]	*International Society for Cellular Therapy criteria*: CD105$^+$, CD73$^+$, CD90$^+$, CD45$^-$, CD34$^-$, CD14$^-$, CD11b$^-$, CD79a$^-$, CD19$^-$, HLA-DR$^-$ *Other additional markers*: Stro-1$^+$, SB-10$^+$(CD166), SH-2$^+$(epitope on CD105), SH-3$^+$(epitope on CD73), SH-4$^+$(epitope on CD73), CD44$^+$, CD29$^+$, CD31$^-$, vWF$^-$ *Markers of most primitive MSCs*: CXCR4, CD133, CD34 (?), p75LNGFR
Multipotent adult progenitor cells (MAPCs)[a]	SSEA-1$^+$, CD13$^+$, Flk-1^{low}, Thy-1^{low}, CD34$^-$, CD44$^-$, CD45$^-$, CD117(c-kit)$^-$, MHC I$^-$, MHC II$^-$
Marrow-isolated adult multilineage inducible (MIAMI) cells[a]	CD29$^+$, CD63$^+$, CD81$^+$, CD122$^+$, CD164$^+$, c-met$^+$, BMPR1B$^+$, NTRK3$^+$, CD34$^-$, CD36$^-$, CD45$^-$, CD117 (c-kit)$^-$, HLA-DR$^-$
Unrestricted somatic stem cells (USSCs) found in cord blood[a]*- neonatal BM derived?*	CD13$^+$, CD29$^+$, CD44$^+$, CD49e$^+$, CD90$^+$, CD105$^+$, vimentin$^+$, cytokeratin (CK8/18)$^+$, CD10^{low}, Flk-1^{low}, HLA-I^{low}, CD14$^-$, CD33$^-$, CD34$^-$, CD45$^-$, CD49 (b, c, d, f)$^-$, CD50$^-$, CD62 (E, L, P)$^-$, CD106$^-$, c-kit (CD117)$^-$, glycophorin$^-$, DLA-DR$^-$
Very small embryonic-like (VSEL) stem cells	CD133$^+$, CXCR4$^+$, CD34$^+$, SSEA-4$^+$(human), Sca-1$^+$, CXCR4$^+$SSEA-1$^+$(mouse), AP$^+$, c-met$^+$, LIF-R$^+$, CD45$^-$, Lin$^-$

AP Fetal alkaline phosphatase, *BMPR1B* Bone morphogenetic protein receptor 1B, *c-met* Receptor for hepatocyte growth factor, *LIF-R* Receptor for leukemia inhibitory factor, *NTRK3* Neurotropic tyrosine kinase receptor 3, *vWF* von Willebrand factor, *BM* Bone marrow

[a] Phenotype of expanded/cultured adherent cells

was described "post factum," after phenotyping clones of already differentiated, in vitro-expanded cells [57, 74, 78, 83]. Nevertheless, many investigators would agree that if early-development stem cells endowed with broader differentiation potential

reside in adult tissues, they are probably closely related and exist at different levels of tissue specification. Most likely, they represent overlapping populations of early-development stem cells that, depending on isolation strategy, ex vivo expansion protocol, and the markers employed for their identification, have been given different names [57, 74, 75, 77, 78, 83–85], such as multipotent adult stem cells (MASC) [74], mesenchymal stem cells (MSCs) [85], multilineage-differentiating stress-enduring cells (Muse cells) [83, 84], multipotent adult progenitor cells (MAPCs) [76], unrestricted somatic stem cells (USSCs) [77], marrow-isolated adult multilineage inducible cells (MIAMIs) [78], multipotent progenitor cells (MPCs) [57, 74], spore-like stem cells [86], and, as described by my team, very small embryonic-like stem cells (VSELs). Some of these cells isolated from adult BM are listed in Table 1.6.

Overall, the presence of pluripotent/multipotent stem cells in adult tissues can be explained by the possibility that early during embryogenesis not all of the earliest-development stem cells disappear from the embryo after giving rise to TSSCs, but some survive in developing organs as a dormant back-up population of more primitive stem cells [87]. These cells could give rise to monopotent TSSCs and thus be involved in tissue/organ rejuvenation and in organ regeneration following organ injury. In support of this notion, evidence has accumulated that adult murine tissues do in fact contain, in addition to rapidly proliferating stem cells, a back-up population of more primitive dormant stem cells [88, 89]. An alternative explanation is that some somatic cells may undergo epigenetic changes during stress situations and revert to the pluripotent state. That cells expressing primitive phenotypes are detected during tissue/organ injuries in peripheral blood (PB) [90–93] and recent observations that somatic cells may be converted into PSCs lend support to this notion [27, 28]. However, this reported by Obokata et al. conversion of adult stem cells exposed to stress conditions to pluripotency [27, 28], requires conformation by other independent laboratories.

In conclusion, the quest for PSCs that could be employed in the clinic continues. In this book, we will focus on stem cells isolated from adult tissues and discuss potential mechanisms responsible for their therapeutic effects.

Acknowledgements This work was supported by NIH grants 2R01 DK074720 and R01HL112788, the Stella and Henry Endowment, and Maestro grant 2011/02/A/NZ4/00035 to MZR.

References

1. Popiela T (2003) Medycyna kliniczna na progu trzeciego millenium—refleksje osobiste. Adv Clin Exp Med 12:405–408
2. O'Farrell PH, Stumpff J, Su TT (2004) Embryonic cleavage cycles: how is a mouse like a fly? Curr Biol 14:35–45
3. Moore KA, Lemischka IR (2006) Stem cells and their niches. Science 311:1880–1884
4. Alison MR, Islam S (2009) Attributes of adult stem cells. J Pathol 217:144–160
5. Kucia M, Ratajczak J, Ratajczak MZ (2005) Are bone marrow stem cells plastic or heterogenous—that is the question. Exp Hematol 33:613–623
6. Lo B, Kriegstein A, Grady D (2008) Clinical trials in stem cell transplantation: guidelines for scientific and ethical review. Clin Trials 5:517–522

7. Lo B, Zettler P, Cedars MI, Gates E, Kriegstein AR, Oberman M et al (2005) A new era in the ethics of human embryonic stem cell research. Stem Cells 23:1454–1459
8. Hipp J, Atala A (2008) Sources of stem cells for regenerative medicine. Stem Cell Rev 4:3–11
9. Leedham SJ, Brittan M, McDonald SAC, Wright NA (2005) Intestinal stem cells. J Cell Mol 9:11–24
10. Ratajczak MZ (2008) Phenotypic and functional characterization of hematopoietic stem cells. Curr Opin Hematol 15:293–300
11. Lo Celso C, Scadden D (2007) Isolation and transplantation of hematopoietic stem cells (HSCs). J Vis Exp 4:157–162
12. Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74
13. Stocum DL, Zupanc GK (2008) Stretching the limits: stem cells in regeneration science. Dev Dyn 237:3648–3671
14. Zhu WZ, Hauch KD, Xu C, Laflamme MA (2009) Human embryonic stem cells and cardiac repair. Transplant Rev 23:53–68
15. Cabrera CM, Cobo F, Nieto A, Concha A (2006) Strategies for preventing immunologic rejection of transplanted human embryonic stem cells. Cytotherapy 8:517–518
16. Blum B, Benvenisty N (2008) The tumorigenicity of human embryonic stem cells. Adv Cancer Res 100:133–158
17. Andrews PW, Matin MM, Bahrami AR, Damjanov I, Gokhale P, Draper JS (2005) Embryonic stem (ES) cells and embryonal carcinoma (EC) cells: opposite sides of the same coin. Biochem Soc Trans 33:1526–1530
18. Hwang WS, Lee BC, Lee CK, Kang SK (2005) Cloned human embryonic stem cells for tissue repair and transplantation. Stem Cell Rev 1:99–109
19. Yang X, Smith SL, Tian XC, Lewin HA, Renard JP, Wakayama T (2007) Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. Nat Genet 39:295–302
20. McHugh PR (2004) Zygote and ‘‘clonote’’ - the ethical use of embryonic stem cells. N Engl J Med 351:209–211
21. Tachibana M, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H et al (2013) Human embryonic stem cells derived by somatic cell nuclear transfer. Cell 153:1228–1238
22. Green RM (2007) Can we develop ethically universal embryonic stem-cell lines? Nat Rev Genet 8:480–485
23. Tsunoda Y, Kato Y (2002) Recent progress and problems in animal cloning. Differentiation 69:158–161
24. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
25. Kim JB, Sebastiano V, Wu G, Araúzo-Bravo MJ, Sasse P, Gentile L et al (2009) Oct4-induced pluripotency in adult neural stem cells. Cell 136:411–419
26. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S (2008) Generation of mouse induced pluripotent stem cells without viral vectors. Science 322:949–953
27. Obokata H, Wakayama T, Sasai Y, Kojima K, Vacanti MP, Niwa H (2014) Stimulus-triggered fate conversion of somatic cells into pluripotency. Nature 505:641–647
28. Obokata H, Sasai Y, Niwa H, Kadota M, Andrabi M, Takata N et al (2014) Bidirectional developmental potential in reprogrammed cells with acquired pluripotency. Nature 505: 676–680
29. Ronen D, Benvenisty N (2012) Genomic stability in reprogramming. Curr Opin Genet Dev 22:444–449
30. Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR (2000) Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 290:1779–1782
31. Quesenberry PJ, Abedi M, Aliotta J, Colvin G, Demers D, Dooner M et al (2004) Stem cell plasticity: an overview. Blood Cells Mol Dis 32:1–4
32. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B et al (2001) Bone marrow cells regenerate infarcted myocardium. Nature 410:701–705

33. Hess DC, Abe T, Hill WD, Studdard AM, Carothers J, Masuya M et al (2004) Hematopoietic origin of microglial and perivascular cells in brain. Exp Neurol 186:134–144
34. Corti S, Locatelli F, Donadoni C, Strazzer S, Salani S, Del Bo R et al (2002) Neuroectodermal and microglial differentiation of bone marrow cells in the mouse spinal cord and sensory ganglia. J Neurosci Res 70:721–733
35. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N et al (1999) Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170
36. Mezey E, Chandross KJ (2000) Bone marrow: a possible alternative source of cells in the adult nervous system. Eur J Pharmacol 405:297–302
37. Prockop DJ (2003) Further proof of the plasticity of adult stem cells and their role in tissue repair. J Cell Biol 160:807–809
38. Wagers AJ, Weissman IL (2004) Plasticity of adult stem cells. Cell 116:639–648
39. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M et al (2004) Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 428:664–668
40. Castro RF, Jackson KA, Goodell MA, Robertson CS, Liu H, Shine HD (2002) Failure of bone marrow cells to transdifferentiate into neural cells in vivo. Science 297:1299
41. Lucas JJ, Terada N (2003) Cell fusion and plasticity. Cytotechnology 41:103–109
42. Vassilopoulos G, Russell DW (2003) Cell fusion: an alternative to stem cell plasticity and its therapeutic implications. Curr Opin Genet Dev 13:480–485
43. Scott EW (2004) Stem cell plasticity or fusion: two approaches to targeted cell therapy. Blood Cells Mol Dis 32:65–67
44. Eisenberg LM, Eisenberg CA (2003) Stem cell plasticity, cell fusion, and transdifferentiation. Birth Defects Res C Embryo Today 69:209–218
45. Janowska-Wieczorek A, Majka M, Kijowski J, Baj-Krzyworzeka M, Reca R, Turner AR et al (2001) Platelet- derived microparticles bind to hematopoietic stem/progenitor cells and enhance their engraftment. Blood 98:3143–3149
46. Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ (2006) Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia 20:1487–1495
47. Ratajczak MZ, Kucia M, Reca R, Majka M, Janowska-Wieczorek A, Ratajczak J (2004) Stem cell plasticity revisited: CXCR4-positive cells expressing mRNA for early muscle, liver and neural cells 'hide out' in the bone marrow. Leukemia 18:29–40
48. Kucia M, Reca R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J et al (2006) A population of very small embryonic-like (VSEL) CXCR4(+) SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 20:857–869
49. McGuckin CP, Forraz N, Baradez MO, Navran S, Zhao J, Urban R et al (2005) Production of stem cells with embryonic characteristics from human umbilical cord blood. Cell Prolif 38:245–255
50. McGuckin C, Jurga M, Ali H, Strbad M, Forraz N (2008) Culture of embryonic-like stem cells from human umbilical cord blood and onward differentiation to neural cells in vitro. Nat Protoc 3:1046–1055
51. Howell JC, Lee WH, Morrison P, Zhong J, Yoder MC, Srour EF (2003) Pluripotent stem cells identified in multiple murine tissues. Ann N Y Acad Sci 996:158–173
52. Ratajczak J, Zuba-Surma E, Klich I, Liu R, Wysoczynski M, Greco N et al (2011) Hematopoietic differentiation of umbilical cord blood-derived very small embryonic/epiblast-like stem cells. Leukemia 25:1278–1285
53. Ratajczak MZ, Zuba-Surma EK, Wojakowski W, Ratajczak J, Kucia M (2008) Bone marrow-home of versatile stem cells. Transfus Med Hemother 35:248–259
54. Kassmer SH, Krause DS (2013) Very small embryonic like cells: biology and function of these potential endogenous pluripotent stem cells in adult tissues. Mol Reprod Dev 80:677–690

55. Virant-Klun I, Stimpfel M, Cvjeticanin B, Vrtacnik-Bokal E, Skutella T (2013) Small SSEA-4-positive cells from human ovarian cell cultures: related to embryonic stem cells and germinal lineage? J Ovarian Res 6:24
56. Anjos-Afonso F, Bonnet D (2007) Nonhematopoietic/endothelial SSEA-1+cells define the most primitive progenitors in the adult murine bone marrow mesenchymal compartment. Blood 109:1298–1306
57. Cesselli D, Beltrami AP, Rigo S, Bergamin N, D'Aurizio F, Verardo R et al (2009) Multipotent progenitor cells are present in human peripheral blood. Circ Res 104:1225–1234
58. Kajstura J, Rota M, Hall SR, Hosoda T, D'Amario D, Sanada F et al (2011) Evidence for human lung stem cells. N Engl J Med 364:1795–1806
59. Atari M, Barajas M, Hernandez-Alfaro F, Gil C, Fabregat M, Ferres Padro E et al (2011) Isolation of pluripotent stem cells from human third molar dental pulp. Histol Histopathol 26:1057–1070
60. Wang X, Ouyang H, Yamamoto Y, Kumar PA, Wei TS, Dagher R et al (2011) Residual embryonic cells as precursors of a Barrett's-like metaplasia. Cell 145:1023–1035
61. d'Aquino R, Tirino V, Desiderio V, Studer M, De Angelis GC, Laino L et al (2011) Human neural crest-derived postnatal cells exhibit remarkable embryonic attributes either in vitro or in vivo. Eur Cell Mater 21:304–316
62. Andreadis D, Bakopoulou A, Leyhausen G, Epivatianos A, Volk J, Markopoulos A et al (2013) Minor salivary glands of the lips: a novel, easily accessible source of potential stem/progenitor cells. Clin Oral Investig [Epub ahead of print]. doi:10.1007/s00784-013-1056-6
63. Roy S, Gascard P, Dumont N, Zhao J, Pan D, Petrie S et al (2013) Rare somatic cells from human breast tissue exhibit extensive lineage plasticity. Proc Natl Acad Sci U S A 110: 4598–4603
64. Murrell W, Palmero E, Bianco J, Stangeland B, Joel M, Paulson L et al (2013) Expansion of multipotent stem cells from the adult human brain. PLoS ONE 8:e71334
65. Stimpfel M, Skutella T, Cvjeticanin B, Meznaric M, Dovc P, Novakovic S et al (2013) Isolation, characterization and differentiation of cells expressing pluripotent/multipotent markers from adult human ovaries. Cell Tissue Res 354:593–607
66. Dyce PW, Liu J, Tayade C, Kidder GN, Betts DH, Li J (2011) In vitro and in vivo germ line potential of stem cells derived from newborn mouse skin. PLoS ONE 6:e20339
67. Song SH, Kumar BM, Kang EJ, Lee YM, Kim TH, Ock SA et al (2011) Characterization of porcine multipotent stem/stromal cells derived from skin, adipose, and ovarian tissues and their differentiation in vitro into putative oocyte-like cells. Stem Cells Dev 20:1359–1370
68. Shirazi R, Zarnani AH, Soleimani M, Abdolvahabi MA, Nayernia K, Ragerdi Kashani I (2012) BMP4 can generate primordial germ cells from bone-marrow-derived pluripotent stem cells. Cell Biol Int 36:1185–1193
69. Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y et al (2005) Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. Cell 122:303–315
70. Selesniemi K, Lee HJ, Niikura T, Tilly JL (2009) Young adult donor bone marrow infusions into female mice postpone age-related reproductive failure and improve offspring survival. Aging 1:49–57
71. Nayernia K, Lee JH, Drusenheimer N, Nolte J, Wulf G, Dressel R et al (2006) Derivation of male germ cells from bone marrow stem cells. Lab Invest 86:654–663
72. Heo YT, Lee SH, Yang JH, Kim T, Lee HT (2011) Bone marrow cell-mediated production of transgenic chickens. Lab Invest 91:1229–1240
73. Hua J, Yu H, Dong W, Yang C, Gao Z, Lei A et al (2009) Characterization of mesenchymal stem cells (MSCs) from human fetal lung: potential differentiation of germ cells. Tissue Cell 41:448–455
74. Beltrami AP, Cesselli D, Bergamin N, Marcon P, Rigo S, Puppato E et al (2007) Multipotent cells can be generated in vitro from several adult human organs (heart, liver, and bone marrow). Blood 110:3438–3446

75. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR et al (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418: 41–49
76. Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM (2002) Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol 30:896–904
77. Kogler G, Sensken S, Airey JA, Trapp T, Muschen M, Feldhahn N et al (2004) A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 200:123–135
78. D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC (2004) Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. J Cell Sci 117: 2971–2981
79. Ratajczak MZ, Zuba-Surma E, Kucia M, Poniewierska A, Suszynska M, Ratajczak J (2012) Pluripotent and multipotent stem cells in adult tissues. Adv Med Sci 57:1–17
80. Jones RJ, Collector MI, Barber JP, Vala MS, Fackler MJ, May WS et al (1996) Characterization of mouse lymphohematopoietic stem cells lacking spleen colony-forming activity. Blood 88:487–491
81. Kassmer SH, Jin H, Zhang PX, Bruscia EM, Heydari K, Lee JH et al (2013) Very small embryonic-like stem cells from the murine bone marrow differentiate into epithelial cells of the lung. Stem Cells [Epub ahead of print]. doi:10.1002/stem.1413
82. Wu JH, Wang HJ, Tan YZ, Li ZH (2012) Characterization of rat very small embryonic-like stem cells and cardiac repair after cell transplantation for myocardial infarction. Stem Cells Dev 21:1367–1379
83. Kuroda Y, Wakao S, Kitada M, Murakami T, Nojima M, Dezawa M (2013) Isolation, culture and evaluation of multilineage-differentiating stress-enduring (Muse) cells. Nat Protoc 8:1391–1415
84. Wakao S, Kitada M, Kuroda Y, Shigemoto T, Matsuse D, Akashi H et al (2011) Multilineage-differentiating stress-enduring (Muse) cells are a primary source of induced pluripotent stem cells in human fibroblasts. Proc Natl Acad Sci U S A 108:9875–9880
85. Le Blanc K, Pittenger M (2005) Mesenchymal stem cells: progress toward promise. Cytotherapy 7:36–45
86. Vacanti MP, Roy A, Cortiella J, Bonassar L, Vacanti CA (2001) Identification and initial characterization of spore-like cells in adult mammals. J Cell Biochem 80:455–460
87. Li L, Clevers H (2010) Coexistence of quiescent and active adult stem cells in mammals. Science 327:542–545
88. Kucia M, Wysoczynski M, Ratajczak J, Ratajczak MZ (2008) Identification of very small embryonic like (VSEL) stem cells in bone marrow. Cell Tissue Res 331:125–134
89. Zuba-Surma EK, Kucia M, Wu W, Klich I, Lillard JW, Ratajczak J et al (2008) Very small embryonic-like stem cells are present in adult murine organs: ImageStream-based morphological analysis and distribution studies. Cytometry A 73A:1116–1127
90. Wojakowski W, Tendera M, Kucia M, Zuba-Surma E, Paczkowska E, Ciosek J et al (2009) Mobilization of bone marrow-derived Oct-4 +SSEA-4 + very small embryonic-like stem cells in patients with acute myocardial infarction. J Am Coll Cardiol 53:1–9
91. Paczkowska E, Kucia M, Koziarska D, Halasa M, Safranow K, Masiuk M et al (2009) Clinical evidence that very small embryonic-like stem cells are mobilized into peripheral blood in patients after stroke. Stroke 40:1237–1244
92. Marlicz W, Zuba-Surma E, Kucia M, Blogowski W, Starzynska T, Ratajczak MZ (2012) Various types of stem cells, including a population of very small embryonic-like stem cells, are mobilized into peripheral blood in patients with Crohn's disease. Inflamm Bowel Dis 18:1711–1722
93. Drukala J, Paczkowska E, Kucia M, Mlynska E, Krajewski A, Machalinski B et al (2012) Stem cells, including a population of very small embryonic-like stem cells, are mobilized into peripheral blood in patients after skin burn injury. Stem Cell Rev 8:184–194

94. Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R et al (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105: 369–377
95. Jones RJ, Wagner JE, Celano P, Zicha MS, Sharkis SJ (1990) Separation of pluripotent haematopoietic stem cells from spleen colony-forming cells. Nature 347:188–189
96. Kassmer SH, Bruscia EM, Zhang PX, Krause DS (2012) Nonhematopoietic cells are the primary source of bone marrow-derived lung epithelial cells. Stem Cells 30:491–499
97. Virant-Klun I, Zech N, Rozman P, Vogler A, Cvjeticanin B, Klemenc P et al (2008) Putative stem cells with an embryonic character isolated from the ovarian surface epithelium of women with no naturally present follicles and oocytes. Differentiation 76:843–856
98. Parte S, Bhartiya D, Telang J, Daithankar V, Salvi V, Zaveri K et al (2011) Detection, characterization, and spontaneous differentiation in vitro of very small embryonic-like putative stem cells in adult mammalian ovary. Stem Cells Dev 20:1451–1464
99. Virant-Klun I, Skutella T, Hren M, Gruden K, Cvjeticanin B, Vogler A et al (2013) Isolation of small SSEA-4-positive putative stem cells from the ovarian surface epithelium of adult human ovaries by two different methods. Biomed Res Int 2013:690415
100. Bhartiya D, Kasiviswananthan S, Shaikh A (2012) Cellular origin of testis-derived pluripotent stem cells: a case for very small embryonic-like stem cells. Stem Cells Dev 21: 670–674
101. Bhartiya D, Unni S, Parte S, Anand S (2013) Very small embryonic-like stem cells: implications in reproductive biology. Biomed Res Int 2013:682326
102. Bhartiya D, Shaikh A, Nagvenkar P, Kasiviswanathan S, Pethe P, Pawani H et al (2012) Very small embryonic-like stem cells with maximum regenerative potential get discarded during cord blood banking and bone marrow processing for autologous stem cell therapy. Stem Cells Dev 21:1–6
103. Halasa M, Baskiewicz-Masiuk M, Dabkowska E, Machalinski B (2008) An efficient two-step method to purify very small embryonic-like (VSEL) stem cells from umbilical cord blood (UCB). Folia Histochem Cytobiol 46:239–243
104. Sovalat H, Scrofani M, Eidenschenk A, Pasquet S, Rimelen V, Henon P (2011) Identification and isolation from either adult human bone marrow or G-CSF-mobilized peripheral blood of CD34(+)/CD133(+)/CXCR4(+)/Lin(-)CD45(-) cells, featuring morphological, molecular, and phenotypic characteristics of very small embryonic-like (VSEL) stem cells. Exp Hematol 39:495–505
105. Havens AM, Shiozawa Y, Jung Y, Sun H, Wang J, McGee S et al (2013) Human very small embryonic-like cells generate skeletal structures, in vivo. Stem Cells Dev 22:622–630
106. Mikhail MA, M'Hamdi H, Welsh J, Levicar N, Marley SB, Nicholls JP et al (2008) High frequency of fetal cells within a primitive stem cell population in maternal blood. Hum Reprod 23:928–933
107. Habich A, Jurga M, Markiewicz I, Lukomska B, Bany-Laszewicz U, Domanska-Janik K (2006) Early appearance of stem/progenitor cells with neural-like characteristics in human cord blood mononuclear fraction cultured in vitro. Exp Hematol 34:914–925
108. Kucia M, Halasa M, Wysoczynski M, Baskiewicz-Masiuk M, Moldenhawer S, E Z-S (2007) Morphological and molecular characterization of novel population of CXCR4+SSEA-4+Oct-4+ very small embryonic-like cells purified from human cord blood: preliminary report. Leukemia 21:297–303

Chapter 2
Novel Therapeutic Approaches in Regenerative Medicine—Adult Tissue-Derived Very Small Embryonic-like Stem Cells and Harnessing Paracrine Signals of Adult Stem Cells

Magda Kucia, Malwina Suszynska, Janina Ratajczak and Mariusz Z. Ratajczak

2.1 Introduction

Regenerative medicine is searching for a reliable source of pluripotent stem cells (PSCs) with potential to give rise to cells from all three germ layers that could be employed for therapy. For almost 20 years, there have been attempts to harness embryonic stem cells (ESCs) isolated from embryos generated by in vitro fertilization [1–4] or by therapeutic cloning [5, 6] in a variety of preclinical animal models. This strategy, however, encounters some ethical considerations [7–9]. A novel promising source of PSCs are induced pluripotent stem cells (iPSCs) generated by genetic modification from adult cells, however again, this strategy is still under development and bares a risk of teratoma formation by injected cells [10, 11] as well as their rejection by a host immune system [12].

Therefore, in the meantime, the various types of adult stem and progenitor cells isolated from bone marrow (BM), mobilized peripheral blood (mPB), umbilical cord blood (UCB), or derived from expanded in vitro cultures of adherent cells such as mesenchymal stem cells (MSCs) or multipotent adult stem cells (MAPCs) are employed in clinical trials to regenerate damaged organs (e.g., heart, kidney, or neural tissues) [13–15]. It is striking that, for a variety of these adult tissues-derived cells, the currently observed final clinical outcomes of cellular therapies are often similar. This fact and the lack of convincing documentation for a robust donor-recipient chimerism in treated tissues in most of the patient studies indicates that a mechanism other than transdifferentiation of cells infused systemically into peripheral blood or injected directly into damaged organs as therapeutics may play an important role [16–18].

Overall, the rare cases of low level of chimerism observed after infusion of donor BM, UCB, or mPB cells reported by some investigators could be explained by cell fusion [19] or presence of rare populations of stem cells present in adult organs that

M. Z. Ratajczak (✉) · M. Kucia · M. Suszynska · J. Ratajczak
Stem Cell Institute, James Graham Brown Cancer Center, University of Louisville, 500 South Floyd Street, Rm. 107, 40202 Louisville, KY, USA
e-mail: mzrata01@louisville.edu

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,* Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_2,

are endowed with multi-tissue differentiation [20–23]. Therefore, one of the most intriguing questions in stem cell biology is whether pluripotent stem cells (PSCs) able to differentiate into cells for all three germ layers or multipotent stem cells (MultSCs) able to differentiate into cells for two different germ layers exist in adult tissues. Several groups of investigators employing (1) various isolation protocols, (2) detection of surface markers, and (3) experimental in vitro and in vivo models have reported the presence of cells that possess a pluripotent/multipotent character in adult tissues. Such cells were assigned various operational abbreviations and names in the literature (e.g., multipotent adult progenitor cells (MAPCs) [22], multipotent adult stem cells (MASCs) [21], unrestricted somatic stem cells (USSCs) [24], or marrow-isolated adult multilineage-inducible (MIAMI) cells [25]) and raised the basic question of whether these are truly distinct or overlapping populations of the same primitive stem cells. In fact, taking into consideration their common features described in the literature, it is very likely that various investigators have described overlapping populations of developmentally early stem cells that are closely related. Unfortunately, these cells were never characterized side-by-side to address this important issue. Moreover, the rare and quiescent population of so called very small embryonic-like stem cells (VSELs) isolated from murine tissues and human UCB initially by our group [26, 27] and subsequently isolated in other laboratories [28–31] expresses several markers of PSCs and shares some of the characteristics with above mentioned cell populations. As shown in Fig. 2.1, we envision that VSELs may contribute to donor-recipient chimerism and in this review we will discuss their potential role in regenerative medicine.

On the other hand, it is striking that, for a variety of stem cells such as hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), or even unpurified mononuclear cells (MNCs), the observed final outcomes of cellular therapies are often similar and there is a lack of convincing documentation for robust donor-recipient chimerism in treated tissues in most of the patient studies. This indicates that a mechanism other than transdifferentiation of cells infused systemically into peripheral blood or injected directly into damaged organs may play an important role. Thus, we will also discuss the involvement of (1) growth factors, cytokines, chemokines, and bioactive lipids and (2) microvesicles (MVs) and exosomes released from cells employed as cellular therapeutics in regenerative medicine (Fig. 2.1). In particular, stem cells are a rich source of these soluble factors and in addition MVs that released from their surface may deliver various species of ribonucleic acid (RNA) and microRNA (miRNA) that are important for cell proliferation and survival into damaged organs [32–34]. Based on these phenomena, paracrine effects probably make major contributions in most of the currently reported positive results in clinical trials employing adult stem cells [35–37]. In this review, we will also discuss different strategies how these paracrine mechanisms could be exploited in regenerative medicine to achieve better therapeutic outcomes. This may yield critical improvements in current cell therapies before true pluripotent stem cells such as VSELs isolated in sufficient quantities from adult tissues and successfully expanded ex vivo will be employed in the clinic.

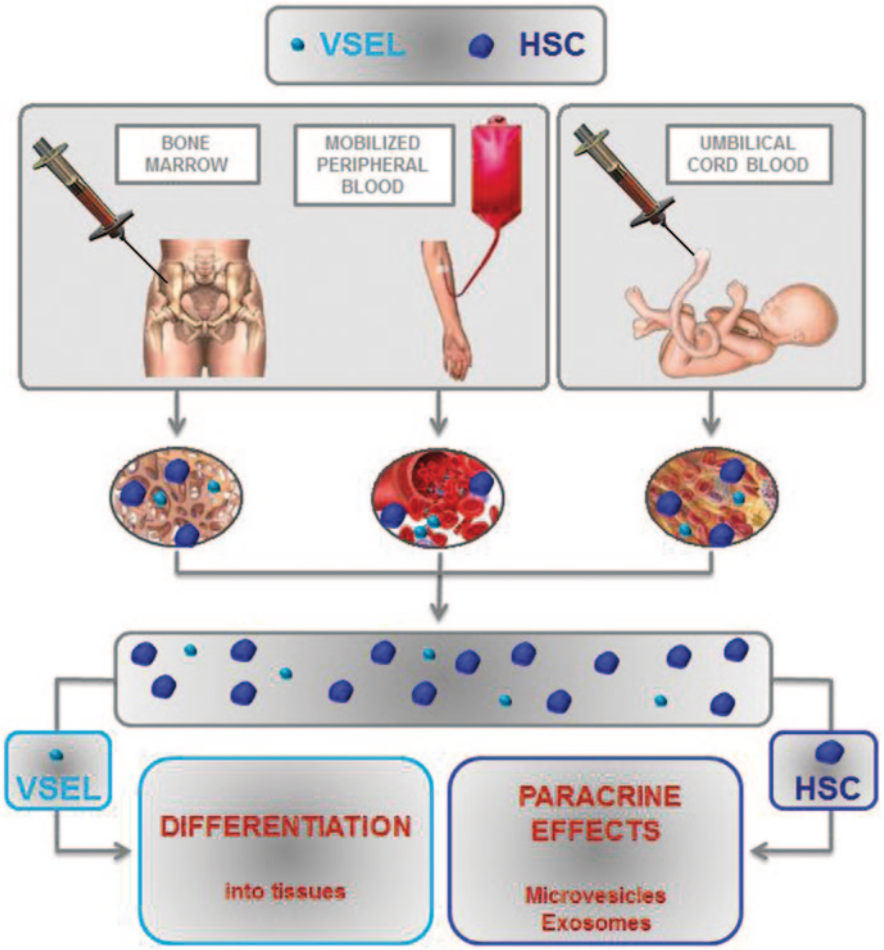

Fig. 2.1 The potential role of cells isolated from bone marrow, mobilized peripheral blood, and umbilical cord blood in regeneration of non-hematopoietic organs. The hematopoietic tissues are a relatively accessible source of cells that can be employed in regenerative medicine (e.g., hematopoietic stem cells (*HSCs*) or MSCs). We propose that the major beneficial effects of these cells, in regeneration of damaged organs, is based mostly on paracrine signals (e.g., secretion of growth factors, cytokines, chemokines, bioactive lipids, and miRNA and RNA transfer by MVs; *right panel*). In addition, cells isolated from adult tissues may contain a small admixture of true pluripotent stem cells (e.g., very small embryonic-like stem cells (*VSELs*)) that can differentiate into organ-specific cells. However, because of epigenetic changes in some of the imprinted genes, these cells have a locked cell cycle and remain quiescent (*left panel*). Without appropriate modulation of the expression of imprinted genes, their contribution to cells in damaged organs is not effective. PSCs must first be purified in therapeutically significant quantities from adult tissues and efficiently expanded ex vivo before they can be successfully employed in the clinic

2.2 Presence of Potential Pluripotent Stem Cells in Cell Preparations Derived from Adult Tissues

From a developmental point of view, an important question is why should PSCs may reside in adult organs? For many years, it has been accepted that adult tissues contain only tissue-committed stem cells (TCSCs), such as epidermal stem cells, hematopoietic stem cells, or skeletal muscle stem cells, that have a limited potential for differentiation. To address this question, we consider two scenarios that could occur during early embryogenesis and the development of lineage-restricted TCSCs [38]. In the first scenario, PSCs present in the inner cell mass of the blastocyst/epiblast, after giving rise to more differentiated lineage-restricted TCSCs, gradually disappear from the growing embryo and are not present in adult tissues. In the second scenario, which we believe is more likely to take place during embryogenesis, some PSCs give rise to TCSCs but some survive in adult tissues as a backup population of PSCs that renews the pool of TCSCs over time. In this second scenario, PSCs are precursors of TCSCs during organ/tissue rejuvenation and a source of these cells in emergency situations when organs are damaged (e.g., heart infarct or stroke). This scenario, however, requires such PSCs population deposited in adult tissues to be kept under control and in a quiescent state, which is essential to preventing uncontrolled proliferation leading to teratoma formation.

We envision that VSELs could be such population of PSCs that play a role as a backup population for more differentiated TCSCs [26]. These rare cells, which are slightly smaller than red blood cells, (1) become mobilized during stress situations into peripheral blood, (2) are enriched in the $Sca1^{+}Lin^{-}CD45^{-}$ cell fraction in mice and the $CD133^{+}Lin^{-}CD45^{-}$ cell fraction in humans, (3) express markers of pluripotent stem cells (e.g., Oct4, Nanog, and Stage Specific Embryonic Antigen (SSEA)), and (4) display a distinct morphology characterized by a high nuclear/cytoplasmic ratio and undifferentiated chromatin [26] called euchromatin. Furthermore, the recent evidence indicates that murine VSELs are kept quiescent in adult tissues and protected from teratoma formation by epigenetic modification of imprinted genes (e.g., erasure of differently methylated regions at Igf2-H19 and Rasgrf1 loci and hypermethylation at KCNQ1 and Igf2R loci) that regulate insulin/insulin-like growth factor signaling (IIS) [39]. As discussed later on in this review, the successful reversal of these epigenetic changes in VSELs that render them quiescent will be crucial for efficient ex vivo expansion of these cells. Moreover, the molecular analysis of adult BM-derived purified VSELs revealed that they share several markers characteristic for epiblast as well as migratory primordial germ cells (PGCs) [40]. In support of this, molecular analysis of murine BM-derived VSELs has revealed that these cells express several genes that are characteristic of epiblast SCs (*Gbx2, Fgf5,* and *Nodal*) and germ line specification (*Stella, Prdm14, Fragilis, Blimp1, Nanos3,* and *Dnd1*) [40].

Based on this, pluripotent VSELs that share epiblast/PGC markers as we hypothesize are deposited at the beginning of gastrulation in developing tissues and play an important role as a backup population for TCSCs [41, 42]. We hypothesize that VSELs play an important role in tissue/organ rejuvenation, and their proliferation

Table 2.1 In vitro and in vivo criteria expected from pluripotent stem cells and pluripotent status of murine VSELs

In vitro criteria for PSCs	*VSELs*
1. Undifferentiated morphology, euchromatin, and high nuclear/cytoplasm ratio	Yes
2. PSC markers (e.g., Oct-4, Nanog, and SSEA), open chromatin at the Oct-4 promoter, bivalent domains, and reactivation of the X chromosome in female PSCs	Yes Yes
3. Broad multilineage differentiation into cells from all three germ layers (meso-, ecto-, and endoderm)	
In vivo criteria for PSCs	*VSELs*
1. Complementation of blastocyst development	No
2. Teratoma formation in immunodeficient mice	No

VSELs very small embryonic-like stem cells, *PSCs* pluripotent stem cells

and potentially premature depletion is negatively controlled by epigenetic changes of imprinted genes that regulate IIS [39, 43]. On the other hand, the same epigenetic changes of imprinted genes keep these cells quiescent in adult tissues unfortunately prevent them from efficient ex vivo expansion in vitro.

The most recent data in vivo from our and other laboratories demonstrated that both murine [44] and human VSELs [45] exhibit some characteristics of long-term repopulating hematopoietic stem cells (LT-HSCs), are at the top of the hierarchy in the mesenchymal lineage [28, 31], may differentiate into organ-specific cells (e.g., cardiomyocytes and lung alveolar epithelium cells) [29, 46, 47], and in gonads are precursors of gamets [48, 49]. Moreover, as recently demonstrated, the number of these cells positively correlates in several murine models with longevity [43, 50, 51].

2.3 VSELs as Potential Pluripotent Stem Cells

There are stringent in vitro and in vivo criteria for classifying a stem cell as a PSC (Table 2.1). These criteria were established by embryologists who are working with ESCs or iPSCs [10, 52, 53]. However, some of these stringent criteria listed as in vivo criteria of pluripotency, such as complementation of blastocyst development and teratoma formation, are not always visible for example in pluripotent epiblast-derived stem cells [54, 55].

Our most recent experimental data support that murine VSELs fulfill all the in vitro criteria listed in Table 1. In particular, as mentioned above murine VSELs not only possess the primitive morphology of early developmental cells and express typical markers for PSCs (e.g., *Oct-4, Nanog,* and *Rex-1*) [26], but we recently confirmed the true expression of *Oct-4* in murine VSELs by the presence of an open-type chromatin in the *Oct-4* promoter by direct analysis of its methylation state [39] and association with transcription-permissive histones [39]. Specifically, we observed that the *Oct-4* promoter in murine VSELs is hypomethylated, and by employing the carrier-ChIP assay, we found that the chromatin in the *Oct-4* promoter

is associated with the gene transcription-promoting histone H3Ac while its association with the transcription-restricting histone H3K9me2 is relatively low [39]. Finally, amplified by reverse transcription polymerase chain reaction (RT-PCR) Oct-4 mitochondrial RNA (mRNA) has been cloned and sequenced to demonstrate that Oct-4 but not any of the Oct-4 pseudogenes has been amplified.

We also evaluated the epigenetic state of another core transcription factor, *Nanog*, and observed that its promoter has a higher level of methylation in VSELs (~50%). In quantitative ChIP experiments performed in parallel, we also observed that the H3Ac/H3K9me2 ratio favors transcription and supports an active state [39]. Based on these results, we conclude that murine VSELs truly express both of the embryonic transcription factors *Oct-4* and *Nanog* [39].

With respect to the other in vitro criteria of pluripotency (Table 1), murine VSELs also possess bivalent domains in promoters that encode developmentally important homeobox-containing transcription factors (*Sox21, Nkx2.2, Dlx1, Lbx1h, Hlxb9, Pax5,* and *HoxA3*) [56]. Furthermore, VSELs derived from female mice reactivate the X-chromosome. Finally, VSELs can differentiate in vitro into cells from all three germ layers [56]. Data published in our and other laboratories demonstrated that in appropriate experimental models murine BM-derived VSELs can be specified in vivo into HSCs [44, 45], mesenchymal stem cells (MSCs) [28, 31], cardiomyocytes [47], pneumocytes [29], and ovary-derived VSELs may give rise to oocytes [49].

However, on other hand taking into consideration the in vivo criteria expected from PSCs (Table 1), murine VSELs do not complete blastocyst development and do not grow teratomas. This discrepancy between in vitro and in vivo criteria of PSCs for VSELs has recently been explained by epigenetic changes in expression of some paternally imprinted genes [39]. Accordingly, we observed that VSELs, in a similar manner as late migratory PGCs, modify the methylation of imprinted genes, preventing them from uncontrolled proliferation and explaining their quiescent state in adult tissues [39].

It is well known that imprinted genes play a crucial role in embryogenesis, fetal growth, the totipotential state of the zygote, and the pluripotency of developmentally early stem cells [57, 58]. It has been demonstrated that VSELs freshly isolated from murine BM erase the paternally methylated imprints at regulatory differentially methylated regions (DMRs) within the *Igf2-H19* and *Rasgrf1* loci; however, they also hypermethylate the maternally methylated imprints at DMRs for the Igf2 receptor (*Igf2R), Kcnq1-p57^{KIP2},* and *Peg1* loci [39]. Because paternally expressed imprinted genes (*Igf2* and *Rasgrf1*) enhance embryonic growth and maternally expressed genes (*H19, p57^{KIP2},* and *Igf2R*) inhibit cell proliferation [59, 60], the unique genomic imprinting pattern observed in VSELs demonstrates the growth-repressive influence of imprinted genes on these cells [39] and may explain their quiescent state.

Thus, these results suggest that epigenetic reprogramming of genomic imprinting maintains the quiescence of Oct4^{+}epiblast/germ line-derived VSELs deposited in the adult body and protects them from premature aging and uncontrolled proliferation (e.g., teratoma formation). On the other hand, reversal of this mechanism

will be crucial to employing VSELs as a population of PSCs in regenerative medicine. Currently, we are testing whether downregulation of the expression of H19 enhances VSEL expansion, as has recently been demonstrated for PSCs derived by parthenogenesis [61].

Taking everything into consideration, we have to pinpoint that while murine BM-derived VSELs have been extensively characterized more work is needed to better characterize these small cells at the molecular level in humans. Nevertheless, similar morphology, pattern of expression of some genes regulating pluripotency (e.g., Oct-4, Nanog, Sal-4) in both murine and human VSELs, as well as differentiation of both murine and human VSELs into HSCs [44, 45] and MSCs [28, 31] suggests that these are corresponding populations of stem cells.

2.4 Paracrine Effects of Cells Employed in Regenerative Medicine

It is known that cells and in particular stem cells secrete a variety of growth factors, cytokines, chemokines, and bioactive lipids that regulate their biology in an autocrine/paracrine-manner and orchestrate interactions with the surrounding microenvironment [33, 62–64]. These factors are secreted, in particular, from activated cells removed from their physiological niches (e.g., aspirated from the BM or mobilized into peripheral blood, PB). Therefore, different types of cells, including HSCs, MSCs, skeletal muscle myoblasts (SMMs), adipose tissue stem cells (ASCs), neural stem cells (NSCs), or cardiac stem cells (CSCs) that are employed in regenerative medicine to rescue damaged organs are potential sources of paracrine factors. These factors may (1) inhibit apoptosis of cells residing in the damaged organs, (2) stimulate proliferation, and (3) promote vascularization of affected tissues to improve oxygen delivery and metabolic exchange. As reported the most important factors secreted from stem cells include vascular endothelial growth factor (VEGF), stem cell factor (SCF), hepatocyte growth factor (HGF), insulin-like growth factor-1 and −2 (IGF-1, -2), and stromal derived factor-1 (SDF-1) [33, 62, 65, 66].

In addition to soluble factors, activated stem cells also secrete MVs, which are small, spherical membrane fragments shed from the cell surface or secreted from the endosomal compartment and seem to play an important and underappreciated role in improving the function of damaged organs [34, 67–71]. A growing body of evidence suggests that MVs secreted, for example, from HSPCs, MSCs, ASCs, NSCs, or CSCs employed in various treatment strategies efficiently inhibit apoptosis of cells residing in the damaged tissues, stimulate their proliferation, and promote vascularization [66, 67, 72, 73]. These pro-regenerative effects mediated by MVs can be explained by the fact that these small, spherical membrane fragments (1) are enriched in bioactive lipids (e.g., sphingosine-1-phosphate [S1P], ceramide-1-phosphate [C1P]), (2) display several anti-apoptoic and pro-stimulatory growth factors or cytokines (e.g., VEGF or SCF) on their surface, and (3) deliver mRNA, regulatory miRNA, and proteins that improve overall cell function [32, 33, 65]. In

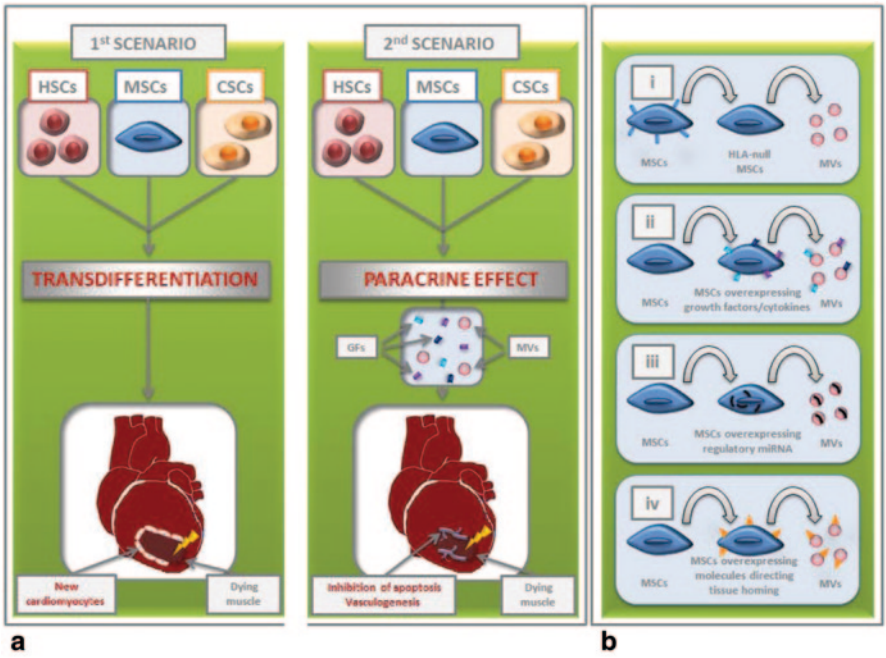

Fig. 2.2 Beneficial effects of hematopoietic stem cells (*HSCs*), mesenchymal stem cells (*MSCs*), or cardiac stem cells (*CSCs*) in regenerative medicine using heart infarct as a model. **a** *Left part, 1st scenario:* Cells employed for therapy may theoretically transdifferentiate into cardiomyocytes. However, if this occurs at all, it is a very rare and random phenomenon and is not well substantiated by current experimental data. *Right part, 2nd scenario:* Cells employed for therapy do not transdifferentiate into cardiomyocytes, but secrete several paracrine factors and shed microvesicles (*MVs*) that inhibit apoptosis in damaged cardiomyocytes, promoting their proliferation and stimulating angiogenesis. Evidence is accumulating that this is a major effect in currently employed stem cell therapies. **b** Different approaches to generating ex vivo more efficient pro-regenerative MVs. MVs could be harvested from large-scale in vitro cultures of MV-producing cell lines. Such cell lines may be modified to obtain MVs that *i* are less immune and do not express human leukocyte antigens (*HLAs*), *ii* are enriched in growth factors, cytokines, and chemokines that promote regeneration of damaged organs, *iii* are enriched in mitochondrial ribonucleic acid (*mRNA*) and regulatory microRNA (*miRNA*) facilitating regeneration of damaged tissues and/or promoting angiogenesis, and *iv* display molecules that direct them to, and subsequently retain them in, damaged tissues

support of this notion, several species of miRNA that regulate cell survival and angiogenesis (e.g., mir126 and mir130) have been identified in $CD34^+$HSPCs-derived MVs [66].

Such cell-derived paracrine signals may explain why, after applying various types of stem cells, the final therapeutic benefits are similar (Fig. 2.2a). For example, coronary infusion or local delivery of $CD34^+$or $CD133^+$HSPCs, MSCs, SMMs, or CSCs following heart infarct often yield a similar improvement in left ventricular ejection fraction [35–37]. This argues against significant transdifferentiation of cells employed for therapy into cardiomyocytes and strongly supports a role for paracrine effects [74].

These observations should prompt investigators to develop novel therapeutic strategies that harness these mechanisms to ensure more efficient outcomes than current therapies. This could be achieved, for example, by modification of cells employed in regenerative medicine to become better sources of paracrine signals. On the other hand, the therapeutic application of large-scale, modified MVs, instead of whole cells, is emerging as an exciting new concept in regenerative medicine. This could be achieved as will be discussed latter in this review by ex vivo manipulation of cells to enhance secretion of pro-regenerative factors and the development of novel therapeutic strategies in which large-scale, ex vivo-generated, modified MVs replace intact cells.

2.4.1 Increase in Release of Pro-regenerative Factors from Cells Employed in Regenerative Medicine

Since autocrine secretion of various soluble factors, as well as MVs, by stem cells can be increased during the stress response, one can envision different approaches to augmenting this phenomenon. Theoretically, this could be accomplished by (1) exposure of cells to hypoxia prior to infusion and delivery to the injured organ, (2) transduction of these cells by expression vectors that increase secretion of pro-angiopoietic factors (e.g., VEGF or FGF-2), (3) modulation of expression of miRNAs that regulate transcription of anti-apoptotic or pro-angiopoietic genes, and (4) exposure of these cells to complement cascade cleavage fragments or cytokines that promote autocrine release of appropriate paracrine peptides or bioactive lipids.

2.4.2 Development of Engineered MVs for Regenerative Medicine Therapies

Based on the fact that MVs have similar beneficial effects in regenerative therapy as the intact cells from which they are derived [67, 68, 72], it should be possible to produce MVs on a large scale and even to modify their composition. Several possibilities for how to modify MVs are shown in Fig. 2.2b**.** Overall, MVs for application in regenerative medicine could be isolated from appropriate generator cells (e.g., MSCs) expanded ex vivo.

First, as depicted in Fig. 2.2b, it should be possible to expand MV-producing cell lines that lack genes encoding histocompatibility antigens. This approach would minimalize the possibility of cross-immunization with donor human leukocyte antigens (HLAs). Second, MV producer cell lines could be transduced with genes that overexpress on the cell surface (1) peptides that protect target cells in damaged organs from apoptosis and stimulate proliferation of residual remaining cell populations (e.g., SCF or Notch ligands) or (2) factors that effectively induce angiogenesis (e.g., VEGF, FGF-2, or SDF-1). Third, we speculate that MVs derived from cells

in hypoxic conditions would be enriched in mRNAs and miRNAs that promote angiogenesis. Furthermore, producer cell lines could be enriched for mRNA and regulatory miRNA species that, after delivery to the damaged tissues, would promote regeneration. Finally, we envision that MV producer cell lines could be enriched for molecules that facilitate their tropism to the damaged organ and subsequently promote retention of MVs in the damaged tissues.

Conclusions

Current therapeutic strategies in regenerative medicine employing adult stem cells for damaged solid organs are mainly based on utilization of their paracrine effects by secreted soluble factors and MVs [72, 73, 75, 76]. Overall, paracrine effects and MV-based therapies also open up new possibilities for clinical applications of iPSCs. Since in vivo application of iPSCs is limited by the high risk of teratoma formation by these cells, MVs from patient-derived iPSCs could be employed as a novel generation of therapeutics to rescue damaged organs and tissues. Based on this possibility, we envision that patient-derived iPSCs could be employed as MV-producing cells. Moreover, taking advantage of the recently proposed epigenetic memory of cells employed for generation of iPSCs [77], one can also envision that, for example, MVs from keratinocyte-derived iPSCs would be preferentially enriched for mRNA and miRNA for epidermis stem cells and thus affect regeneration of damaged skin (e.g., after burns), or similarly MVs isolated from supernatants of cardiomyocyte-derived iPSCs would have by similar mechanisms advantages in regeneration of damaged myocardium.

However, beside paracrine effects of more differentiated adult stem/progenitor cells it is also a hope for clinical application of pluripotent/multipotent isolated from the adult tissues and new data from our group [26, 44, 45] and other groups [28, 47, 49] has provided more evidence on the existence of primitive embryonic-like stem cells in murine adult tissues and their potential role in (1) tissue organ rejuvenation, (2) longevity, and (3) regeneration/repair of damaged tissues. Nevertheless, while murine BM-derived VSELs have been extensively characterized, we are aware that more work is needed to better characterize small $CD133^{+}Lin^{-}$ $CD45^{-}$ cells at the molecular level in humans. We need to determine whether human VSELs have the same molecular signature (e.g., an open chromatin structure at the *Oct4* promoter, modification of somatic imprinting, and the presence of bivalent domains) as their murine counterparts. If we can confirm that a similar imprinting-related mechanism operates for human VSELs, perhaps a controlled modulation of the somatic imprinted state to produce proper de novo methylation of somatic imprinted genes on the maternal and paternal chromosomes could increase the regenerative power of these cells [39] and lead to their broader application in the clinic. Of note, recently National Institutes of Health (NIH) sponsored a first clinical trial to employ human BM-derived VSELs for treatment of periodontitis lesions in patients [http://www.neostem.com/news/vsel1mgrant.html].

Acknowledgments This work was supported by NIH grant 2R01 DK074720, the Stella and Henry Endowment and grant Maestro 2011/02/A/NZ4/00035 to MZR.

References

1. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156
2. 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 U S A 78:7634–7638
3. Bradley A, Evans M, Kaufman MH, Robertson E (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 309:255–256
4. Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA et al (1995) Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A 92:7844–7848
5. Wakayama T, Tabar V, Rodriguez I, Perry AC, Studer L, Mombaerts P (2001) Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science 292:740–743
6. Wakayama T (2003) Cloned mice and embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Oncol Res 13:309–314
7. Condic ML, Rao M (2008) Regulatory issues for personalized pluripotent cells. Stem Cells 26:2753–2758
8. Lo B, Parham L, Alvarez-Buylla A, Cedars M, Conklin B, Fisher S et al (2010) Cloning mice and men: prohibiting the use of iPS cells for human reproductive cloning. Cell Stem Cell 6:16–20
9. Blanpain C, Daley GQ, Hochedlinger K, Passegue E, Rossant J, Yamanaka S (2012) Stem cells assessed. Nat Rev 13:471–476
10. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
11. Takahashi K, Okita K, Nakagawa M, Yamanaka S (2007) Induction of pluripotent stem cells from fibroblast cultures. Nature Protoc 2:3081–3089
12. Zhao T, Zhang ZN, Rong Z, Xu Y (2011) Immunogenicity of induced pluripotent stem cells. Nature 474:212–215
13. Lalu MM, McIntyre L, Pugliese C, Fergusson D, Winston BW, Marshall JC et al (2012) Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. PLoS ONE 7:e47559
14. Cantaluppi V, Biancone L, Quercia A, Deregibus MC, Segoloni G, Camussi G (2013) Rationale of mesenchymal stem cell therapy in kidney injury. Am J Kidney Dis 61:300–309
15. Park JH, Kim DY, Sung IY, Choi GH, Jeon MH, Kim KK et al (2012) Long-term results of spinal cord injury therapy using mesenchymal stem cells derived from bone marrow in humans. Neurosurgery 70:1238–1247
16. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M et al (2004) Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 428:664–668
17. Wagers AJ, Sherwood RI, Christensen JL, Weissman IL (2002) Little evidence for developmental plasticity of adult hematopoietic stem cells. Science 297:2256–2259
18. Castro RF, Jackson KA, Goodell MA, Robertson CS, Liu H, Shine HD (2002) Failure of bone marrow cells to transdifferentiate into neural cells in vivo. Science 297:1299
19. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K et al (2003) Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 425:968–973
20. D'Ippolito G, Howard GA, Roos BA, Schiller PC (2006) Isolation and characterization of marrow-isolated adult multilineage inducible (MIAMI) cells. Exp Hematol 34:1608–1610

21. Beltrami AP, Cesselli D, Bergamin N, Marcon P, Rigo S, Puppato E et al (2007) Multipotent cells can be generated in vitro from several adult human organs (heart, liver, and bone marrow). Blood 110:3438–3446
22. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR et al (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49
23. Kucia M, Reca R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J et al (2006) A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 20:857–869
24. Kogler G, Sensken S, Airey JA, Trapp T, Muschen M, Feldhahn N et al (2004) A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 200:123–135
25. D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC (2004) Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. J Cell Sci 117:2971–2981
26. Kucia M, Wojakowski W, Reca R, Machalinski B, Gozdzik J, Majka M et al (2006) The migration of bone marrow-derived non-hematopoietic tissue-committed stem cells is regulated in an SDF-1-, HGF-, and LIF-dependent manner. Arch Immunol Ther Exp 54:121–135
27. Kucia M, Halasa M, Wysoczynski M, Baskiewicz-Masiuk M, Moldenhawer S, Zuba-Surma E et al (2007) Morphological and molecular characterization of novel population of CXCR4+SSEA-4+Oct-4+ very small embryonic-like cells purified from human cord blood: preliminary report. Leukemia 21:297–303
28. Taichman RS, Wang Z, Shiozawa Y, Jung Y, Song J, Balduino A et al (2010) Prospective identification and skeletal localization of cells capable of multilineage differentiation in vivo. Stem Cells Dev 19:1557–1570
29. Kassmer SH, Bruscia EM, Zhang PX, Krause DS (2012) Nonhematopoietic cells are the primary source of bone marrow-derived lung epithelial cells. Stem Cells 30:491–499
30. Bhartiya D, Shaikh A, Nagvenkar P, Kasiviswanathan S, Pethe P, Pawani H et al (2012) Very small embryonic-like stem cells with maximum regenerative potential get discarded during cord blood banking and bone marrow processing for autologous stem cell therapy. Stem Cells Dev 21:1–6
31. Havens AM, Shiozawa Y, Jung Y, Sun H, Wang J, McGee S et al (2013) Human very small embryonic-like cells generate skeletal structures, in vivo. Stem Cells Dev 22:622–630
32. Collino F, Deregibus MC, Bruno S, Sterpone L, Aghemo G, Viltono L et al (2010) Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs. PLoS ONE 5:e11803
33. Majka M, Janowska-Wieczorek A, Ratajczak J, Ehrenman K, Pietrzkowski Z, Kowalska MA et al (2001) Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. Blood 97:3075–3085
34. Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ (2006) Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia 20:1487–1495
35. Tendera M, Wojakowski W, Ruzyllo W, Chojnowska L, Kepka C, Tracz W et al (2009) Intracoronary infusion of bone marrow-derived selected CD34+CXCR4+ cells and non-selected mononuclear cells in patients with acute STEMI and reduced left ventricular ejection fraction: results of randomized, multicentre Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction (REGENT) Trial. Eur Heart J 30:1313–1321
36. Howe AJ, Shand JA, Menown IB (2011) Advances in cardiology: clinical trial update. Future Cardiol 7:299–310
37. Wojakowski W, Landmesser U, Bachowski R, Jadczyk T, Tendera M (2012) Mobilization of stem and progenitor cells in cardiovascular diseases. Leukemia 26:23–33

38. Ratajczak MZ, Machalinski B, Wojakowski W, Ratajczak J, Kucia M (2007) A hypothesis for an embryonic origin of pluripotent Oct-4(+) stem cells in adult bone marrow and other tissues. Leukemia 21:860–867
39. Shin DM, Zuba-Surma EK, Wu W, Ratajczak J, Wysoczynski M, Ratajczak MZ et al (2009) Novel epigenetic mechanisms that control pluripotency and quiescence of adult bone marrow-derived Oct4(+) very small embryonic-like stem cells. Leukemia 23:2042–2051
40. Shin DM, Liu R, Klich I, Wu W, Ratajczak J, Kucia M et al (2010) Molecular signature of adult bone marrow-purified very small embryonic-like stem cells supports their developmental epiblast/germ line origin. Leukemia 24:1450–1461
41. Ratajczak MZ, Liu R, Ratajczak J, Kucia M, Shin DM (2011) The role of pluripotent embryonic-like stem cells residing in adult tissues in regeneration and longevity. Differentiation 81:153–161
42. Ratajczak MZ, Zuba-Surma E, Kucia M, Poniewierska A, Suszynska M, Ratajczak J (2012) Pluripotent and multipotent stem cells in adult tissues. Adv Med Sci 57:1–17
43. Kucia M, Masternak M, Liu R, Shin DM, Ratajczak J, Mierzejewska K et al (2013) The negative effect of prolonged somatotrophic/insulin signaling on an adult bone marrow-residing population of pluripotent very small embryonic-like stem cells (VSELs). Age (Dordr) 35:315–330
44. Ratajczak J, Wysoczynski M, Zuba-Surma E, Wan W, Kucia M, Yoder MC et al (2011) Adult murine bone marrow-derived very small embryonic-like stem cells differentiate into the hematopoietic lineage after coculture over OP9 stromal cells. Exp Hematol 39:225–237
45. Ratajczak J, Zuba-Surma E, Klich I, Liu R, Wysoczynski M, Greco N et al (2011) Hematopoietic differentiation of umbilical cord blood-derived very small embryonic/epiblast-like stem cells. Leukemia 25:1278–1285
46. Zuba-Surma EK, Guo Y, Taher H, Sanganalmath SK, Hunt G, Vincent RJ et al (2011) Transplantation of expanded bone marrow-derived very small embryonic-like stem cells (VSEL-SCs) improves left ventricular function and remodelling after myocardial infarction. J Cell Mol Med 15:1319–1328
47. Dawn B, Tiwari S, Kucia MJ, Zuba-Surma EK, Guo Y, Sanganalmath SK et al (2008) Transplantation of bone marrow-derived very small embryonic-like stem cells attenuates left ventricular dysfunction and remodeling after myocardial infarction. Stem Cells 26:1646–1655
48. Bhartiya D, Kasiviswananthan S, Shaikh A (2012) Cellular origin of testis-derived pluripotent stem cells: a case for very small embryonic-like stem cells. Stem Cells Dev 21:670–674
49. Parte S, Bhartiya D, Telang J, Daithankar V, Salvi V, Zaveri K et al (2011) Detection, characterization, and spontaneous differentiation in vitro of very small embryonic-like putative stem cells in adult mammalian ovary. Stem Cells Dev 20:1451–1464
50. Kucia M, Shin DM, Liu R, Ratajczak J, Bryndza E, Masternak MM et al (2011) Reduced number of VSELs in the bone marrow of growth hormone transgenic mice indicates that chronically elevated Igf1 level accelerates age-dependent exhaustion of pluripotent stem cell pool: a novel view on aging. Leukemia 25:1370–1374
51. Ratajczak J, Shin DM, Wan W, Liu R, Masternak MM, Piotrowska K et al (2011) Higher number of stem cells in the bone marrow of circulating low Igf-1 level Laron dwarf mice-novel view on Igf-1, stem cells and aging. Leukemia 25:729–733
52. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS et al (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147
53. Yamanaka S (2012) Induced pluripotent stem cells: past, present, and future. Cell Stem Cell 10:678–684
54. Brons IG, Smithers LE, Trotter MW, Rugg-Gunn P, Sun B, Chuva de Sousa Lopes SM et al (2007) Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448:191–195
55. Tesar PJ, Chenoweth JG, Brook FA, Davies TJ, Evans EP, Mack DL et al (2007) New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448:196–199

56. Shin DM, Liu R, Wu W, Waigel SJ, Zacharias W, Ratajczak MZ et al (2012) Global gene expression analysis of very small embryonic-like stem cells reveals that the Ezh2-dependent bivalent domain mechanism contributes to their pluripotent state. Stem Cells Dev 21:1639–1652
57. Kono T, Obata Y, Wu Q, Niwa K, Ono Y, Yamamoto Y et al (2004) Birth of parthenogenetic mice that can develop to adulthood. Nature 428:860–864
58. Surani MA, Hayashi K, Hajkova P (2007) Genetic and epigenetic regulators of pluripotency. Cell 128:747–762
59. Reik W, Walter J (2001) Genomic imprinting: parental influence on the genome. Nat Rev Genet 2:21–32
60. Keniry A, Oxley D, Monnier P, Kyba M, Dandolo L, Smits G et al (2012) The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r. Nat Cell Biol 14:659–665
61. Ragina NP, Schlosser K, Knott JG, Senagore PK, Swiatek PJ, Chang EA et al (2012) Down-regulation of H19 improves the differentiation potential of mouse parthenogenetic embryonic stem cells. Stem cells Dev 21:1134–1144
62. Janowska-Wieczorek A, Majka M, Ratajczak J, Ratajczak MZ (2001) Autocrine/paracrine mechanisms in human hematopoiesis. Stem Cells 19:99–107
63. Ratajczak J, Kijowski J, Majka M, Jankowski K, Reca R, Ratajczak MZ (2003) Biological significance of the different erythropoietic factors secreted by normal human early erythroid cells. Leuk Lymphoma 44:767–774
64. Kim C, Schneider G, Abdel-Latif A, Mierzejewska K, Sunkara M, Borkowska S et al (2013) Ceramide-1-phosphate regulates migration of multipotent stromal cells and endothelial progenitor cells-implications for tissue regeneration. Stem Cells 31:500–510
65. Lataillade JJ, Clay D, Bourin P, Herodin F, Dupuy C, Jasmin C et al (2002) Stromal cell-derived factor 1 regulates primitive hematopoiesis by suppressing apoptosis and by promoting G(0)/G(1) transition in CD34(+) cells: evidence for an autocrine/paracrine mechanism. Blood 99:1117–1129
66. Sahoo S, Klychko E, Thorne T, Misener S, Schultz KM, Millay M et al (2011) Exosomes from human CD34(+) stem cells mediate their proangiogenic paracrine activity. Circ Res 109:724–728
67. Camussi G, Deregibus MC, Tetta C (2010) Paracrine/endocrine mechanism of stem cells on kidney repair: role of microvesicle-mediated transfer of genetic information. Curr Opin Nephrol Hypertens 19:7–12
68. Quesenberry PJ, Dooner MS, Aliotta JM (2010) Stem cell plasticity revisited: the continuum marrow model and phenotypic changes mediated by microvesicles. Exp Hematol 38:581–592
69. Beaudoin AR, Grondin G (1991) Shedding of vesicular material from the cell surface of eukaryotic cells: different cellular phenomena. Biochim Biophys Acta 1071:203–219
70. Barry OP, FitzGerald GA (1999) Mechanisms of cellular activation by platelet microparticles. Thromb Haemost 82:794–800
71. Fevrier B, Raposo G (2004) Exosomes: endosomal-derived vesicles shipping extracellular messages. Curr Opin Cell Biol 16:415–421
72. Gatti S, Bruno S, Deregibus MC, Sordi A, Cantaluppi V, Tetta C et al (2011) Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. Nephrol Dial Transplant 26:1474–1483
73. Aliotta JM, Sanchez-Guijo FM, Dooner GJ, Johnson KW, Dooner MS, Greer KA et al (2007) Alteration of marrow cell gene expression, protein production, and engraftment into lung by lung-derived microvesicles: a novel mechanism for phenotype modulation. Stem Cells 25:2245–2256
74. VanWijk MJ, VanBavel E, Sturk A, Nieuwland R (2003) Microparticles in cardiovascular diseases. Cardiovasc Res 59:277–287

75. Herrera MB, Fonsato V, Gatti S, Deregibus MC, Sordi A, Cantarella D et al (2010) Human liver stem cell-derived microvesicles accelerate hepatic regeneration in hepatectomized rats. J Cell Mol Med 14:1605–1618
76. Camussi G, Cantaluppi V, Deregibus MC, Gatti E, Tetta C (2011) Role of microvesicles in acute kidney injury. Contrib Nephrol 174:191–199
77. Ohi Y, Qin H, Hong C, Blouin L, Polo JM, Guo T et al (2011) Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human iPS cells. Nat Cell Biol 13:541–549

Chapter 3
Cord Blood Stem Cells

Marta Adamiak, Zbigniew Madeja and Ewa K. Zuba-Surma

3.1 Introduction

Umbilical cord represents a vast tissue structure connecting developing fetus with placenta and contains three major blood vessels including one vein and two arteries harboring fetal blood routinely called as umbilical cord blood (UCB) [1]. The major function of UCB is to supply the developing fetus with oxygen and important nutrition elements required for proper fetal development as well as removes metabolites and fetal waste products [1]. Cord blood as a body fluid of such type contains heterogeneous mixture of cells including standard morphometric elements of blood such as red blood cells—with fetal type of hemoglobin, white blood cells, platelets, and plasma [1].

Importantly, because of a unique fetal origin, UCB may be greatly enriched in immature cells including stem and progenitor cells (SPCs). Therefore, the UCB has become an interesting subject for potential therapeutic applications, which has been initially reported in 1972 when UCB-derived cells were employed in treatment of lymphoblastic leukemia by US clinicians [2]. In addition to UCB, other components of umbilical cord, such as a gelatinous substance called the Wharton's jelly [3] or even placenta itself have been indicated as valuable sources of stem cells (SCs) [4, 5].

Currently, the main sources of SPCs for clinical auto- and allogenic transplantations include human bone marrow (BM) tissue and mobilized peripheral blood harvested following SPC mobilization with granulocyte colony-stimulating factor (G-CSF) treatment and leukapheresis products collection [6, 7]. However, more recently UCB has been also included into these clinical specimens as alternate source of SCs and may offer multiple advantages in practical applications in allogenic transplants [6]. Advantages of fetal blood SPCs include their non-invasive and rapid availability, immaturity, which may play a significant role in reduced rejection after

E. K. Zuba-Surma (✉) · M. Adamiak · Z. Madeja
Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, 7 Gronostajowa St., 30-387 Krakow, Poland
e-mail: ewa.zuba-surma@uj.edu.pl

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,* Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_3,

transplantation into a mismatched host, and their ability to produce large quantities of homogenous tissue or cells during proliferation and differentiation [8].

Due to volume restriction, single UCB unit typically offers a limited number of cells. However, a growing interest in the scientific community focuses on methods to develop safe and reliable technologies for ex vivo expansion, effective cryopreservation of UCB-derived SCs as well as pooling of SPCs derived from few UCB units [9]. Moreover, the applications of whole multiple pooled units have been also examined as a method of use CB-derived SCs in allogenic adult patients [10, 11].

3.2 Cord Blood Stem Cells

SCs may be distinguished from other cell types by two important characteristics. First, SCs represent unspecialized, early-stage cells capable of renewing through cell division often following long stage of quiescence. Second, under certain physiological or experimental conditions, SPCs may give rise into another type of cell with a more specialized function including muscles, blood or different types of neuronal cell and other mature cells. The heterogeneous compartment of SCs existing during mammalian ontogenesis may be organized in a hierarchical way from the most primitive (totipotent and pluripotent SC) to already differentiated tissue-committed (monopotent) SCs [12]. Initially, while the monopotent, tissue-committed SCs have been described in adult tissues, the SPCs with pluripotent and multipotent capacity were thought to be restricted to the early embryonic stages [12]. However, the presence of cells that possess a pluripotent and multipotent character in adult tissues was recently confirmed. Indeed, several groups of investigators have identified different populations of SCs closely related to the compartment of pluripotent SCs in adult tissues, mainly in BM and postnatal material such as UCB [13–19].

In 1974, Knudtzon and colleagues demonstrated that UCB contains an abundant population of hematopoietic stem cells (HSCs) [28]. Such HSCs may be defined by two key functional properties: (1) multipotency, represented by their ability to generate committed progenitors of both the myeloid and lymphoid compartments, and (2) long-term self-renewal. Consequently, early HSCs themselves are capable of constant repopulating and renewing whole hematopoietic and immunological system within the human lifespan. HSCs represent a heterogeneous population of immature precursor cells at different stages of hematopoietic commitment in UCB. Interestingly, in case of UCB, it has been shown that it harbors greater primitive HSC content than either BM or mobilized peripheral blood, which exhibit high proliferative and expansion potential with an extended cell lifespan and longer telomeres [29]. CB HSCs may be characterized by the expression of certain hematopoietic antigens, such as CD34 and CD133, and the absence of hematopoietic lineage-specific antigens, which phenotype confirms their early stage of tissue commitment [30]. Importantly, Majeti et al. have demonstrated that the $Lin^{-}CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$ UCB population contains human HSCs and may reside at the top of the human hematopoietic developmental hierarchy [31].

Morover, in vitro studies confirmed, that UCB HSCs may be selectively induced into specific hematopoietic lineages including erythroid, megakaryocytic and monocytic cells [30]. Several other subpopulations of SPCs with hematopoietic differentiation potential have been already identified [1, 32–34].

3.3 Non-hematopoietic Stem Cells Present in Cord Blood

In addition to well-known and characterized HSCs, other SPC fractions, such as very small embryonic-like stem cells (VSELs) [15, 20, 21], endothelial progenitor cells (EPCs) [19, 23], neural stem cells (NSCs) [18, 24], unrestricted somatic stem cells (USSCs) [14], mesenchymal stem cells (MSCs) [25, 26], multipotent progenitor cells (MPCs) [27], and other have been isolated from human UCB and characterized according to their morphology, immunophenotype, proliferation and differentiation potential (Table 3.1). In some cases, similar or overlapping populations of primitive SCs in the UCB have probably been detected by using different experimental strategies and hence have been assigned with different names [1, 32–34]. In this chapter, we briefly summarize the characteristics of human UCB-derived stem and progenitor SCs indicating their similarities and great differences.

3.3.1 Very Small Stem Cell with Embryonic-like Characteristics

VSELs have been initially described by Kucia et al. in adult murine BM as a rare (~0.02% of BM mononuclear cells) population of SCs with embryonic characteristics [35]. Such murine population of VSEL SCs may be characterized by (1) their small size (~4 μm), (2) a high nuclear/cytoplasmic ratio, (3) open-type of chromatin (euchromatin), (4) a membrane phenotype including expression of Sca-1 and lack of hematopoietic lineages markers and CD45 antigen (Sca-1$^+$Lin$^-$CD45$^-$), and (5) migratory response to stromal cell-derived factor 1 (SDF-1) stimulation [35, 36]. Importantly, such murine VSELs express several embryonic stem cell (ESC) markers such as surface marker stage-specific embryonic antigen 1 (SSEA-1) and transcription factors Oct-4, Nanog and Rex-1 on both at the mitochondrial ribonucleic acid (mRNA) and protein level as well as possess unique epigenetic status and may be found not only in BM but also in other tissues [35–37]. VSELs are capable to differentiate into selected cell types derived from all three germ-layer lineages. It has been hypothesized that the population of Sca-1$^+$Lin$^-$CD45$^-$ VSELs was deposited in tissues early during development and could be a source of early SCs with some pluripotent characteristics for tissue regeneration [38–40].

SC population with similar morphology, electron microscopic ultrastructure as well as Oct-4, Nanog, and other embryonic marker expression has been also identified in human UCB [20]. UCB-derived VSELs (CB-VSELs) are (1) smaller than human erythrocytes and resemble small lymphocytes (6–7 μm in diameter), (2) are

Table 3.1 Selected populations of versatile non-HSCs described in CB

Stem cells	Antigenic phenotype	Other features	References
Very small embryonic-like stem cells (VSELs)	$CD45^-$ Lin^- (CD235a-CD2-CD3-CD14-CD16-CD19-CD20-CD56-CD66b) $CD133/1^+$ or $CD34^+$ $SSEA\text{-}4^+Oct\text{-}4^+$ $Nanog^+SSEA\text{-}1^-$	6–7 μm in size; isolated directly from UCB by MACS ($CD133^+$or $CD34^+$) and FACS ($Lin^-CD45^-CD133/1^+$)	[20, 21] Fig. 3.1
Cord-blood-derived embryonic-like stem cells (CBEs)	$CD45^-CD33^-$, $CD7^-$, $CD235a^-$ $CD34^-CD133/1^+$ $TRA\text{-}1\text{–}60^+TRA\text{-}1\text{–}81^+SSEA\text{-}4^+SSEA\text{-}3^+Oct\text{-}4^+Sox2^+SSEA\text{-}1^-$	2–3 μm in size; isolated directly from UCB by MACS ($CD45^-CD235a^-CD33^-CD7^-$)	[15, 22]
Endothelial progenitor cells (EPCs)	$CD34^+CD45^-$ or $CD31^+CD34^+CD133/1^+$ $CD45^{dim}$	Form colonies of adherent ECs in vitro and possess angiogenic potential in vivo	[19, 23]
Human UCB-neural stem cells (UCB-NSCs)	$CD34^-CD45^-CD133^+Nestin^+$ $Oct3/4^+Sox2^+Rex1^+$	Isolated directly from UCB-derived MNCs by MACS ($CD34^-$)	[18, 24]
Unrestricted somatic stem cells (USSCs)	$CD13^+CD29^+CD90^+CD105^+$ $CD49e^+CD44^+VIM^+CK8^+$ $CK18^+CD10^{dim}FLK1^{dim}CD14^-CD33^-CD34^-CD45^-CD49b^-CD49c^-CD49d^-CD49f^-CD50^-CD62E^-CD62L^-CD62P^-CD106^-$ $CD117^-CD235a^-HLA\text{-}DR^-$	Selected within MNCs-derived adherent cells cultured in presence of DXM; differentiate into cells from 3 germ layers	[14]
Mesenchymal stem cells (MSCs)	$CD105^+CD73^+CD166^+CD34^-$ $CD45^-CD86^-$	Selected in culture as adherent fraction of UCB cells	[25, 26]
Multilineage progenitor cells	$CD14^+CD31^+CD44^+CD45^+CD54^+CD49a^-CD62E^-CD73^-CD90^-$ $CD104^-$	Selected within MNCs-derived adherent cells cultured in presence of GM-CSF	[27]

VIM vimentin, *CK* cytokeratin, *ECs* endothelial cells, *MNCs* mononuclear cells, *MACS* magnetic-activated cell sorting, *FACS* fluorescence-activated cell sorting, *DXM* dexamethasone, *GM-CSF* granulocyte-macrophage colony-stimulating factor

highly enriched in a population of $Lin^-CD45^-CD133^+$ cells, (3) possess large nuclei containing immature open-type euchromatin, (4) express nuclear embryonic transcription factors Oct-4A and Nanog, and (5) stain positively for surface embryonic antigen SSEA-4 [20, 21, 41, 42], Fig. 3.1. These neonatal VSEL SCs are supposedly mobilized from the BM niche and other tissues into neonatal peripheral blood due to the stress related to delivery [20, 21, 41, 42]. Because of their small size and density, UCB-derived VSELs may be lost at various steps of classical, standardized UCB preparation prior to its banking [21, 41]. Accordingly, a significant number of these cells, which are smaller than erythrocytes, may be lost during gradient centrifugation over Ficoll-Paque as well as during routine volume depletion of UCB units before freezing. Taking this fact under consideration, and to preserve these precious cells in final UCB preparations, Zuba-Surma et al. proposed a relatively short

and economical three-step isolation protocol that allows recovery of approximately 60 % of the initial number of $Lin^-CD45^-CD133^+$ UCB-VSEL SCs present in freshly harvested UCB units [21]. In this approach, UCB is typically treated with hypotonic ammonium chloride solution to deplete erythrocytes, then CD34 or CD133-positive cells including VSEL SCs are enriched by employing immunomagnetic beads (magnetic-activated cell sorting, MACS) and subsequently $Lin^-CD45^-CD133^+$ cells are sorted by employing fluorescence-activated cell sorting (FACS) [21, 43]. The published data indicate that subpopulation of $Lin^-CD45^-CD133^+$ cells coexpressing SSEA-4 antigen may represent the most primitive compartment of VSELs and be enriched in cells sharing embryonic-like features [21]. Importantly, by employing deoxyribonucleic acid (DNA) cytogenetic matrix array, it has been recently confirmed that such purified $Lin^-CD45^-CD133^+$ CB-VSELs represent population of healthy diploid cells with normal karyotype (Fig. 3.1). While murine BM-derived VSELs have been extensively characterized, more work is still required to better characterize these small cells at the molecular level in humans.

Importantly, several independent groups worldwide have provided evidence on the existence of primitive VSEL or VSEL-like SCs residing in human UCB, BM, mobilized peripheral blood, and other human specimens, reporting their unique features and indicating their potential role in tissue and organ rejuvenation, longevity and regeneration [41, 44–49]. Notably, growing evidence confirm the existence of similar small embryonic-like SCs in UCB, which express CD133 antigen, Oct-4, and other pluripotency markers; however, such SPC fractions may be identified in literature under different names given by their inventors [15, 18, 22, 24, 50]. Selected populations of such SPCs will be described in further parts of this chapter.

Although there are strong data indicating presence of such very small SCs exhibiting several features of embryonic-like cells in UCB, some pitfalls in their isolation have been currently reported [51]. Although it has been strongly emphasized by several investigators that most primitive UCB-derived SCs are predominantly enriched infractions expressing CD34 and CD133 antigens, Danova-Alt and colleagues exclusively focused on the subset of $CD45^-Lin^-CXCR4^+$ UCB cells and improperly referred this fraction to "VSELs" [51]. Interestingly, these authors concluded that $CD45^-/Lin^-$ cells from CB essentially lack CD34 and CD133 antigens while these cells have been described before and more importantly are even visible on the histograms shown in the paper [51]. Unfortunately, the authors inexpediently overlooked the both rare $CD45^-Lin^-CD34^+$ and $CD45^-Lin^-CD133^+$ subpopulations including VSELs and most likely CB-derived embryonic-like stem cells (CBEs) [15, 21, 22], which they actually detected in their samples [51].

Thus, the right protocol for UCB-derived human VSELs, which has been widely provided, should be followed [21, 43]. The isolation would include a two-step strategy involving both MACS and FACS strategy and focus on $CD45^-Lin^-CD133^+$ cells as the most enriched in cells with embryonic-like features [21, 43]. This may be an example to emphasize difficulties, which may be faced during identification and isolation of not only VSELs but indeed any rare SC fraction and to stress that some discrepancies in data collection and analysis may result in their improper interpretation or even in loss of quested SC fractions during isolation procedures.

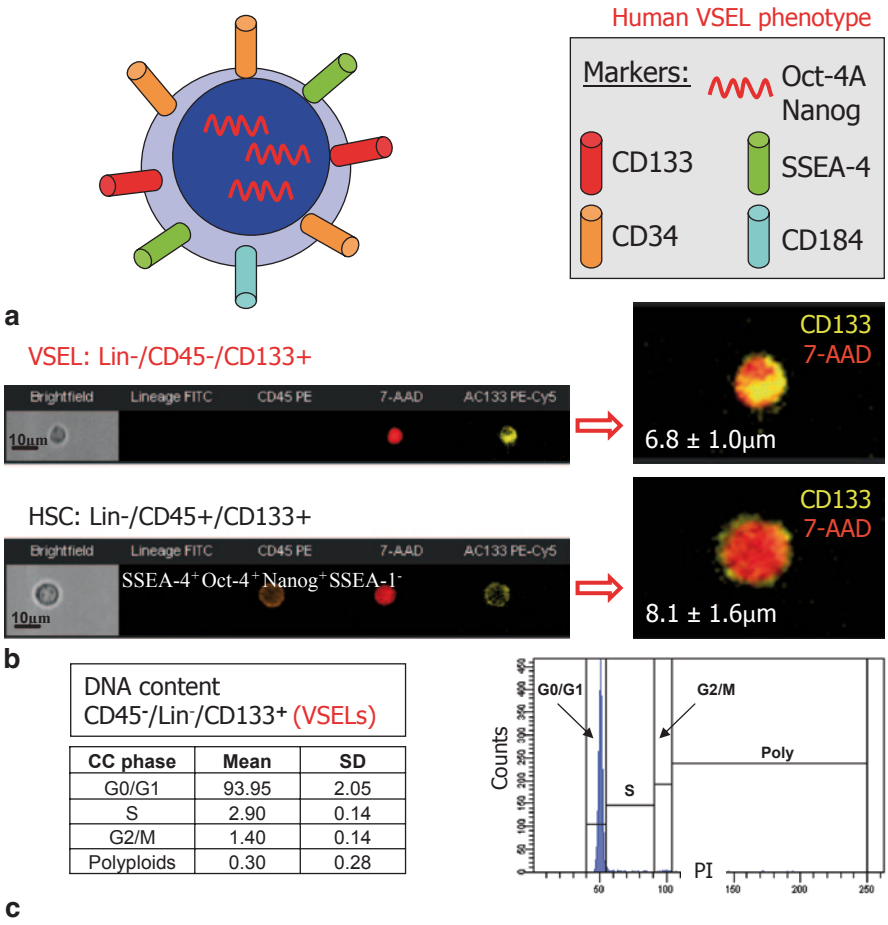

Fig. 3.1 Phenotype of CB-derived very small embryonic-like stem cells (VSELs). Panel A—Scheme of major multiantigenic phenotype of CB-VSELs including surface markers (CD133, CD34, CXCR4, CD184) and intranuclear antigens (e.g., Oct-4A, Nanog). Panel B—Representative images of CB-derived VSEL and hematopoietic stem cell (HSC) by imaging cytometry (ImageStream system, Amnis Corp.). Each photograph indicates expression of selected antigens ("Lineage"—including markers of several major hematopoietic lineages (green); CD45 (orange); CD133 (AC133, yellow)). Nuclear image is shown in red as indicated by staining with 7-amino-actinomycin D (7-AAD). Combined images of both VSEL and HSC show expression of CD133 and include average size of each population calculated as minor axis of acquired cells computed based on brightfield images by IDEAS software (Amnis Corp.). Panel C—Deoxyribonucleic acid (DNA) content of purified CD45−/Lin−/CD133+ CB−-derived VSELs measured by classical flow cytometry (LSR II flow Cytometer; Becton Dickinson) following the staining with propidium iodide (PI). Majority of CB-VSELs are represented as quiescent cells in G0/G1 phase of cell cycle

Importantly, similar cell fractions of primitive embryonic-like SCs isolated from human UCB have also been reported by other investigators and described under different names. Interestingly, McGuckin and colleagues have demonstrated the

presence of very small SCs in UCB and estimated their size at 2–3 μm that is even smaller that size calculated for CB-VSELs [15, 22]. Similarly to VSELs, these cells were described asCBEs due to their expression of similar markers to the embryonic SCs derived from embryonic tissues [15]. CBEs represent a very rare population of mononucleated cells present in human UCB (0,1–1 % of total UCB cells) that are negative for hematopoietic lineage markers such as CD45, glycophorin-A, CD7, CD33, and CD34, but are positive CD133 [15, 22]. CBEs may form embryoid body-like colonies in vitro, which progressively increase in both size and number during the culture. The CBE colonies express the ESC transcription factors Oct-4 and Sox2, and are positive for the embryonic stage-specific antigens SSEA-3 and SSEA-4, but lack the expression of embryonic antigen-1 (SSEA-1), which confirm their undifferentiated phenotype [15, 22]. Interestingly, this exceptionally immature and small SC fraction has been proved to bear differentiation potential into neural tissue [22].

Importantly, the work published by McGuckin and coworkers strongly confirms the existence of SCs smaller than erythrocytes in human UCB specimens, which cannot be underappreciated, especially when the functional potential of these cells has been confirmed.

3.3.2 Endothelial Progenitor Cells

Over a decade ago, Asahara and colleagues have reported that human CD34-positive cells isolated from peripheral blood are capable of incorporating into the sites of active angiogenesis and may contribute to the formation of new blood vessels by vasculogenesis [13]. Since then, postnatal vasculogenesis has been purported to be an important mechanism by which the adult vasculature may be augmented via EPCs.

Currently, there is no uniform definition of an EPC and the discussion regarding the right antigenic phenotype of such cell is still ongoing [52, 53]. In most studies, EPCs are identified and enumerated by using flow cytometric protocols focusing on cells expressing CD34, CD133, or VEGF receptor 2 (kinase insert domain receptor, KDR) [19, 54]. Importantly, since these molecules may be also expressed on hematopoietic stem and progenitor populations, the presence of hematopoietic contamination of EPCs should be taken under consideration. EPCs are also often quantitated by counting and employing commercially available kit that identifies endothelial cell colony-forming units (CFU-ECs). However, Yoder et al. have indicated that CFU-ECs are not in fact EPCs but are the proangiogenic hematopoietic progenitor cells or their myeloid progeny, whereas endothelial colony-forming cells (ECFC) are vessel-forming EPCs [19].

Numerous studies show that populations of cells termed EPCs may be also isolated from human UCB by culturing sorted $CD34^+$ cells or mononuclear cells (MNCs) in defined culture conditions [55, 56]. However, only a rare population of EPCs with ECFC is considered as true endothelial progenitors capable to form vessels in vivo [19].

In order to obtain EPCs, UCB-derived MNCs are typically harvested and seeded onto collagen-coated tissue culture plates in endothelial growth media (e.g., EGM-2) [19]. Nonadherent cells are discarded after 24 h of culture and only adherent cells are further propagated. ECFC colonies may be identified within adherent cells between days 5 to 22 of culture [19]. Importantly, ECFCs and their progeny differ from HSCs in their membrane antigenic phenotype and clonal growth in proangiogenic medium (EGM-2). Thus, ECFCs are capable of extensive proliferation when grown in EGM-2 medium and possess typical functional properties of endothelial cells, both in vitro and in vivo [19]. The most stringent method to assess whether the ECFCs display the ability to function as postnatal vasculogenic cells was their implantation followed by counting of new blood vessels in vivo [19].

Although, the angiogenic activity of ECFCs has been confirmed, the antigenic phenotype of UCB cells generating ECFCs remains still unclear. It has been reported that either the $CD34^{+}CD45^{-}$ [23] or the $CD34^{+}CD133^{+}CD45^{dim}$ [57] UCB populations fraction may in fact contain ECFCs. Interestingly, recent study by Lee et al. has indicated the vast supportive role of CD34- compartment of UCB-derived MNCs in promoting activity of $CD34^{+}$ progenitors to form ECFC colonies in co-cultures system [58]. Despite the unquestionable impact of these results on future EPC propagation strategies, this data also indicate that some of the discussions and discrepancies in terms of EPC definition, marker expression, and genuine endothelial potential may results from nonoptimal conditions employed for evaluating of proangiogenic potential of such isolated "candidate" subpopulations of EPCs.

Thus, the "EPC story" is still ongoing and developing, and presence of such progenitors in human UCB may potentially create a future opportunities to obtain large quantities of autologous or allogenic ECs for therapeutic vascularization and tissue engineering. However, the isolation of UCB-derived EPCs with bona fide blood vessel-forming ability has not been straightforward because of the low concentration of EPCs in fresh and stored UCB and the lack of a set of specific EPC markers. For these reasons, the isolation of UCB-derived EPCs by flow cytometry or other optimized techniques remains still a challenging problem [59].

3.3.3 Human Umbilical Cord Blood—Neural Stem Cells

NSCs naturally present in central nervous system of adult mammals are clonogenic, self-renewing cells with the potential to differentiate into each of the three cell types of human brain, including neurons, astrocytes, and oligodendrocytes [60].

Buzanska and colleagues have demonstrated that such NSC subpopulation may be also harvested from human UCB samples and expanded in vitro [18, 61]. Such population of UCB-derived NSC (UCB-NSCs) was isolated from the CB mononuclear cell fraction through combination of (1) negative selection of $CD34^{-}$ fraction of MNCs following staining against CD34 antigen by immunomagnetic cell sorting, (2) selection of population of $CD34^{-}$ cells with adhesive properties to culture dish, and (3) subsequent propagation of adherent fraction in the presence of defined media and growth factors [18, 61].

Interestingly, freshly isolated $CD34^-$ UCB-NSCs may express markers of embryonic cell including Oct3/4, Sox2, and Rex1, which strongly supports the existence of SCs with pluripotent characteristics in human UCB [24]. Moreover, the authors indicated that the frequency of Oct3/4 immunopositive cells significantly increased with parallel enlargement of "side population" cells and $CD133^+$ cell appearance following 24 h of UCB-NSCs culture [24].

The outgrown clones obtained during prolonged UCB-NSCs culture have been shown to lose CD45 antigen as well as their hematopoietic or angiogenic properties and exhibited the expression of Nestin—a neurofilament protein that represents one of the most specific markers of multipotent NSCs [18, 24]. In the presence of selected growth factors or rat brain-derived astrocytes in the co-culture system, such purified CB-derived NSCs started to differentiate along the three major central nervous system lines, which may be identified by morphological features and specific protein expression patterns including a class of III β-tubulin as a neuronal marker, glial fibrillar acidic protein (GFAP) and S100β as markers of astrocytes, and galactosylceramidase protein (GALC) as a marker of oligodendrocytes [24]. It has been also shown that majority of stem and neural-related genes were upregulated in UCB-NSCs as compared to the initial nonselected mononuclear cell population [24]. In addition to activation of early neural genes including NeuroD1, Otx1, and Msi, the upregulation in genes characterizing pluripotent ESCs (e.g., Oct4, Sox2, Mdr1, Rex1) was also described in these cells [24]. Thus, HUCB-NSC may retain their potential pluripotency while executing exclusively. Moreover, Sun et al. have showed that HUCB-NSC express numerous neurotransmitter receptors and several functional voltage-gated channels identified in neuronal systems that may probably allow them to function within a neural network and respond to neurotransmitters released from neighboring neurons [62]. Indeed, it was demonstrated that blood-cells-derived neurons may be capable to generate and transmit action potential in a similar pattern to that of primary cultures of neurons [63]. Other functional neuronal properties such as calcium-influx have also been confirmed in UCB-derived neuron-like cells [64].

Human UCB-derived and neural-committed SCs, due to their capacity for self-renewal, expression of several markers of pluripotent cells, multilineage differentiation, proliferation potential, and ability for long-lasting culturing in vitro, confirm the existence of early SCs with embryonic-like characteristics in UCB that may be an useful tool for future cell therapy applicable not only for treatment of human central nervous system diseases but also other tissues disorders.

3.3.4 Unrestricted Somatic Stem Cells

In 2004, the group of Kögler and colleagues described, in human UCB, a population of CD45-negative and human leukocyte antigen (HLA) class II-negative cells with intrinsically pluripotent features, which they called USSCs [14]. Such SPC population has been isolated by growth of total UCB MNCs in presence of dexamethasone

[14]. The selected rare, adherent cell population was highly proliferative and easily expandable in serum-free conditions.

USSCs have been well described in terms of surface marker expression and they are characterized by (1) negativity for following antigens: CD14, CD33, CD34, CD45, CD49b, CD49c, CD49d, CD49f, CD50, CD62E, CD62L CD62P, CD106, CD117, glycophorin-A, HLA-DR, by (2) the high expression of CD13, CD29, CD90, CD105, CD49e, CD44, vimentin, cytokeratins (8 and 18), as well as (3) low level of CD10 and FLK1 (KDR) [14]. Moreover, USSCs actively express various transcripts for cell surface markers, transcription factors, and cytokine receptors, including epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), insulin-like growth factor receptor (IGFR), runt-related transcription factor (RUNX1), Y-box-binding protein (YB1), CD49e, and CD105 [14].

The cells were shown to be negative for chondrogenic extracellular protein (chondroadherin), bone-specific markers (collagenase X and sialoprotein), liver- and pancreas-specific markers (e.g., Cyp1A1, PDX-1), and neural markers such as neurofilament, synaptophysin, tyrosine hydroxylase, and GFAP [65].

On the other hand, in vitro and in vivo experiments revealed that USSCs possess the capacity of differentiation into cell types found in mesodermal, endodermal, and ectodermal lineages, that is, hematopoietic cells, osteoblasts, chondroblasts, adipocytes, neurons, and astrocytes, thus indicating their pluripotent properties [14].

The multilineage potential of USSCs remains unclear and, unlike in human ESCs, is not based on expression of the major SC factors including Oct4, Sox2, and Nanog [66]. Interestingly, it has been reported that none of the major listed SC factors exhibits significant expression in USSC and the subsequent DNA methylation analysis revealed partial demethylation of the regulatory region of Oct-4 and a demethylated state of Nanog and Sox2 promoter/enhancer regions in USSCs [66]. Thus, the molecular signature of USSCs may indicate that they hold an epigenetic state in between that of an ESC and a terminally differentiated cell type [66].

USSCs are considered as early precursors to MSCs with greater differentiation capacity. Although, selective antigenic markers of USSCs are still unknown, these cells may be distinguished from MSCs by their higher expansion capacity, broader differentiation ability, and distinct level of expression of selected genes including δ-like 1/preadipocyte factor 1 (DLK1) and the homeobox (HOX) gene clusters [67]. It has been demonstrated that particularly adipogenic differentiation potential may be a functional marker to discriminate between USSCs and CB-derived MSCs [68]. In opposite to MSCs, USSCs do not exhibit any adipogenic differentiation potential except for those passing through a high number of population doublings [68].

Based on their biological features, USSCs may represent interesting candidates for regenerative and cell replacement purposes. Indeed, therapeutic potential of these cells has been already tested in bone healing, in the repair of myocardial infarction, and reduction of graft-versus-host disease [69]. However, a vast condition to employ USSC for such purposes in a more controlled manner would be a better understanding of the molecular mechanisms underlying establishment and maintenance of USSC stemness.

3.3.5 Umbilical-Cord-Blood-derived Mesenchymal Stem Cells

MSCs are defined as multipotent cells capable of differentiating into osteogenic, adipogenic, and chondrogenic lineages as well as cardiomyocytes and non-mesodermal lineages, including neural and hepatic lineages [70, 71]. Currently, the most common source of MSCs for scientific purposes and clinical applications is represented by BM tissue [71]. However, UCB comprises an important alternative source for harvesting of MSCs with high proliferative and differentiation potentials [72, 73]. The isolation of MSCs may be performed from mononuclear UCB cells by growth of adherent cells under specific culture conditions, such as special culture media containing defined growth factors (i.e., basic fibroblast growth factor, bFGF) [26].

UCB-derived MSCs exhibit high morphological and molecular similarities to BM-derived MSCs, including the cell surface antigen profile. They may proliferate in culture with an attached spindle-shaped morphology and are strongly positive for MSC-specific cell surface markers such as CD105, CD73, and CD166, but negative for CD14 (monocyte antigen), CD31 (endothelial cell antigen), CD34 (HSC antigen), CD45 (leukocyte common antigen), and CD86 (a costimulatory molecule) [33]. Isolation of MSCs from UCB has been shown to be challenging because of the low number of such SPCs when compared to BM tissue. However, UCB-derived MSCs demonstrate higher proliferation capabilities than BM MSCs [33].

MSCs can be isolated not only from UCB but also from the umbilical cord matrix—Wharton's jelly, placenta, as well as from amnion and amniotic fluid itself [3, 73]. Umbilical cord Wharton's jelly-derived MSCs may be isolated by mechanical dissection and enzymatic digestion and in general exhibit immunophenotypic and immunological properties similar to those reported for marrow-derived MSCs [74].

Wang et al. in their study have demonstrated that Wharton's jelly-derived mesenchymal cells expanded in in vitro culture may express several mesenchymal antigens including adhesion molecules (CD44, CD105), integrin markers (CD29, CD51), and MSC markers (SH2, SH3) but not markers of hematopoietic differentiation (CD34, CD45) [3]. Importantly, such human umbilical cord matrix MSCs may not only be expanded in culture but also induced to form several different types of cells [3]. Umbilical cord Wharton's jelly-derived MSCs are being considered as more robust than those from UCB and less invasive than those from the BM as well as secrete more angiogenesis-stimulating growth factors [75, 76]. They may therefore be a new source of cells for practical applications.

Wide-ranging differentiation and in vitro expansion potential, immunomodulatory effects, and the ability to enhance an engraftment rate make MSCs very promising candidates to develop new cell-based therapeutic strategies. MSCs have been already shown to provide hematopoietic engraftment support through angiogenic and neurogenic mechanisms. This has led to the thought-provoking possibility that co-infusion of MSCs and hematopoietic cells can shorten the time to engraftment and reduce graft failure after transplant. MSCs may also play a vast role in repair

of injured tissue by differentiating into damaged cell types, releasing appropriate cytokines and growth factors, and undergoing cell fusion [33, 72]. Clinical trials employing UCB-derived MSCs are currently ongoing for treatment of knee articular cartilage injury or defect, as well as for other disorders, including hematologic malignancies, diabetes mellitus, for reduction of the incidence of graft versus host disease, liver cirrhosis, and bronchopulmonary dysplasia [34]. The potential of this SC population may be therefore greatly utilize for practical applications in regenerative medicine.

3.3.6 Multilineage Progenitor Cells Derived from Umbilical Cord Blood

Group of Lee and colleagues has reported successful isolation of a cell line driven from SC fraction of human UCB and introduced these cells as UCB-derived MPCs [27]. MPCs have been obtained in prolonged cultures (over 4–6 weeks) of UCB-derived mononuclear fraction where initially plated UCB-derived MNCs were found to form adherent colonies gradually developing into adherent monolayer with homogeneous fibroblast-like morphology [27]. Interestingly, such multipotent progenitor cells were negative for CD49a, CD62E, CD73 (SH3, SH4), CD90, CD104 and expressed high levels of CD14, CD31, CD44, CD45, CD54, with variable expression of CD104, CD105 (SH2), and CD166 (ALCAM) [27].

MPCs have been characterized as morphologically and phenotypically novel SC type, distinct from other primitive adherent populations including MSCs [27]. They are also typically different from USSCs and CBEs. Indeed, CD45 is expressed in MPCs, but not in human USSCs or CBEs and VSELs [15, 20, 27]. Interestingly, HSC-specific surface markers such as CD34 and CD133 are fully expressed in the early adherent cells forming MPC colonies, but not at late-cultured cells [27]. Thus, the distinct phenotype may indicate that MPCs could be derived from HSCs or their precursors in UCB. Interestingly, such MPCs with more "hematopoietic" antigenic profile were shown to maintain proliferative capacity with intrinsic and directable potential to develop into three germinal tissue-specific cell types including osteoblasts, myoblasts, endothelial cells, hepatocytes, and neuronal cells after exposure to appropriate conditions [27, 77]. Moreover, functional recovery in rats with spinal cord injury was promoted by the transplantation of neurally induced multipotent progenitor SCs [78]. Similar results were observed when MPCs differentiated into functional hepatocytes were transplanted into liver in rat models of hepatic injury [79]. To sum up, MPCs may also provide an interesting source of primitive precursor cells source for research and applications in the area of regenerative medicine and may be potentially expected to facilitate the development of SC-based therapies.

3.4 The Potential of Umbilical Cord Blood Stem Cells for Practical Applications in Tissue Regeneration

SC-based therapy allowing for efficient treatment of various diseases and injuries or even potential replacement of damaged organ fragments constitutes a promising approach in regenerative medicine and tissue engineering. Successful development of therapeutic strategies employing SPCs could greatly change medical approach in several areas of non-hematopoietic tissue treatment including cardiology, neurology, diabetology, and organ transplantation. Pluripotent and multipotent SCs, which are naturally endowed with the property of self-renewal and wide ability to differentiate into cell types from multiple germ lineages, seem to be optimal candidates for such appraches. Both pluripotent and multipotent SCs may be widely recognized in embryonic tissues. However, potential use of SCs isolated from embryonic tissues for therapy remains still controversial and may not always be safe because of teratogenic potential of pluripotent cells of such origin. Thus, current approach in SC biology has been focused on questing for an alternative non-embryonic source of potential pluripotent and multipotent non-HSCs. Importantly, several populations of SCs with such differentiation capacity have been already described in human UCB as presented in this chapter.

USB has been initially described as a rich source of hematopoietic SPCs and was successfully transplanted for BM reconstitution in hematology following hematological malignancy or BM failure. Clinical applications of UCB were initially employed in early 1970s, when it was used in lymphoblastic leukemia patients' treatment. Subsequent multitude discoveries of several populations of UCB-derived non-HSCs with ability to differentiate into distinct cell types greatly highlighted the potential use of this vast material as a source of cells for potential treatment of several diseases and disorders. Importantly, some of these SC populations, such as MSCs or UCB-NSCs, have been already employed in clinical studies. This anticipates that also other vast UCB-derived SC populations would be eventually optimized for potential clinical applications in the future.

3.5 Summary

In conclusion, stem and progenitors cells derived from fresh or cryopreserved UCB may be considered as promising candidates for use in practical applications in regenerative medicine. Such primitive cells with multipotent and pluripotent characteristics attract special interest due to their multiple advantages over ESC counterparts. Compared to primitive cells from other sources, CB-derived SCs are ethically suitable, easily available, and applicable in both autologous and allogeneic venues. Moreover, most of UCB non-HSCs exhibit a high level of proliferation in vitro and phenotypic plasticity facilitating differentiation into various tissue-specific cells. Supported by intense experimental investigation in vitro and preclinical studies

using animal models, UCB has been utilized in many different clinical studies aiming to treat not only hematological disorders but also other organ diseases. Although such applications are still at early stage, especially in treatment of non-hematological disorders, current results based on preclinical studies and early clinical experience are very promising. Therefore, further studies of umbilical-cord-derived SCs would be required to enforce their therapeutic potential and optimize them for practical applications.

Acknowledgments Supported by the European Union structural funds, Innovative Economy Operational Programme, grant No. POIG 01.02-00-109/09 "Innovative methods of stem cells applications in medicine" and Team grant (TEAM/2012-9/6) from the Foundation for Polish Science to EZS.3.6.

References

1. Ali H, Al-Mulla F (2012) Defining umbilical cord blood stem cells. Stem Cell Discov 2:15–23
2. Ende M, Ende N (1972) Hematopoietic transplantation by means of fetal (cord) blood: a new method. Va Med Mon (1918) 99:276–280
3. Wang HS, Hung SC, Peng ST et al (2004) Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells 22:1330–1337
4. Semenov OV, Koestenbauer S, Riegel M et al (2010) Multipotent mesenchymal stem cells from human placenta: critical parameters for isolation and maintenance of stemness after isolation. Am J Obstet Gynecol 202:193.e1–193.e13
5. Vellasamy S, Sandrasaigaran P, Vidyadaran S et al (2012) Isolation and characterisation of mesenchymal stem cells derived from human placenta tissue. World J Stem Cells 4:53–61
6. Chevallier P, Robillard N, Illiaquer M et al (2013) Characterization of various blood and graft sources: a prospective series. Transfusion 53:2020–2026
7. Michejda M (2004) Which stem cells should be used for transplantation? Fetal Diagn Ther 19:2–8
8. Zhong XY, Zhang B, Asadollahi R et al (2010) Umbilical cord blood stem cells: what to expect. Ann N Y Acad Sci 1205:17–22
9. Mayani H (2011) Umbilical cord blood: lessons learned and lingering challenges after more than 20 years of basic and clinical research. Arch Med Res 42:645–651
10. Brunstein CG, Gutman JA, Weisdorf DJ et al (2010) Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. Blood 116:4693–4699
11. Scaradavou A, Brunstein CG, Eapen M et al (2013) Double unit grafts successfully extend the application of umbilical cord blood transplantation in adults with acute leukemia. Blood 121:752–758
12. Ratajczak MZ, Zuba-Surma EK, Machalinski B et al (2007) Bone-marrow-derived stem cells-our key to longevity? J Appl Genet 48:307–319
13. Asahara T, Murohara T, Sullivan A et al (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967
14. Kogler G, Sensken S, Airey JA et al (2004) A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 200:123–135
15. McGuckin CP, 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. Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74

17. Zuba-Surma EK, Kucia M, Ratajczak J et al (2009) “Small stem cells” in adult tissues: very small embryonic-like stem cells stand up! Cytometry A 75:4–13
18. Buzanska L, Machaj EK, Zablocka B et al (2002) Human cord blood-derived cells attain neuronal and glial features in vitro. J Cell Sci 115:2131–2138
19. Yoder MC, Mead LE, Prater D et al (2007) Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 109:1801–1809
20. Kucia M, Halasa M, Wysoczynski M et al (2007) Morphological and molecular characterization of novel population of CXCR4+SSEA-4+Oct-4+very small embryonic-like cells purified from human cord blood: preliminary report. Leukemia 21:297–303
21. Zuba-Surma EK, Klich I, Greco N et al (2010) Optimization of isolation and further characterization of umbilical-cord-blood-derived very small embryonic/epiblast-like stem cells (VSELs). Eur J Haematol 84:34–46
22. McGuckin C, Jurga M, Ali H et al (2008) Culture of embryonic-like stem cells from human umbilical cord blood and onward differentiation to neural cells in vitro. Nat Protoc 3:1046–1055
23. Case J, Mead LE, Bessler WK et al (2007) Human CD34+AC133+VEGFR-2+cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. Exp Hematol 35:1109–1118
24. Habich A, Jurga M, Markiewicz I et al (2006) Early appearance of stem/progenitor cells with neural-like characteristics in human cord blood mononuclear fraction cultured in vitro. Exp Hematol 34:914–925
25. Hou L, Cao H, Wang D et al (2003) Induction of umbilical cord blood mesenchymal stem cells into neuron-like cells in vitro. Int J Hematol 78:256–261
26. Lee OK, Kuo TK, Chen WM et al (2004) Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 103:1669–1675
27. Lee MW, Moon YJ, Yang MS et al (2007) Neural differentiation of novel multipotent progenitor cells from cryopreserved human umbilical cord blood. Biochem Biophys Res Commun 358:637–643
28. Knudtzon S (1974) In vitro growth of granulocytic colonies from circulating cells in human cord blood. Blood 43:357–361
29. Vaziri H, Dragowska W, Allsopp RC et al (1994) Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. Proc Natl Acad Sci U S A 91:9857–9860
30. Mayani H, Lansdorp PM (1998) Biology of human umbilical cord blood-derived hematopoietic stem/progenitor cells. Stem Cells 16:153–165
31. Majeti R, Park CY, Weissman IL (2007) Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell 1:635–645
32. Buchheiser A, Liedtke S, Looijenga LH et al (2009) Cord blood for tissue regeneration. J Cell Biochem 108:762–768
33. Lee MW, Jang IK, Yoo KH et al (2010) Stem and progenitor cells in human umbilical cord blood. Int J Hematol 92:45–51
34. Pelosi E, Castelli G, Testa U (2012) Human umbilical cord is a unique and safe source of various types of stem cells suitable for treatment of hematological diseases and for regenerative medicine. Blood Cells Mol Dis 49:20–28
35. Kucia M, Reca R, Campbell FR et al (2006) A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 20:857–869
36. Zuba-Surma EK, Kucia M, Abdel-Latif A et al (2008) Morphological characterization of very small embryonic-like stem cells (VSELs) by ImageStream system analysis. J Cell Mol Med 12:292–303
37. Zuba-Surma EK, Kucia M, Wu W et al (2008) Very small embryonic-like stem cells are present in adult murine organs: ImageStream-based morphological analysis and distribution studies. Cytometry A 73A:1116–1127

38. Ratajczak MZ, Zuba-Surma EK, Wysoczynski M et al (2008) Hunt for pluripotent stem cell—regenerative medicine search for almighty cell. J Autoimmun 30:151–162
39. Zuba-Surma EK, Wojakowski W, Ratajczak MZ et al (2011) Very small embryonic-like stem cells: biology and therapeutic potential for heart repair. Antioxid Redox Signal 15:1821–1834
40. Kucia M, Zuba-Surma EK, Wysoczynski M et al (2007) Adult marrow-derived very small embryonic-like stem cells and tissue engineering. Expert Opin Biol Ther 7:1499–1514
41. Bhartiya D, Shaikh A, Nagvenkar P et al (2012) Very small embryonic-like stem cells with maximum regenerative potential get discarded during cord blood banking and bone marrow processing for autologous stem cell therapy. Stem Cells Dev 21:1–6
42. Ratajczak J, Zuba-Surma E, Klich I et al (2011) Hematopoietic differentiation of umbilical cord blood-derived very small embryonic/epiblast-like stem cells. Leukemia 25:1278–1285
43. Zuba-Surma EK, Ratajczak MZ (2010) Overview of very small embryonic-like stem cells (VSELs) and methodology of their identification and isolation by flow cytometric methods. Current protocols in cytometry/editorial board, J Paul Robinson, managing editor [et al] Chap. 9:Unit9 29
44. Anand S, Bhartiya D, Sriraman K et al (2013) Quiescent very small embryonic-like stem cells resist oncotherapy and can restore spermatogenesis in germ cell depleted mammalian testis. Stem Cells Dev Sept 30
45. Sovalat H, Scrofani M, Eidenschenk A et al (2011) Identification and isolation from either adult human bone marrow or G-CSF-mobilized peripheral blood of CD34(+)/CD133(+)/CXCR4(+)/Lin(-)CD45(-) cells, featuring morphological, molecular, and phenotypic characteristics of very small embryonic-like (VSEL) stem cells. Exp Hematol 39:495–505
46. Iwaki R, Nakatsuka R, Matsuoka YM et al (2012) Development of a highly efficient method for isolating very small embryonic-like stem cells identified in adult mouse bone and their stem cell characteristics. (Abstract #2345). 54th ASH annual meeting (8–11 Dec 2012). Atlanta, GA, USA
47. Wojakowski W, Tendera M, Kucia M et al (2009) Mobilization of bone marrow-derived Oct-4+SSEA-4+ very small embryonic-like stem cells in patients with acute myocardial infarction. J Am Coll Cardiol 53:1–9
48. Paczkowska E, Kucia M, Koziarska D et al (2009) Clinical evidence that very small embryonic-like stem cells are mobilized into peripheral blood in patients after stroke. Stroke 40:1237–1244
49. Havens AM, Shiozawa Y, Jung Y et al (2013) Human very small embryonic-like cells generate skeletal structures, in vivo. Stem Cells Dev 22:622–630
50. Howe M, Zhao J, Bodenburg Y et al (2009) Oct-4 A isoform is expressed in human cord blood-derived CD133 stem cells and differentiated progeny. Cell Prolif 42:265–275
51. Danova-Alt R, Heider A, Egger D et al (2012) Very small embryonic-like stem cells purified from umbilical cord blood lack stem cell characteristics. PLoS ONE 7:e34899
52. Basile DP, Yoder MC (2014) Circulating and tissue resident endothelial progenitor cells. J Cell Physiol 229:10–16
53. Sieveking DP, Buckle A, Celermajer DS et al (2008) Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. J Am Coll Cardiol 51:660–668
54. Rehman J (2011) Chipping away at the surface of the endothelial progenitor cell (EPC) mystery. J Mol Med (Berl) 89:943–945
55. Phuc PV, Ngoc VB, Lam DH et al (2012) Isolation of three important types of stem cells from the same samples of banked umbilical cord blood. Cell Tissue Bank 13:341–351
56. Shin JW, Lee DW, Kim MJ et al (2005) Isolation of endothelial progenitor cells from cord blood and induction of differentiation by ex vivo expansion. Yonsei Med J 46:260–267
57. Estes ML, Mund JA, Mead LE et al (2010) Application of polychromatic flow cytometry to identify novel subsets of circulating cells with angiogenic potential. Cytometry A 77:831–839
58. Lee JH, Lee SH, Yoo SY et al (2013) CD34 hybrid cells promote endothelial colony-forming cell bioactivity and therapeutic potential for ischemic diseases. Arterioscler Thromb Vasc Biol 33:1622–1634

59. Lin RZ, Dreyzin A, Aamodt K et al (2011) Functional endothelial progenitor cells from cryopreserved umbilical cord blood. Cell Transplant 20:515–522
60. Ladran I, Tran N, Topol A et al (2013) Neural stem and progenitor cells in health and disease. Wiley interdisciplinary reviews systems biology and medicine
61. Domanska-Janik K, Buzanska L, Lukomska B (2008) A novel, neural potential of non-hematopoietic human umbilical cord blood stem cells. Int J Dev Biol 52:237–248
62. Sun W, Buzanska L, Domanska-Janik K et al (2005) Voltage-sensitive and ligand-gated channels in differentiating neural stem-like cells derived from the nonhematopoietic fraction of human umbilical cord blood. Stem Cells 23:931–945
63. Jurga M, Lipkowski AW, Lukomska B et al (2009) Generation of functional neural artificial tissue from human umbilical cord blood stem cells. Tissue Eng Part C Methods 15:365–372
64. Zangiacomi V, Balon N, Maddens S et al (2008) Cord blood-derived neurons are originated from CD133+/CD34 stem/progenitor cells in a cell-to-cell contact dependent manner. Stem Cells Dev 17:1005–1016
65. Kogler G, Radke TF, Lefort A et al (2005) Cytokine production and hematopoiesis supporting activity of cord blood-derived unrestricted somatic stem cells. Exp Hematol 33:573–583
66. Santourlidis S, Wernet P, Ghanjati F et al (2011) Unrestricted somatic stem cells (USSC) from human umbilical cord blood display uncommitted epigenetic signatures of the major stem cell pluripotency genes. Stem Cell Res 6:60–69
67. Buchheiser A, Houben AP, Bosch J et al (2012) Oxygen tension modifies the 'stemness' of human cord blood-derived stem cells. Cytotherapy 14:967–982
68. Kluth SM, Buchheiser A, Houben AP et al (2010) DLK-1 as a marker to distinguish unrestricted somatic stem cells and mesenchymal stromal cells in cord blood. Stem Cells Dev 19:1471–1483
69. Handschel J, Naujoks C, Langenbach F et al (2010) Comparison of ectopic bone formation of embryonic stem cells and cord blood stem cells in vivo. Tissue Eng Part A 16:2475–2483
70. Myers TJ, Granero-Molto F, Longobardi L et al (2010) Mesenchymal stem cells at the intersection of cell and gene therapy. Expert Opin Biol Ther 10:1663–1679
71. Chugh AR, Zuba-Surma EK, Dawn B (2009) Bone marrow-derived mesenchymal stems cells and cardiac repair. Minerva Cardioangiol 57:185–202
72. Bieback K, Kern S, Kluter H et al (2004) Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 22:625–634
73. Flynn A, Barry F, O'Brien T (2007) UC blood-derived mesenchymal stromal cells: an overview. Cytotherapy 9:717–726
74. Carvalho MM, Teixeira FG, Reis RL et al (2011) Mesenchymal stem cells in the umbilical cord: phenotypic characterization, secretome and applications in central nervous system regenerative medicine. Curr Stem Cell Res Ther 6:221–228
75. Balasubramanian S, Venugopal P, Sundarraj S et al (2012) Comparison of chemokine and receptor gene expression between Wharton's jelly and bone marrow-derived mesenchymal stromal cells. Cytotherapy 14:26–33
76. Forraz N, McGuckin CP (2011) The umbilical cord: a rich and ethical stem cell source to advance regenerative medicine. Cell Prolif 44(Suppl 1):60–69
77. Moon YJ, Lee MW, Yoon HH et al (2008) Hepatic differentiation of cord blood-derived multipotent progenitor cells (MPCs) in vitro. Cell Biol Int 32:1293–1301
78. Cho SR, Yang MS, Yim SH et al (2008) Neurally induced umbilical cord blood cells modestly repair injured spinal cords. Neuroreport 19:1259–1263
79. Moon YJ, Yoon HH, Lee MW et al (2009) Multipotent progenitor cells derived from human umbilical cord blood can differentiate into hepatocyte-like cells in a liver injury rat model. Transplant Proc 41:4357–4360

Chapter 4
Human CD34-negative Hematopoietic Stem Cells

Yoshiaki Sonoda

4.1 Introduction

During the past two decades, fluorescence-activated cell sorting (FACS) technology has dramatically improved, and it is now possible to prospectively isolate murine and human hematopoietic stem cells (HSCs) using various cell surface markers. In fact, the surface immunophenotype of murine HSCs has been increasingly refined, resulting in the success of single cell-based transplantation analyses as described later. However, our understanding of the primitive human HSC compartment is far behind that of the murine HSC system. In this chapter, I focus on current knowledge concerning the human primitive HSC compartment, with a particular focus on the identification of human CD34-negative ($CD34^-$) HSCs.

HSCs in mammals, including mice, rhesus monkeys, and humans, have long been believed to be CD34 antigen (Ag)-positive ($CD34^+$). CD34 is a surface glycoprotein expressed on early lymphomyeloid hematopoietic stem/progenitor cells (HSPCs) (1). In fact, immunomagnetically separated $CD34^+$ cell populations obtained from bone marrow (BM) and peripheral blood (PB) can form all types of hematopoietic progenitor cell (HPC)-derived colonies, and achieve long-term donor-derived lymphohematopoietic reconstitution following hematopoietic stem celltransplantation (HSCT) in the clinical setting [2–4]. These experimental and clinical data demonstrate the presence of HSCs with a long-term repopulating ability within $CD34^+$ cells. Based on these data, the usefulness/significance of CD34 Ag as a reliable positive marker of human hematopoietic stem/progenitor cells (HSPCs) has been established. However, the functional significance of CD34 Ag in early hematopoiesis has not yet been fully elucidated.

On the other hand, the colony-forming ability (CFA) of HPCs derived from yolk sacs or fetal livers isolated from CD34-null embryos demonstrates decreased

Y. Sonoda (✉)
Department of Stem Cell Biology and Regenerative Medicine,
Graduate School of Medical Science, Kansai Medical University,
Shinmachi, Hirakata, Osaka 573-1010, Japan
e-mail: sonoda@hirakata.kmu.ac.jp

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,*
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_4,

numbers of these progenitors [5]. Moreover, the CFA of HPCs derived from both BM and spleen is significantly reduced in adult CD34-null mice [5]. In addition, CD34-deficient HPCs cannot be expanded in liquid cultures in the presence of a combination of cytokines [5]. Regardless of these apparent deficiencies, CD34-null mice exhibit similar kinetics of erythroid, myeloid, and platelet recovery following sublethal irradiation compared with wild-type mice [5]. These data strongly suggest that adult CD34-null mice have a potential reservoir of HSPCs that allows for a normal hematopoietic recovery following sublethal irradiation. In addition, a number of previous reports have indicated the absence of CFA in the lineage-negative, CD34-negative (Lin^-CD34^-) cell fraction [6–11]. The CFA is generated following short- and long-term culture based on CD34 Ag induction [6, 7, 9, 11]. However, Gallacher et al. demonstrated that a rare population of $AC133^+$ cells within $Lin^-CD34^-CD38^-$ cells possess a CFA [12]. We recently provided clear evidence that cord blood (CB)-derived highly purified $18Lin^-CD34^-$ cell populations exhibit a significant CFA that is almost equivalent to that of $18Lin^-CD34^+$ cell populations [13]. Collectively, these experimental findings provide a strong evidence that murine and human hematopoietic tissue-derived Lin^-CD34^- cell populations contain HSPCs.

4.2 Identification of Murine CD34-Negative Hematopoietic Stem Cells (HSCs)

4.2.1 Isolation and Functional Properties of Side Population(SP) Cells

Goodell et al. demonstrated that a unique class of HSCs (side population (SP) cells) expressing either low or undetectable levels of CD34 Ag exist in multiple species, including mice, rhesus monkeys, and humans, using the fluorescent deoxyribonucleic acid (DNA)-binding dye, Hoechst 33342 [14]. These Hoechst-unstained SP cells have been shown to protect recipient mice from lethal irradiation and contribute to long-term lymphomyeloid cell repopulation. The distinct staining pattern of SP cells results from a high level of dye efflux activity mediated by the adenosine triphosphate (ATP)-binding cassette (ABC) transporter, ABCG2 [15]. Collectively, these studies imply the existence of a hitherto unidentified population of primitive murine/human HSCs that lack an expression of CD34 Ag.

4.2.2 A Single $CD34^{low/negative}$ Murine HSC can Reconstitute Long-Term Lymphohematopoiesis

As mentioned above, primitive murine HSCs have been reported to be CD34 Ag-positive [1]. However, Nakauchi and colleagues challenged this long-standing

dogma. They reported that primitive adult murine BM-derived HSCs are detected in the murine CD34 low to negative cell fraction [16]. First, the authors developed a monoclonal antibody (Ab) to the murine homologue of CD34. Using this monoclonal Ab, they purified BM-derived c-kit$^+$Sca-1$^+$Lin$^-$ (KSL) cells into CD34$^+$, CD-34^{low}, and CD34$^-$ cell fractions. An in vivo analysis of the HSC activity in these three subpopulations clearly demonstrated that the CD34$^{low/-}$ populations support long-term lymphomyeloid reconstitution. In contrast, CD34$^+$ cells support only short-term hematopoietic cell reconstitution. Surprisingly, the injection of a single CD34$^{low/-}$ KSL cell results in long-term lymphomyeloid reconstitution in 22 % (9/41) of recipient mice. Because the marrow seeding efficiency of murine repopulating stem cells has been reported to be approximately 20 % [17], CD34$^{low/-}$ KSL cells appear to comprise an almost purified murine HSC population. Therefore, the self-renewal and multilineage differentiation potential of individual HSCs has been extensively investigated using this purified HSC population [18]. Accordingly, the surface immunophenotype of primitive murine HSCs has been established to be CD34$^-$ KSL cells.

4.2.3 Reversible Expression of CD34 Antigens on Primitive Murine HSCs

As mentioned above, primitive long-term lymphomyeloid reconstituting HSCs in mice lack a CD34 expression [16]. However, Ogawa and colleagues performed a series of experiments and demonstrated that the expression of surface CD34 antigens on murine primitive HSCs is under the influence of developmental stages and the kinetic state of the HSCs [19–21]. Sato et al. first reported that the majority of long-term reconstituting BM cells in adult mice are CD34$^-$ [19]. Interestingly, following in vivo 5-fluorouracil (FU) treatment, high-level engraftment is achieved with both CD34$^+$ and CD34$^-$ HSCs five months posttransplantation, thus suggesting a phenotypic change due to the activation of HSCs by 5-FU. In addition, in vitro cultures of CD34$^-$ HSCs seeded in the presence of early-acting cytokines [22, 23], such as interleukin (IL)-11 and stem cell factor (SCF), have been observed to produce CD34$^+$ cells. These CD34$^+$ cells generate long-term, multilineage engraftment in lethally irradiated mice following the transplantation. This phenotypic change indicates that CD34$^-$ HSCs can develop into CD34$^+$ HSCs after in vitro culture. The authors also showed that mobilized adult mice PB-derived HSCs obtained using granulocyte colony-stimulating factor (G-CSF) express CD34 [21]. These results suggest that the CD34 expression on murine primitive HSCs reflects an activation state caused by 5-FU or in vitro cytokine exposure. Moreover, the CD34 expression on murine HSCs has been reported to change developmentally [20]. Namely, BM-derived long-term repopulating HSCs in fetal, newborn, and young mice are CD34$^+$, whereas CD34$^-$ HSCs emerge in 7–10-week-old mice and increase thereafter. These findings give rise to the concept that the expression of CD34 antigens by murine HSCs is reversible, and suggest the possibility that the surface phenotype of murine HSCs is also reversible.

4.3 Identification of Human CD34-Negative HSCs

4.3.1 Evidence for the Presence of CD34-Negative HSCs

A number of studies concerning human $CD34^-$ primitive HSCs have suggested that the $CD34^{low/-}$ cell population contains long-term lymphohematopoietic repopulating HSCs [6, 7, 10, 14]. One of the assay systems that can be used to measure the repopulation and differentiation capacities of human HSCs is the severe combined immunodeficiency (SCID)-repopulating cell (SRC) assay [24–26]. Using this system, Bhatia et al. first reported that SRCs are present in human BM- and CB-derived Lin^-CD34^- cells [6]. In that study, the multilineage reconstituting analyses of $CD34^-$ SRCs demonstrated that the vast majority of $CD45^+$ human cells in murine BMs are $CD19^+$ B cells. In addition, a limiting dilution analysis indicated that there is one SRC in 125,000 Lin^-CD34^- cells. On the other hand, the frequency of $CD34^-$ SRCs increases to 1 in 38,000 cells after 4 days of short-term culture of Lin^-CD34^- cells in the presence of a cocktail of cytokines or human umbilical vein endothelial cell-conditioned medium. These findings suggest that unidentified cells, termed "pre-SRCs," present in the $CD34^-$ cell population, acquire homing molecules necessary for homing/redistribution to nonobese diabetic (NOD)/SCID murine BM following tail vein injection (TVI) (6).

The existence of long-term repopulating $CD34^-$ HSCs in human BM-derived Lin^- cells is also supported by other reported data, in which the $CD34^-$ fraction of normal human BM has been found to contain cells capable of engraftment and differentiation into $CD34^+$ progenitors as well as multiple lymphohematopoietic lineages using the human/sheep competitive engraftment model [10]. However, the precise analysis of human $CD34^-$ HSCs has been hindered by the lack of (1) a positive marker, comparable to Sca-1 in mice, and (2) a simple and reliable assay system for evaluating these rare cells. Further attempts to characterize the $CD34^-$ SRCs present in human CB have revealed the presence of unidentified "pre-SRCs" using our previously developed highly sensitive SRC assay system [27].

4.3.2 Development of a Highly Efficient SCID-Repopulating Cell (SRC) Assay System Using the Intra-bone Marrow Injection (IBMI) Technique

The repopulation and differentiation capabilities of primitive human HSCs have been assayed using the xenograft transplantation system employing NOD/SCID mice with SRC activity developed by Dick and colleagues [6, 24, 25, 28]. The authors originally injected cells using TVI. When candidate human HSCs are transplanted via TVI, the cells circulate in blood vessels into the right atrium, ventricle, and lungs in which most of the cells (over 95%) are trapped, although a small portion of the cells enter the systemic circulation. Finally, a small fraction of the injected

cells lodge in the BM niche. In fact, the seeding efficiency of human CB-derived $CD34^+$ cells in NOD/SCID mice is 4.4% by week 6 cobblestone area-forming cell (CAFC) assays [17]. Therefore, the SRC assay using TVI possibly underestimates the proportion of SRCs in target cell populations.

Previously, a novel technique for obtaining BM cells via aspiration from the femurs of living mice was reported [29]. Thereafter, the development of the intrabone marrow injection (IBMI)technique for the treatment of intractable autoimmune diseases in MRL/lpr mice was reported [30]. Using this IBMI technique, we successfully developed a highly sensitive SRC assay system [27]. According to the IBMI technique, the frequency of SRCs in CB-derived $Lin^-CD34^+CD38^-$ cells is 1/40 cells [31]. When these cells are injected using TVI, the frequency of SRC decreases to approximately 1/600 cells. These results demonstrate that the IBMI technique is associated with a seeding efficiency 15 times greater than that of TVI. Therefore, the SRC assay system using the IBMI technique is very useful for investigating the HSC activity of certain target populations.

4.3.3 Identification of Human CD34-Negative HSCs Using IBMI

First, the lineage-negative (Lin^-) cells were separated from CB-derived mononuclear cells using an immunomagnetic beads system. The cells were further labeled with a mixture of 13 lineage-specific monoclonal antibodies (mAbs) and then subdivided into three distinct populations based on their surface CD34 Ag expression (Fig. 4.1a). The three fractions were sorted and transplanted into NOD/SCID mice using TVI, as previously reported [27]. All 13 mice transplanted with Lin^-CD34^{high} cells were engrafted with human cells. The level of human $CD45^+$ cells in the murine BM was 3.0–70.8% (median, 26.2%; Fig. 4.1b(a), left column). In contrast, neither the nine mice transplanted with Lin^-CD34^{low} cells nor the 10 mice transplanted with Lin^-CD34^- cells were engrafted with human cells (Fig. 4.1b (b, c), left columns). The phenotypic and functional characterizations of these three fractions were further determined using the co-culture of these cells with hematopoietic-supportive stromal (HESS)-5 cells [32], and in the presence of SCF, flt3 ligand (FL), thrombopoietin (TPO), IL-3, IL-6, and G-CSF. After 7 days of coculture of Lin^-CD34^{high} cells with HESS-5, 30% of the cells remained $CD34^+$ cells. With respect to the Lin^-CD34^- cells, 18% of these cells were $CD34^+$. On the other hand, the flow cytometric pattern of Lin^-CD34^{low} cells was very different from the other two patterns. Only 1.6% of this population was $CD34^+$ after the co-culture. The three fractions of cells recovered from the co-cultures were then transplanted into five NOD/SCID mice each using TVI. Very interestingly, the cultured Lin^-CD34^- cells repopulated all five recipient mice. These results clearly indicate that the cultured Lin^-CD34^- cell fraction contains SRCs, which were not able to home into the BM niche following TVI before the co-culture. However, none of the five mice transplanted with cultured Lin^-CD34^{low} cells were repopulated [27].

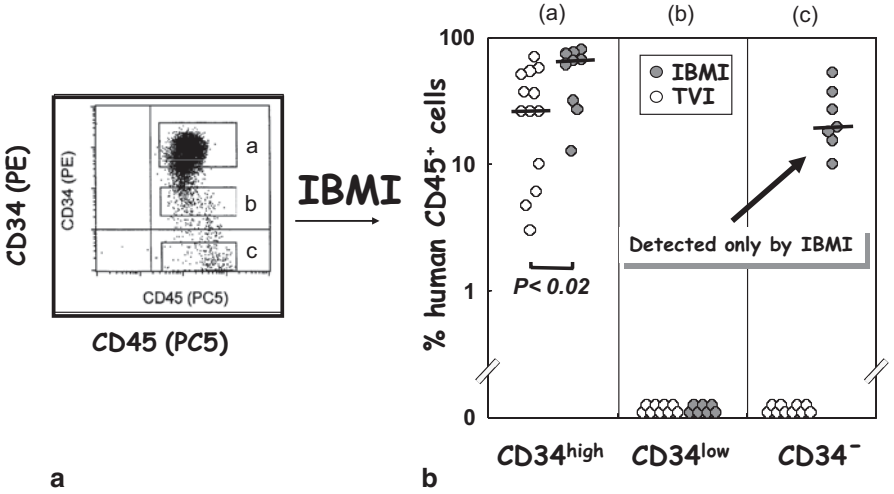

Fig. 4.1 Characterization of purified CB-derived Lin$^-$CD34$^{high/low/-}$ cells and their SRC activities. **a** The FACS profile of immunomagnetically separated lineage-negative cells, demonstrating CD34^{high} (*a*), CD34^{low} (*b*), and CD34$^-$ (*c*) cells. **b** Human CD45$^+$ cell engraftment in the NOD/SCID mice receiving a transplant of 5×10^4 Lin$^-$CD34^{high} (*a*), Lin$^-$CD34^{low} (*b*) and Lin$^-$CD34$^-$ (*c*) cells. The *open and closed circles* represent the repopulation rates in the total murine BM following conventional tail vein injection (*TVI; left column*) and by intra-bone marrow injection (*IBMI; right column*), respectively

Lapidot and colleagues clearly demonstrated that chemokine stromal cell-derived factor (SDF)-1 (CXCL12) and its receptor, CXCR4, play a pivotal role in the homing and repopulation of CD34$^+$ SRCs in NOD/SCID mice [33, 34]. It has recently been reported that CXCR4, very late antigen (VLA)-4, and VLA-5 play important roles in the homing of CD34$^+$ SRCs following TVI and IBMI [35]. Moreover, the homing of HSCs to the BM is a multistep process, in which various adhesion molecules present on both HSCs and BM endothelial cells are involved [35–37]. Accordingly, the expression patterns of CXCR4 and other adhesion molecules on the surface of CB-derived Lin$^-$CD34^{high}, Lin$^-$CD34^{low}, and Lin$^-$CD34$^-$ cells were analyzed using flow cytometry. Significant numbers of CB-derived Lin$^-$CD34^{high} cells were found to express CXCR4, CD31, CD49d, CD54, CD62L, and CD106. However, Lin$^-$CD34$^-$ cells were found to express lower levels of CXCR4, CD62L, and CD106 [27]. This suggests that very primitive repopulating HSCs that lack a CD34 Ag expression may not home into the BM niche following TVI, since Lin$^-$CD34$^-$ cells express low levels of these homing receptors. Therefore, the IBMI technique [27] was used to determine the SRC activity of these three fractions of cells (Fig. 4.1).

When Lin$^-$CD34^{high} cells were transplanted using IBMI, all mice were repopulated, and the level of human cell engraftment was 12.8–80.0% (median, 64.8%; Fig. 4.1b(a), right column). Very interestingly, the repopulating rate was significantly higher than that achieved with conventional TVI ($P<0.02$). Next, Lin$^-$CD34$^-$ cells were transplanted using IBMI. Surprisingly, all seven mice were

repopulated, and the level of human cell engraftment was 10.0–52.6% (median, 19.3%; Fig. 4.1b(c), right column). On the other hand, none of the seven mice transplanted with Lin^-CD34^{low} cells using IBMI were repopulated with human cells (Fig. 4.1b(b), right column). These results clearly indicate that the CB-derived Lin^-CD34^- cell population contains SRCs detected with IBMI only. A limiting dilution analysis demonstrated the frequency of SRCs in the CB-derived Lin^-CD34^{high} and Lin^-CD34^- cells to be 1/1010 and 1/24,100, respectively [27, 38].

$CD34^-$ SRCs cannot home into the BM niche when transplanted using TVI. This is partly explained by their lower expression levels of homing receptors, including CXCR4. A transwell migration assay towards a gradient of SDF-1 clearly indicated that $CD34^-$ SRCs possess poor SDF-1/CXCR4-mediated migration and homing abilities [27]. An analysis of the in vivo migration ability of HSCs demonstrated that a significant proportion of $CD34^-$ SRCs as well as $CD34^+$ SRCs are redistributed from the injected left tibia to other bones and proliferate at the migrated sites at which both SRCs generate significant numbers of $CD34^+$ progenies [27, 38]. However, it remains unknown whether $CD34^-$ SRCs migrate to other bones with either the $CD34^-$ immunophenotype or after their conversion to the $CD34^+$ state. Our recent data suggest that $CD34^-$ SRCs convert to the $CD34^+$ state under the influence of BM-derived mesenchymal stromal cells in vitro (Takahashi, Matsuoka, and Sonoda, unpublished data). However, the molecular mechanisms involved in this migratory (redistribution and homing) process have yet to be clarified.

Secondary transplantation studies of sorted $CD45^+CD34^+$ and $CD45^+CD34^-$ cells obtained from primary recipient mice transplanted with either $CD34^-$ SRCs or $CD34^+$ SRCs have demonstrated that only $CD34^+$ cells can repopulate secondary recipient mice. These results indicate that $CD34^-$ SRCs generate $CD34^+$ SRCs in vivo, consistent with reported data that human CB-derived Lin^-CD34^- cells generate a large number of $CD34^+$ stem cells in ex vivo culture systems using HESS-5 and various human cytokines [7, 27]. More importantly, secondary transplantation studies have demonstrated for the first time that CB-derived $CD34^-$ SRCs possess a long-term (up to 28 weeks) human cell repopulating capacity in NOD/SCID mice [27].

In contrast to murine BM-derived HSCs [19], human CB-derived $CD34^+$ SRCs do not convert to $CD34^-$ SRCs for at least 24 weeks after transplantation [27] (Matsuoka and Sonoda, unpublished data). However, a longer period of observation (over 1 year) is required to elucidate the possibility of reversion of CD34 antigens expressed on human CB-derived $CD34^+$ HSCs, as demonstrated by Zanjani et al. using human BM-derived HSCs [39]. In addition, it remains unclear whether the $CD34^-$ cell population obtained from BM cells of primary recipient mice transplanted with Lin^-CD34^- cells contains $CD34^-$ HSCs, which would indicate the self-renewal of $CD34^-$ HSCs in NOD/SCID mice. There is a possibility that the self-renewing $CD34^-$ HSCs cannot home into the BM niche in secondary recipients, even following IBMI, or that human CB-derived $CD34^-$ HSCs cannot be maintained in the xenogeneic mouse BM niche. Further studies are required to clarify this important issue.

4.3.4 *Surface Immunophenotype and Functional Characteristics of Human CD34-Negative HSCs*

Identifying the cell surface immunophenotypes of HSCs is an important issue in both clinical HSCT and basic research. Transplantation of pure populations of HSCs should eliminate the occurrence of graft-vs-host disease (GVHD) in allogeneic HSCT and may reduce the recurrence rate of leukemia in patients undergoing autologous HSCT due to the elimination of minimal residual disease (MRD).

As described in the previous section, the surface immunophenotype of the most primitive human HSCs is believed to be $Lin^{-}CD34^{+}CD38^{-}$. However, functional studies, including analyses of the multilineage reconstituting ability, kinetics of engraftment, productivity of $CD34^{+}$ progenies, proliferative and migratory potential, and secondary repopulating ability have revealed that CB-derived $CD34^{-}$ SRCs possess different HSC characteristics from $CD34^{+}$ SRCs [13, 27, 38]. Based on these data, the identified CB-derived $CD34^{-}$ SRCs may therefore be a novel class of primitive repopulating HSCs that can be detected using the sensitive IBMI technique only [13, 27, 38].

It is well documented that the tyrosine kinase receptors, c-kit and flt3, are expressed and function in early murine and human hematopoiesis [4, 40, 41]. Moreover, their respective ligands, SCF and FL, synergistically act with each other while also playing an important role in the regulation (generation, maintenance, proliferation, differentiation, and expansion) of early stages of murine and human candidate HSCs [40–42]. In a murine model, $flt3^{-}$ KSL cells have recently been reported to support long-term multilineage hematopoietic reconstitution [43]. In contrast, $flt3^{+}$ KSL cells are progenitors for the common lymphoid stage [43]. These $flt3^{+}$ KSL cells have also been shown to lack erythro-megakaryocytic potential [43]. This notion is supported by the findings of other reports demonstrating that mice deficient in the expression of flt3 or FL exhibit deficient lymphopoiesis [44].

In contrast to murine candidate HSCs ($CD34^{-}$ KSL cells) (16), the identified $CD34^{-}$ SRCs do not express detectable levels of c-kit tyrosine kinase receptor according to flow cytometry [45]. However, the degree to which flt3 is expressed on human HSCs, including $CD34^{+}$ and $CD34^{-}$ SRCs, which are capable of in vivo lymphomyeloid reconstitution, has not yet been fully elucidated. A number of studies have reported that flt3 is expressed and functions in human $CD34^{+}$ hematopoietic progenitor cells [41], including long-term culture-initiating cell (LTC-ICs) (41). However, only two reports have demonstrated, using the conventional TVI method, that human CB- and BM-derived $CD34^{+}$ HSCs capable of multilineage reconstitution in NOD/SCID mice express flt3 tyrosine kinase receptor [46, 47].

Recently, we reported the function of flt3 in the identified very primitive human CB-derived $CD34^{-}$ SRCs [45] as well as more committed $CD34^{+}$ SRCs using the sensitive IBMI method. These data clearly demonstrate that a portion of human CB-derived $CD34^{+}$ SRCs express flt3. However, only $CD34^{+}flt3^{-}$ cells displayed a significant secondary repopulating ability, even when a comparable number of $CD34^{+}flt3^{-}$ cells as $CD34^{+}flt3^{+}$ cells were transplanted. Moreover, the $CD34^{-}flt3^{-}$

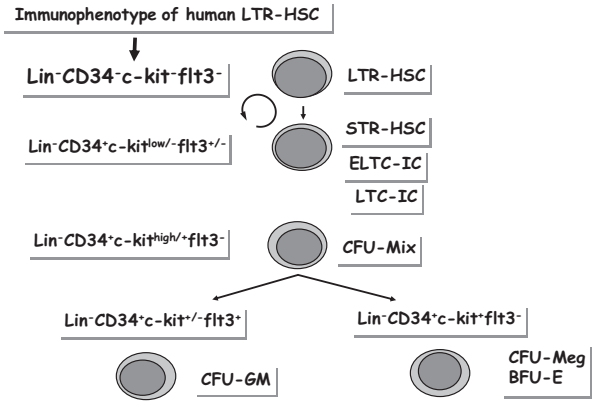

Fig. 4.2 Summary of the immunophenotypes of human hematopoietic stem cells (*HSCs*)/hematopoietic progenitor cells (*HPCs*). The figure illustrates the expression patterns of CD34, c-kit, and flt3 on various classes of HSCs/HPCs detected using flow cytometry, as described in the text. Because most HSCs/HPCs populations continue to be heterogeneous and hard to purify, it is not possible to exclude the CD34, c-kit, and flt3 expression on a minority of cells in the different cell populations. Therefore, this figure indicates the c-kit and flt3 receptor expression status on the majority of cells within the $CD34^{+/-}$ HSCs/HPCs populations based on our and other reported studies concerning the expression of these receptors and/or the findings of functional studies

cells exhibited a distinct and potent SRC activity following IBMI while also demonstrating a high and efficient secondary repopulating ability in comparison to $CD34^{+}flt3^{-}$ cells. $CD34^{-}flt3^{-}$ cells also produce $CD34^{+}flt3^{-}$ and $CD34^{+}flt3^{+}$ SRCs following coculture with HESS-5 [32] in vitro, thus suggesting that $CD34^{-}flt3^{-}$ cells contain very primitive human CB-derived HSCs. These observations suggest that the surface immunophenotype of very primitive human long-term repopulating (LTR)-HSCs is $Lin^{-}CD34^{-}c\text{-}kit^{-}flt3^{-}$ [45]. As illustrated in Fig. 4.2, primitive LTR-HSCs express lower levels of c-kit and flt3 receptors on their surface when they commit to more mature short-term repopulating (STR)-HSCs. It remains unclear, however, whether such a distinct pattern of the c-kit and flt3 expression can be used to identify distinct subpopulations of either LTR-HSC or STR-HSC within the human HSC hierarchy (Fig. 4.3). These results are consistent with the findings of recent studies regarding the significance of the flt3 expression in murine primitive hematopoiesis [43, 48] while also providing a new concept of a hierarchy in the human primitive HSC compartment.

4.3.5 Development of a High-Resolution Purification Method for Human CD34-Negative HSCs

In our previous studies [27, 31, 38, 45], 13 lineage (Lin)-specific monoclonal antibodies (mAbs) against CD2, CD3, CD4, CD7, CD10, CD14, CD16, CD19, CD20,

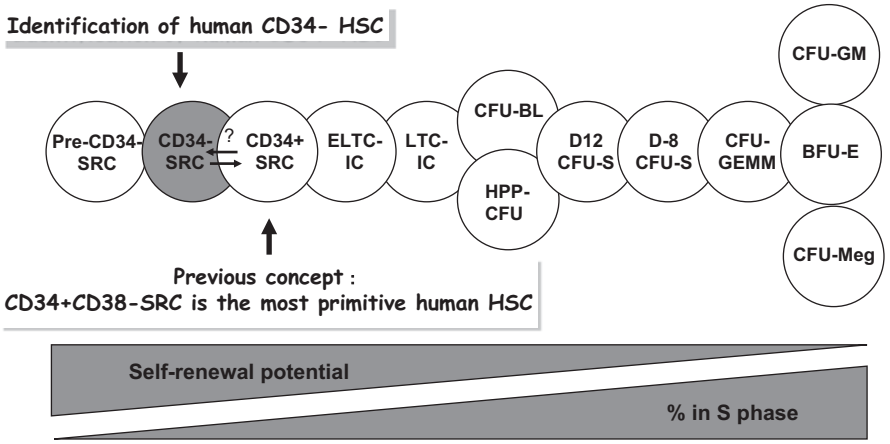

Fig. 4.3 Hierarchical organization of the human/mouse hematopoietic stem cells (*HSCs*)/hematopoietic progenitor cells (*HPCs*) compartment as identified using currently available bioassays. The compartments are shown in relationship to the self-renewal potential and cell-cycle status under steady-state hematopoiesis. In the primitive human HSC compartment, the reversion of CD34 antigens and the presence of pre-CD34$^-$ SRC (origin of CD34$^-$ SRC) have not been fully elucidated

CD24, CD41, CD56, and CD235a were used to purify CD34$^-$ SRCs. An in vivo limiting dilution analysis using the IBMI demonstrated the frequency of CD34$^-$ SRCs in the 13Lin$^-$CD34$^-$ cells to be approximately 1/25,000 [27, 31, 38]. The SRC assay system using the IBMI technique is very sensitive and useful for investigating the HSC activity of certain target populations; however, the SRC frequency in the 13Lin$^-$CD34$^-$ cells was still very low in comparison to the frequency of SRC in the 13Lin$^-$CD34$^+$CD38$^-$ cells (1/40) (31). Therefore, it is crucial to further purify CD34$^-$ SRCs in order to more precisely characterize CD34$^-$ SRCs. We herein describe the development of a high-resolution method for purifying the rare class of primitive human CD34$^-$ SRCs (HSCs) using mAbs to 18Lin-specific Ags, including the above-mentioned 13 Ags and 5 additional Ags (CD11b, CD33, CD45RA, CD66c, and CD127). Employing these 18Lin mAbs, we succeeded in highly purifying human CB-derived CD34$^-$ SRCs, as presented in Fig. 4.4 [13].

As shown in Fig. 4.4e and f, the 18Lin$^-$CD34$^-$ (R8) fraction was highly purified (0.32 % of R4-gated cells) in comparison to the 13Lin$^-$CD34$^-$ (R6) fraction (3.63 % of R3-gated cells). The 13Lin$^-$CD34^{high}CD38$^-$ (R10) fraction comprised 0.83 % of the R5-gated cells (Fig. 4.4g). A morphological analysis showed that almost all of the 18Lin$^-$CD34$^-$ cells were immature blast-like in appearance, with a high nuclear/cytoplasmic ratio, fine chromatin network, few nucleoli, and scant basophilic cytoplasm (data not shown).

We subsequently investigated the SRC activity of the 18Lin$^-$CD34$^-$ cells using NOD/SCID mice. As expected, a limiting dilution analysis using the IBMI demonstrated the frequency of the CD34$^-$ SRCs in the 18Lin$^-$CD34$^-$ cells to be approximately 1/1000 [13]. The frequency of the CD34$^-$ SRCs in the 13Lin$^-$CD34$^-$ cells was 1/25,000, as previously reported [27, 31, 38]. Therefore, these results clearly demonstrate that the application of 18Lin mAbs in addition to the IBMI

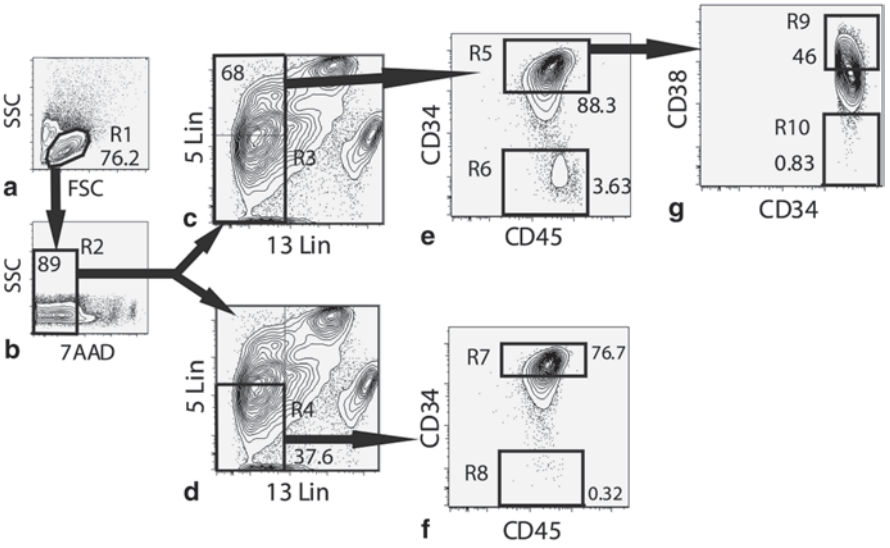

Fig. 4.4 Representative FACS profiles of CB-derived 13Lin$^-$CD45$^+$CD34^{high}CD38$^-$ and 18Lin$^-$CD45$^+$CD34$^{high/-}$ cells. **a** The forward scatter/side scatter (*FSC/SSC*) profile of immunomagnetically separated Lin$^-$ cells. The R1 gate was set on the blast-lymphocyte window. **b** The R2 gate was set on the living cells. **c**, **d** The expressions of the FITC-conjugated 13 Lin markers, including CD2, CD3, CD4, CD7, CD10, CD14, CD16, CD19, CD20, CD24, CD41, CD56, and CD235a, and the PE-conjugated 5 Lin markers, including CD11b, CD33, CD45RA, CD66c, and CD127 are presented. **e** The 13Lin$^-$ (R3) cells were subdivided into two fractions: 13Lin$^-$CD45$^+$CD34^{high} (R5) and CD34$^-$ (R6) cells, according to the expression level of CD34. The definition of CD34$^{high/-}$ cells is as follows: the CD34^{high} fraction contains cells expressing maximum apophycocyanin (APC) fluorescence intensity (FI) up to 5% of the level of FI and the CD34$^-$ fraction contains cells expressing $<0.5\%$ of the level of FI. **f** The 18Lin$^-$ cells residing in the R4 gate were further subdivided into two fractions, 18Lin$^-$CD45$^+$CD34^{high} (R7) andCD34$^-$(R8) cells, according to the expression level of CD34. The definition of CD34$^{high/-}$ cells is as follows: the CD34^{high} fraction contains cells expressing maximum APC FI up to 15% of the level of FI and the CD34$^-$ fraction contains cells expressing $<0.2\%$ of the level of FI. **g** The R5-gated cells were further subdivided into two fractions: 13Lin$^-$CD45$^+$CD34^{high}CD38$^+$ (R9) and CD38$^-$ (R10) cells. The definition of CD38$^{+/-}$ cells is as follows: the CD38$^+$ fraction contains cells expressing maximum PE-Cy7 FI up to 5% of the level of FI and the CD38$^-$ fraction contains cells expressing $<0.5\%$ of the level of FI

technique is associated with a seeding efficiency 25 times greater than that of the previously described method.

4.3.6 Characterization of Highly Purified Human CD34-Negative HSCs

The next approach to characterize the self-renewal and long-term repopulating potential of the CD34$^-$ SRCs was to serially analyze the kinetics of BM engraftment for 24 weeks in NOD/Shi-scid/IL-2Rγ_c^{null} (NOG) mice that received transplants of

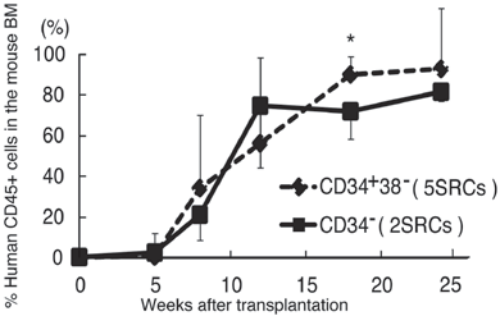

Fig. 4.5 Long-term human hematopoietic cell reconstitution in NOG mice. Five $CD34^{+}CD38^{-}$ SRCs (*dotted line*) or two $CD34^{-}$ SRCs (*solid line*) were injected into the left tibiae of NOG mice using IBMI. The number of SRCs transplanted was estimated based on the limiting dilution analysis data. The human $CD45^{+}$ cell rates in the contralateral sites of the recipient mice were serially analyzed according to the bone marrow (*BM*) aspiration method 5, 8, 12, 18, and 24 weeks after transplantation using 6-color flow cytometry. The data represent the median ± SD of the results obtained from six mice at each time point, except 8 weeks ($n=3$). *$P<0.05$

2×10^3 $18Lin^{-}CD34^{-}$ cells (containing two SRCs). The findings were compared with the repopulating patterns in NOG mice that received 200 $13Lin^{-}CD34^{high}CD38^{-}$ cells (containing five SRCs). The number of SRCs transplanted was estimated based on the data obtained from the limiting dilution analyses and recently reported data [31].

In these experiments, both the mice that received transplants of $CD34^{-}$and $CD34^{+}CD38^{-}$ SRCs showed signs of human cell repopulation at 5 weeks after transplantation. The repopulation levels in all six mice that received transplants of two $CD34^{-}$ SRCs were 0.4–28.9% (median 2.5%). Five of the six mice that received transplants of five $CD34^{+}CD38^{-}$ SRCs also showed a comparable level of human cell repopulation (0.2–28.7%, median 0.4%). The mice that received transplants of $CD34^{+}CD38^{-}$ SRCs exhibited a peak level of repopulation at 18 weeks after the transplantation (74.2–98.5%, median 90.0%), and the repopulation rates were maintained at this level until 24 weeks after transplantation. On the other hand, the repopulation rates of the mice that received transplants of $CD34^{-}$ SRCs gradually increased over 5–12 weeks, then reached high levels (74.9–88.0%, median 81.6% at 24 weeks after transplantation) that were maintained during the observation period. These results demonstrate for the first time that $CD34^{-}$ SRCs possess potent human cell repopulation potential that is almost equivalent to that of $CD34^{+}CD38^{-}$ SRCs (Fig. 4.5).

All of the secondary recipient mice that received the transplants from the primary recipient mice (receiving the transplants of two $CD34^{-}$ SRCs or five $CD34^{+}CD38^{-}$ SRCs) showed signs of human cell repopulation at 12 weeks after transplantation. The human $CD45^{+}$ cell rates in all secondary recipient mice reflected comparable levels of human cell repopulation (0.1–10.8% vs. 0.1–10%). We confirmed that all of the secondary recipient mice that received transplants of $CD34^{-}$ and $CD34^{+}CD38^{-}$

SRCs displayed multilineage human hematopoiesis, including $CD34^+$, $CD19^+$, and $CD33^+$ cells (data not shown).

These results demonstrate that $CD34^-$ and $CD34^+CD38^-$ SRCs both have comparable levels of secondary reconstituting ability, and are capable of sustaining long-term (up to 36 weeks) human hematopoiesis in NOG and NOD/SCID mice, suggesting that both SRCs possess a significant self-renewing potential (Fig. 4.5).

To further evaluate the functional differences between the $CD34^-$ and $CD34^+CD38^-$ SRCs, we studied the multilineage reconstitution ability of these cells in various organs, including the BM, PB, and spleen, in NOG mice that were transplanted with either 2×10^3 $18Lin^-CD34^-$ cells (two SRCs) or 200 $18Lin^-CD34^{high}CD38^-$ cells (five SRCs) by IBMI. The BM repopulation patterns are shown in Fig. 4.6. The analyses of the two representative mice that were transplanted with either $CD34^-$ SRCs (Fig. 4.6, right column) or $CD34^+CD38^-$ SRCs (Fig. 4.6, left column) demonstrated that both the $CD34^-$ and $CD34^+CD38^-$ SRCs generated $CD34^+$ stem/progenitor cells and that both SRCs had a comparable in vivo differentiation capacity to the B-lymphoid, myeloid, monocytic, megakaryocytic, and erythroid lineages 24 weeks after transplantation.

4.3.7 Reversible Expression of CD34 Antigens on Primitive Human HSCs

As described in the previous section, the expression of CD34 antigens by murine HSCs is reversible [19–21]. On the other hand, human BM-derived $CD34^+$ HSCs have been reported to convert to $CD34^-$ HSCs in vivo [39, 49]. These studies demonstrated that the conversion of the CD34 expression occurs at least 8–15 months following transplantation of purified Lin^-CD34^+ or $CD34^+CD38^-$ cells in preimmune fetal sheep and homozygous bg.bg/nu.nu/xid.xid (bnx) mice. These studies indicate the possibility that the human BM-derived $CD34^+$ cell population can act as a reservoir for the generation of $CD34^-$ cells. Conversely, the human BM-derived $CD34^-$ cell population generates $CD34^+$ repopulating cells in vivo, thus indicating that the expression of CD 34 antigens on human BM-derived HSCs is reversible [39, 49]. As we previously reported [27], human CB-derived $CD34^-$ SRCs generate $CD34^+$ SRCs in vitro as well as in vivo. In contrast to BM-derived HSCs [39, 49], human CB-derived $CD34^+$ SRCs do not convert to $CD34^-$ SRCs for at least 24 weeks after transplantation ([27], Matsuoka and Sonoda, unpublished data). However, it remains to be determined whether human CB-derived $CD34^+$ SRCs can convert to $CD34^-$ SRCs. Therefore, much longer observation periods (more than 1 year) are needed, as suggested in previous reports [39, 49].

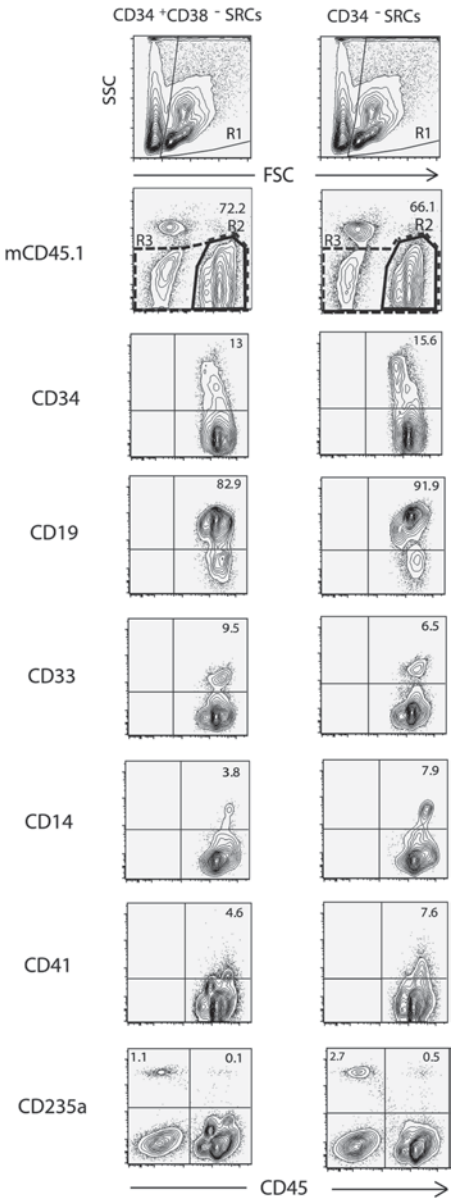

Fig. 4.6 Long-term multilineage reconstitution abilities of $CD34^{+}CD38^{-}$, and $CD34^{-}$SRCs. First, the R1 gate was set on the total murine BM cells obtained from two representative NOG mice that received five $CD34^{+}CD38^{-}$ SRCs (*left column*) or two $CD34^{-}$ SRCs (*right column*) 24 weeks after transplantation. Then, the human $CD45^{+}$ cells were gated as R2 (*solid line*). The expression of surface markers, including CD34, CD19, CD33, CD14 and CD41, on the R2 gated cells was analyzed using 6-colorflow cytometry. Only the expression of CD235a was analyzed using the R3 gate (*dotted line*) containing $CD45^{+/-}$ cells. The percentage of positive cells in each scattergram (CD45, CD34, CD19, CD33, CD14, and CD41) is presented in the *right upper corner*. The percentage of CD235a positive cells is presented in the *bilateral upper corners*

4.4 Candidate Positive Markers for Isolation of Human CD34-Negative HSCs

As previously described, we developed a high-resolution purification method for efficiently isolating human CB-derived $CD34^{-}$ SRCs [13]. However, the frequency of CB-derived $CD34^{-}$ SRCs is 1/1000 [13], which is still very low in comparison

to that of $CD34^+CD38^-$ SRCs (1/40 cells) (31). In order to more effectively enrich/purify human $CD34^-$ LTR-HSCs, it is very important to identify reliable positive markers of human LTR-HSCs, such as Sca-1 and CD150, for murine $CD34^-$ KSL cells. In this section, possible/useful reported positive markers for human HSCs are briefly described.

4.4.1 AC133 (CD133)

The cell-surface glycoprotein CD133 (AC133) is expressed on primitive human HSPCs. Bhatia and colleagues reported that, in their study, $AC133^+CD7^-$ cells were identified at a frequency of 0.2% of human CB-derived $Lin^-CD34^-CD38^-$ cells [12]. Interestingly, the $AC133^+CD7^-$ cells were highly enriched for HPC activity, which was almost equivalent to that of $CD34^+$ cells. These observations are consistent with our recent results [13]. Moreover, the in vitro cultured $AC133^+CD7^-$ cells exhibited a significant SRC activity. More recently, it was reported that human CB-derived $CD133^+G_0$ cells, most of which are CD38-negative and aldehyde dehydrogenase (ALDH)-positive, demonstrate significant multilineage reconstituting activity when they are transplanted into NOD/SCID mice [50]. Collectively, these results suggest that AC133 (CD133) is a candidate positive marker of human HSPC identified in human CB-derived Lin^-CD34^- cells. Very recently, we succeeded in highly purifying human CB-derived $CD34^-$ SRCs using the CD133 expression in an $18Lin^-CD34^-$ cell population [51]. The limiting dilution analysis demonstrated that the incidence of $CD34^-$ SRCs in $18Lin^-CD34^-CD133^+$ cells was 1/142, which is the highest level of purification of these unique $CD34^-$ HSCs to date. In addition, the incidence of $CD34^+$ SRCs in $18Lin^-CD34^+CD133^+$ cells was 1/98.9 (Fig. 4.7). Furthermore, CD133 expression clearly segregated the SRC activities of $18Lin^-CD34^-$ cells, as well as $18Lin^-CD34^+$ cells, in their positive fractions, indicating its functional significance as a common cell surface maker to effectively isolate both $CD34^+$ and $CD34^-$ SRCs.

4.4.2 C1qRp (CD93)

A receptor for the complement molecule C1q (C1qRp), the human homologue of the murine stem cell antigen AA4 [52], has been reported to be useful as a marker for isolating/purifying human HSCs [53]. AA4 has been identified to be the antigen recognized by the murine AA4.1 monoclonal Ab. Danet et al. reported that C1qRp is a positive marker of all BM-repopulating HSCs because it is expressed on both $CD34^-$ and $CD34^+$ HSCs obtained from CB and adult BM [53]. They showed that as few as 5000 $Lin^-CD34^-CD38^-C1qRp^+$ cells can result in engraftment in NOD/SCID mice. In contrast, as many as 840,000 $Lin^-CD34^-CD38^-C1qRp^-$ cells are incapable of repopulating recipient mice. Interestingly, $Lin^-CD34^{+\text{ or }-}CD38^-C1qRp^+$ cells not only exhibit SRC activity but are also able to differentiate into human hepatocytes in vivo [53].

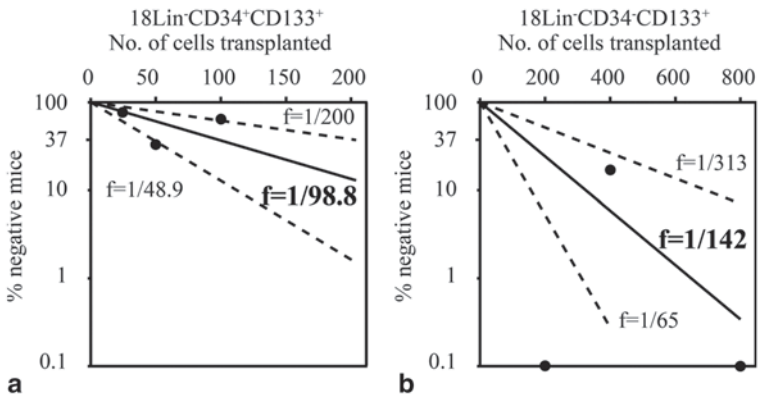

Fig. 4.7 The frequency of SRCs in the 18Lin$^-$CD34$^+$CD133$^+$ and 18Lin$^-$CD34$^-$CD133$^+$ cells. Various numbers of 18Lin$^-$CD34$^+$CD133$^+$ cells (20, 50, and 100, $n=20$) (**a**) and 18Lin$^-$CD34$^-$CD133$^+$ cells (200, 400, 800, $n=15$) (**b**) were transplanted to NOG mice. The human CD45$^+$ cell repopulation in the mouse BM was analyzed by FCM 12 weeks after transplantation. The frequency of SRCs was one per 98.8 18Lin$^-$CD34$^+$CD133$^+$ cells, and one per 142 18Lin$^-$CD34$^-$CD133$^+$ cells, respectively. For the frequency determination, the *middle solid line* represents the estimated weighted mean frequency (f_{WM}). The *lower and upper dotted lines* represent the 95% confidence interval of the f_{WM}

Very recently, Bonnet and colleagues reported that CD93 is a positive marker of human CB-derived very primitive CD34$^-$ HSCs [54]. They transplanted Lin$^-$CD34$^-$CD93$^+$ or Lin$^-$CD34$^-$CD38$^-$CD93$^+$ cells, which did not express CD133, by TVI, and demonstrated their SRC activity. In contrast, our identified CB-derived CD34$^-$ SRCs could only be detected by IBMI [13, 21–27, 31, 38]. Furthermore, CD93 was only marginally expressed on our highly purified 18Lin$^-$CD34$^-$ cells. When we back-gated these cells into the FSC/SSC scattergram, the 18Lin$^-$CD34$^-$CD93$^+$ cells were scattered inside and around the blast window. We further analyzed the expression of CD93 on the CB-derived 18Lin$^-$CD34$^-$CD133$^+$ cells, which contained all of the CD34$^-$ SRC activity in the CB demonstrated by our recent study [51]. As reported, we did not detect any CD93$^+$ cells in our 18Lin$^-$CD34$^-$CD38$^{-/lo}$CD133$^+$ cell population. Their Lin$^-$CD34$^-$CD38$^-$CD93$^+$ cells did not express CD133. However, almost all of the Lin$^-$CD34$^-$CD38$^-$CD93$^+$ cells were included in the 18Lin$^+$ cell fraction. This is not surprising because they only depleted mature cells from their target cells by immunomagnetic beads using the StemSep (Stem Cell Technologies), which only removed strongly Lin$^+$ cells. These immunomagnetically Lin-depleted cells still contained many lymphocytes, monocytes, granulocytes, and NK cells, which expressed CD93 [51]. Human CB-derived CD45RA$^+$ naïve T-lymphocytes were previously reported to express CD93 [55]. Indeed, their incidence of CD34$^-$ SRCs in the Lin$^-$CD34$^-$CD38$^-$CD93$^+$ cells was 1/~6100 cells, which is very low compared to our recent data (1/142) (51). Overall, their data are exactly the opposite of our data, thus suggesting the possibility that there may be different classes of CD34$^-$ HSCs in the primitive human HSC compartment. Further studies will therefore be required to clarify this important issue.

4.4.3 Thy-1 (CD90) and CD49f

Thy-1 (CD90) is a heavily glycosylated cell surface protein that can be used as a marker for a variety of stem cells, including murine and human HSCs. McCune et al. identified human HSC activity in $Lin^-CD34^+Thy\text{-}1^+$ cells using an SCID-hu mouse model [56]. It has also been reported that the human fetal BM-derived $Lin^-CD34^+Thy\text{-}1^+$ cell population contains pluripotent HSCs [57] and that $CD34^+$ cells derived from the human fetal liver, CB, and BM express this protein [58]. These in vivo studies suggest that human HSCs are contained in the $Lin^-CD34^+Thy\text{-}1^+$ cell fraction. Majeti et al. reported that the human CB-derived $Lin^-CD34^+CD38^-Thy\text{-}1^+CD45RA^-$ cell population contains human HSCs and isolated this activity to as few as 10 purified cells [59]. They also reported that the loss of the Thy-1 expression in the CB-derived $Lin^-CD34^+CD38^-CD45RA^-$ cell population can be used to separate HSCs from multipotent progenitor cells (MPPs; 59). However, in that study, more than one-third of the primary recipient mice that received $Lin^-CD34^+CD38^-CD45RA^-Thy\text{-}1^-$ cells gave rise to engraftment in the secondary mice, raising uncertainty regarding whether Thy-1 can completely segregate HSCs from MPPs. Very recently, Notta et al. reported the findings of an elegant study showing that human HSCs and MPPs are demarcated by the CD49f. expression on human CB-derived $Lin^-CD34^+CD38^-Thy\text{-}1^{+/-}$ cells [60]. Interestingly, the $Lin^-CD34^+CD38^-Thy\text{-}1^+CD49f^+$ cells exhibited a high repopulating capacity and were capable of being serially transplanted. A limiting dilution analysis revealed that 9.5% (1/10.5) of the $Thy\text{-}1^+CD49f^+$ cells possessed a long-term repopulating activity. The authors performed a single cell transplantation analysis using $Lin^-CD34^+CD38^-Thy\text{-}1^+Rho^{low}CD49f^+$ cells. Five of the 18 recipients mice displayed multilineage repopulation for 20 weeks. Engraftment of a single $Lin^-CD34^+CD38^-Thy\text{-}1^+Rho^{low}CD49f^+$ cell provides evidence that human HSCs express CD49f. [60]. Based on these reports, we tested the expression of Thy-1 and CD49f. on human CB-derived $18Lin^-CD34^-$ cells in which $CD34^-$ HSCs are highly enriched [13]. The data clearly demonstrated that a portion of $18Lin^-CD34^-$ cells marginally express these two markers [51].

4.4.4 MPL (CD110)

In the murine primitive HSC compartment, including $LSKCD34^-Flt3^-Tie2^+$ and $LSKCD48^-CD150^+$ cells, MPL/thrombopoietin (THPO) signaling plays an important role in the maintenance of adult quiescent HSCs [61, 62]. However, the role of MPL/THPO signaling in the human primitive HSC compartment has not yet been elucidated. We previously identified very primitive human CB-derived $CD34^-$SRCs using the IBMI method [27]. Recently, we developed a high-resolution method for purifying primitive $CD34^-$ SRCs using 18Lin-specific antibodies [13]. Therefore, we investigated the functional significance of the MPL expression in human primitive $CD34^-$ SRCs (HSCs). First, we sorted the $18Lin^-CD34^{+/-}MPL^{+/-}$

cells using FACS. Then, the four fractions of cells were transplanted using IBMI into NOG mice in order to investigate the long-term repopulating capacity of the cells. We performed primary, secondary, and tertiary transplantations for up to 18 months. The results clearly indicated that both the $CD34^{+/-}$ SRCs not expressing MPL sustained a long-term (>1 year) human cell repopulation in the NOG mice. Therefore, these findings suggest, for the first time, that the functional significance of the MPL expression in human CB-derived primitive HSCs is different from that observed in adult murine primitive HSCs [63] (Takahashi and Sonoda, manuscript in preparation).

4.5 Proposal of a Novel Model of the Human HSC Hierarchy

Lifelong blood cell production is supported by rare HSCs that replenish mature hematopoietic cells via a series of Lin-restricted HPCs. As described in an earlier section, the most primitive HSCs in mammals, including mice and humans, are believed to be $CD34^+$. Moreover, $CD34^+CD38^-CD45RA^-CD90^+$ HSCs are defined as being the most primitive human HSCs [60, 64, 65]. However, it has been elucidated that murine long-term repopulating HSCs are lineage-marker negative (Lin^-) c-$kit^+Sca\text{-}1^+CD34^{low/-}$ [16]. In addition, we previously identified human CB-derived $CD34^-$ SRCs with an extensive lymphoid and myeloid repopulating ability using the IBMI technique [27].

In order to investigate the differentiation pathway of $CD34^-$ SRCs (HSCs), it is necessary to highly purify the $CD34^-$SRCs. We recently succeeded in highly purifying $CD34^-$ SRCs (incidence 1/142) using CD133 as a positive marker [51]. On the other hand, it has been suggested that a human HSC-supportive microenvironment exists in the BM that plays a pivotal role in the maintenance of the self-renewal capacity and dormancy of primitive HSCs. It has been reported that osteoblasts, mesenchymal stromal cells (MSCs)and vascular endothelial cells play an important role in organizing HSC niches [66, 67]. Very recently, we succeeded in isolating three MSC lines derived from human BM cells according to the expression levels of CD271 and SSEA-4 using FACS [68]. Approximately one of six $CD271^+SSEA\text{-}4^+$ (DP) cells were able to form CFU-F-derived colonies. The DP cell-derived MSCs were able to differentiate into osteoblasts and chondrocytes; however, they could not differentiate into adipocytes. In contrast, the $CD271^+SSEA\text{-}4^-$ cell- and $CD271^-SSEA\text{-}4^-$ cell-derived MSCs were able to differentiate into three lineages. Then, we assessed the $CD34^-$ SRC-supportive activity of the three MSC lines, and found that the highest level of $CD45^+$ human cell engraftment was observed in the mice that received $18Lin^-CD34^-$ cells cocultured with DP cell-derived MSCs. Using the highly purified $18Lin^-CD34^-$ cells [13] and DP cell-derived MSCs [68], we investigated the differentiation pathway of the $CD34^-$ SRCs (HSCs) in vitro.

Interestingly, $Lin^-CD34^+CD38^-CD45RA^-CD90^+$ cells, which contain the most primitive human $CD34^+CD38^-$ SRCs [60], were generated using the above mentioned cocultures. Moreover, these cells exhibited a distinct SRC activity by IBMI. These results indicate that human CB-derived $18Lin^-CD34^-$ cells are able to generate very primitive $Lin^-CD34^+CD38^-CD45RA^-CD90^+$ SRCs in vitro. In addition, we confirmed that very primitive $Lin^-CD34^+CD38^-CD45RA^-CD90^+$ SRCs were produced in vivo 24 weeks after transplantation in long-term repopulating NOG mice that received limited numbers of $CD34^-$ SRCs (Matsuoka and Sonoda, manuscript in preparation).

These findings clearly demonstrate that human CB-derived $CD34^-$ SRC (HSC) are able to produce very primitive $Lin^-CD34^+CD38^-CD45RA^-CD90^+$ SRCs in vitro as well as in vivo. Based on these data, we propose that $CD34^-$SRCs are more immature than the previously believed most primitive $CD34^+CD38^-$ SRCs. These results provide a new concept of the hierarchy in the human primitive HSC compartment (Fig. 4.8).

4.6 Clinical Significance of Non-migrating Human CD34-Positive and CD34-Negative HSCs and Intra-bone Marrow HSCT

In clinical HSCT, the number of cord blood stem cell transplantations (CBSCTs) has increased rapidly [69]. However, the cells are difficult to transplant in adult patients, due to the limited number of HSCs in a single CB unit, resulting in a higher risk of graft failure as well as a delayed neutrophil and platelet recovery. Therefore, the ability to successfully engraft adults on a routine basis using the IBMI technique would greatly expand the clinical applicability of CBSCT for the following reasons: (1) IBMI achieves more efficient engraftment of $CD34^+$ SRCs (HSCs), which thus contributes to the early phase of BM reconstitution; (2) the identified non-migrating $CD34^+$ SRCs (HSCs) can efficiently home into the BM niche only when transplanted using IBMI; (3) $CD34^-$ SRCs (HSCs) are also able to efficiently home into the BM niche following IBMI. The cells exhibit a high HSC activity and produce a large number of $CD34^+$ HSCs in vivo following transplantation [13, 27, 31, 38, 51]. It is therefore imperative that $CD34^-$ SRCs (HSCs) be transplanted with migrating and non-migrating $CD34^+$ SRCs (HSCs) in clinical HSCT using the IBMI technique. As expected, several clinical trials have already reported that direct intra-BM CBSCT overcomes the problems of graft failure even when low numbers of HLA-mismatched CB cells are transplanted [70–72]. In the near future, therefore, the use of the IBMI technique is expected to have a great impact on CBSCT, particularly in overcoming the above-mentioned problems, including graft failure and a delayed hematopoietic recovery.

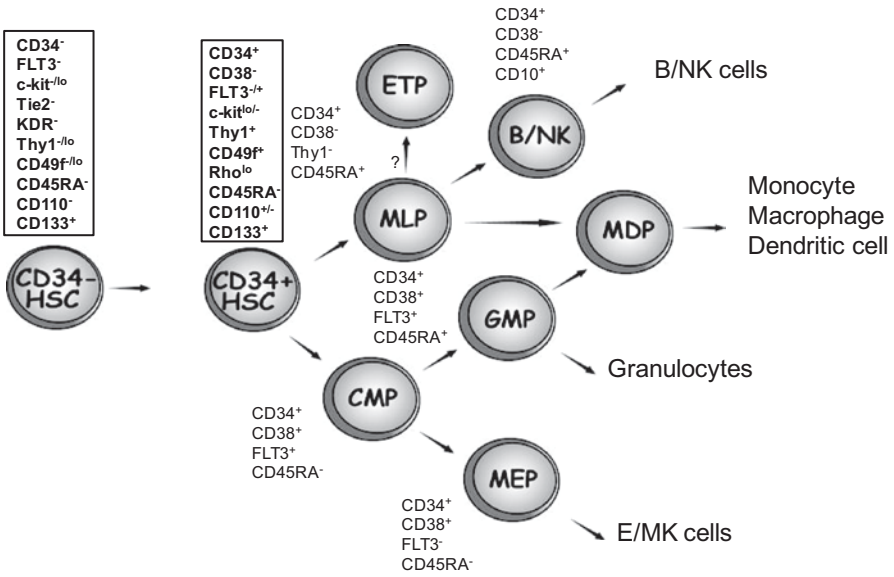

Fig. 4.8 Proposed model of the human hematopoietic hierarchy. It has previously been proposed that $CD34^+CD38^-Thy1^+$ HSCs give rise to various types of HPCs, as shown in this figure. Our identified very primitive $CD34^-$ HSCs are able to produce primitive $CD34^+CD38^-Thy1^+$ HSCs in vitro as well as in vivo, suggesting their primitive nature. Based on our current data obtained using flow cytometric and serial transplantation analyses, we propose that the immunophenotype of $CD34^-$ HSC is $CD34^-FLT3^-c\text{-}kit^{-/low}Tie2^-KDR^-Thy1^{low/-}CD49f^{low/-}CD45RA^-CD110^-CD133^+$. (Modified from supplementary Fig. 10 in [64])

Conclusions and Future Prospects

The relationship of primitive HSCs within the human HSC hierarchy has been difficult to clarify due to the heterogeneity of the stem cell compartment. This heterogeneity results in major problems in the isolation/purification of most primitive human HSCs. We successfully developed a sensitive SRC assay system using the IBMI technique to identify a new class of CB-derived $CD34^-$ HSCs [13, 27, 31, 38, 51]. Based on our current data obtained using flow cytometry and serial transplantation analyses, we propose that the surface immunophenotype of the most primitive human LTR-HSCs is $Lin^-CD34^-FLT3^-c\text{-}kit^{-/low}Tie2^-KDR^-Thy1^{low/-}CD49f^{low/-}CD45RA^-CD110^-CD133^+$ (Fig. 4.8). However, the frequency of CB-derived $CD34^-$ SRCs is 1/142 in $18Lin^-CD34^-CD133^+$ cells [51] at the present time, which is still low in comparison to that of $CD34^+CD38^-$ SRCs (1/40) (31). In order to more effectively enrich/purify human $CD34^-$ LTR-HSCs at the single cell level, it is very important to identify another reliable positive marker for human LTR-HSCs beside CD133. Our goal is to achieve the complete purification of most primitive human HSCs, enabling the development of stem cell-based cellular and genetic therapies for various hematological and non-hematological diseases.

The molecular mechanisms that regulate the self-renewal and/or differentiation of human HSPCs are poorly understood because our understanding of these mechanisms, which control hematopoiesis, has primarily been obtained from mouse models. However, the cellular properties of murine and human HSCs appear to differ. Therefore, these analyses should be performed using purified human HSCs to confirm the data obtained from mouse models.

The application of our sensitive SRC assay system using the IBMI technique may make it possible to discover other hitherto unidentified HSCs in various organs and/or identify new markers for LTR-HSCs. It is also important to clarify whether the BM or mobilized PB contains an equivalent class of $CD34^-$ SRCs. From another point of view, the candidate positive markers for primitive human HSCs, such as CD133 and MPL, are expressed differently on the surface of CB- and BM-derived $18Lin^-CD34^-$ cells [51](Matsuoka and Sonoda, unpublished data). These results suggest that human CB and BM contain different classes of HSCs. As described in this review, $CD34^-$ HSCs continue to be heterogeneous populations and are not completely purified at the single cell level. Therefore, an important issue, the developmental origin of these primitive $CD34^-$HSCs, has not been fully clarified and requires further investigation.

Acknowledgements The author is grateful to Drs. Y. Matsuoka, M. Takahashi, R. Nakatsuka, K. Sumide, T. Fujioka, H. Kohno, and Y. Sasaki, for their contributions. The author also thanks Kirin Brewery Co. Ltd. (Tokyo, Japan) for providing the various growth factors. Ms. K. Ishino is also acknowledged for her valuable assistance in preparing the manuscript.

This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (Grant No. 15039227) and for Scientific Research C (Grant Nos. 15591015, 19591144, 21591251, and 24591432) from the Ministry of Education, Science and Culture of Japan, a grant from Haiteku Research Center of the Ministry of Education, a grant from the Science Frontier Program of the Ministry of Education, a grant from the Twenty-first Century Center of Excellence (COE) program of the Ministry of Education, a grant from the Promotion and Mutual Aid Corporation for Private Schools of Japan, a grant from Kansai Medical University (Research grant B), grants from MEXT-supported Program for the Strategic Research Foundation at Private Universities (2011–2016 and 2012–2017), a grant from the Adaptable and Seamless Technology Transfer Program through target-driven R&D, JST, a grant fromthe Japan Leukemia Research Foundation, a grant from the Mitsubishi Pharma Research Foundation, a grant from the Terumo Life Science Foundation, and a grant from the SENSHIN Medical Research Foundation.

References

1. Krause DS, Fackler MJ, Civin CI, May WS (1996) CD34: structure, biology, and clinical utility. Blood 87:1–13
2. Vogel W, Scheding S, Kanz L, Brugger W (2000) Clinical applications of $CD34^+$peripheral blood progenitor cells (PBPC). Stem Cells 18:87–92
3. Civin C, Trischmann T, Kadan NS et al (1996) Highly purified CD34-positive cells reconstitute hematopoiesis. J Clin Oncol 14:2224–2233
4. Sonoda Y, Sakabe H, Ohmisono Y, Tanimukai S, Yokota S, Nakagawa S, Clark SC, Abe T (1994) Synergistic actions of stem cell factor and other burst-promoting activities on proliferation of $CD34^+$highly purified blood progenitors expressing HLA-DR or different levels of c-kit protein. Blood 84:4099–4106

5. Cheng J, Baumhueter S, Cacalano G, Carver-Moore K, Thibodeaux H, Thomas R, Broxmeyer HE, Cooper S, Hague N, Moore M, Lasky LA (1996) Hematopoietic defects in mice lacking the sialomucin CD34. Blood 87:479–490
6. Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE (1998) A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat Med 4:1038–1045
7. Nakamura Y, Ando K, Chargui J, Kawada H, Sato T, Tsuji T, Hotta T, Kato S (1999) Ex vivo generation of CD34+ cells from CD34−hematopoietic cells. Blood 94:4053–4059
8. Ando K (2002) Human $CD34^-$ hematopoietic stem cells: basic futures and clinical relevance. Int J Hematol 75:370–375
9. Guo Y, Lubbert M, Engelhardt M (2003) $CD34^-$ hematopoietic stem cells: current concepts and controversies. Stem Cells 21:15–20
10. Zanjani ED, Almeida-Porada G, Livingston AG, Flake AW, Ogawa M (1998) Human bone marrow $CD34^-$ cells engraft in vivo and undergo multilineage expression that includes giving rise to $CD34^+$cells. Exp Hematol 26:353–360
11. Fujisaki T, Berger MG, Rose-John S, Eaves CJ (1999) Rapid differentiation of a rare subset of adult human $Lin^-CD34^-CD38^-$ cells stimulated by multiple growth factors in vitro. Blood 94:1926–1932
12. Gallacher L, Murdoch B, Wu DM, Karanu FN, Keeney M, Bhatia M (2000) Isolation and characterization of human $CD34^-Lin^-$ and $CD34^+Lin^-$ hematopoietic stem cells using cell surface markers AC133 and CD7. Blood 95:2813–2820
13. Ishii M, Matsuoka Y, Sasaki Y, Nakatsuka R, Takahashi M, Nakamoto T, Yasuda K, Matsui K, Asano H, Uemura Y, Tsuji T, Fukuhara S, Sonoda Y (2011) Development of a high-resolution purification method for precise functional characterization of primitive human cord-blood-derived CD34-negative SCID-repopulating cells. Exp Hematol 39:203–213
14. Goodell MA, Rosenzweig M, Kim H, Marks DF, Demaria M, Paradis G, Grupp S, Sieff CA, Mulligan RC, Jahnson P (1997) Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 3:1337–1345
15. Zhou S, Morris JJ, Barnes Y, Lan L, Schuetz JD, Sorrentino BP (2002) Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. Proc Natl Acad Sci U S A 99:12339–12344
16. Osawa M, Hanada K, Hamada H, Nakauchi H (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic cell. Science 273:242–245
17. van derLJC, Ploemacher RE (1995) Marrow- and spleen-seeding efficiencies of all murine hematopoietic stem cell subsets are decreased by preincubation with hematopoietic growth factors. Blood 85:2598–2606
18. Seita J, Ema H, Ooehara J, Yamazaki S, Tadokoro Y, Yamasaki A, Eto K, Takaki S, Takatsu K, Nakauchi H (2007) Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin-mediated signal transduction. Proc Natl Acad Sci U S A 104:2349–2354
19. Sato T, Laver JH, Ogawa M (1999) Reversible expression of CD34 by murine hematopoietic stem cells. Blood 94:2548–2554
20. Ito T, Tajima F, Ogawa M (2000) Developmental changes of CD34 expression by murine hematopoietic stem cells. Exp Hematol 28:1269–1273
21. Tajima F, Sato T, Laver JH, Ogawa M (2000) CD34 expression by murine hematopoietic stem cells mobilized by granulocyte colony-stimulating factor. Blood 96:1989–1993
22. Ogawa M (1993) Differentiation and proliferation of hematopoietic stem cells. Blood 81:2844–2853
23. Moore MAS (1991) Clinical implications of positive and negative hematopoietic cell regulators. Blood 78:1–19
24. Larochelle A, Vormoor J, Hanenberg H, Wang JCY, Bhatia M, Lapidot T, Moritz T, Murdoch B, Xiao XL, Kato I, Williams DA, Dick JE (1996) Identification of primitive human hemato-

poietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. Nat Med 2:1329–1337
25. Guenechea G, Gan OI, Dorrell C, Dick JE (2001) Distinct classes of human stem cells that differ in proliferative and self-renewal potential. Nat Immunol 2:75–82
26. Hogan CJ, Shpall EJ, Keller G (2002) Differential long-term and multilineage engraftment potential from subfractions of human CD34$^+$ cord blood cells transplanted into NOD/SCID mice. Proc Natl Acad Sci U S A 99:413–418
27. Wang J, Kimura T, Asada R, Harada S, Yokota S, Kawamoto Y, Fujimura Y, Tsuji K, Ikehara S, Sonoda Y (2003) SCID-repopulating cell activity of human cord blood-derived CD34$^-$ cells assured by intra-bone marrow injection. Blood 101:2924–2931
28. Wang JCY, Doedens M, Dick JE (1997) Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by quantitative in vivo SCID-repopulating cell assay. Blood 89:3919–3924
29. Verlinden SEF van Es HHG, van Bekkum DW (1998) Serial bone marrow sampling for long-term follow up of human hematopoiesis in NOD/SCID mice. Exp Hematol 26:627–630
30. Kushida T, Inaba M, Hisha H, Ichioka N, Esumi T, Ogawa R, Iida H, Ikehara S (2001) Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. Blood 97:3292–3299
31. Kimra T, Matsuoka Y, Murakami M, Kimura T, Takahashi M, Nakamoto T, Yasuda K, Matsui K, Kobayashi K, Imai S, Asano H, Nakatsuka R, Uemura Y, Sasaki Y, Sonoda Y (2010) In vivo dynamics of human cord blood-derived CD34- SCID-repopulating cells using intra-bone marrow injection. Leukemia 24:162–168
32. Tsuji T, Ogasawara H, Aoki Y, Tsurumaki Y, Kodama H (1996) Characterization of murine stromal cell clones established from bone marrow and spleen. Leukemia 10:803–812
33. Peled A, Petit I, Kollet O, Magid M, Ponomaryov T, Byk T, Nagler A, Ben-Hur H, Many A, Shultz L, Lider O, Alon R, Zipori D, Lapidot T (1999) Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 283:845–848
34. Kollet O, Petit I, Kahn J, Samira S, Dar A, Peled A, Deutsch V, Gunetti M, Piacibello W, Nagler A, Lapidot T (2002) Human CD34$^+$CXCR4$^-$ sorted cells harbor intracellular CXCR4, which can be functionally expressed and provide NOD/SCID repopulation. Blood 100:2778–2786
35. Yahata T, Ando K, Sato T, Miyatake H, Nakamura Y, Muguruma Y, Kato S, Hotta T (2003) A highly sensitive strategy for scid-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. Blood 101:2905–2913
36. Papayannopoulou T, Priestley GV, Nakamoto B (1998) Anti-VLA4/VCAM-1-induced mobilization requires cooperative signaling through the kit/kit ligand pathway. Blood 91:2231–2239
37. Peled A, Kollet O, Ponomaryov T, Petit I, Franitza S, Grabovsky V, Slav MM, Nagler A, Lider O, Alon R, Zipori D, Lapidot T (2000) The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34$^+$cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. Blood 95:3289–3296
38. Kimura T, Wang J, Matsui K, Imai S, Yokoyama S, Nishikawa S, Ikehara S, Sonoda Y (2004) Proliferative and migratory potentials of human cord blood-derived CD34$^-$ severe combined immunodeficiency repopulating cells that retain secondary reconstituting capacity. Int J Hematol 79:328–333
39. Zanjani ED, Almeida-Porada G, Livingston AG, Zeng H, Ogawa M (2003) Reversible expression of CD34 by adult human bone marrow long-term engrafting hematopoietic stem cells. Exp Hematol 31:406–412
40. Sonoda Y, Kimura T, Sakabe H, Tanimukai S, Ogmizono Y, Nakagawa S, Yokota S, Lyman SD, Abe T (1997) Human flt3 ligand acts on myeloid as well as multipotential progenitors derived from purified CD34$^+$ blood progenitors expressing different levels of c-kit protein. Eur J Hematol 58:257–264
41. Lyman SD, Jacobsen SEW (1998) c-kit ligand and flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. Blood 91:1101–1134

42. Kondo M, Wagers AJ, Manz MG, Prohaska SS, Scherer DC, Beihack GF, Shizuru JA, Weissman IL (2003) Biology of hematopoietic stem cells and progenitors: implications for clinical application. Annu Rev Immunol 21:759–806
43. Adolfsson J, Borge OJ, Bryder D, Theilgaard-Monch K, Astrand-Grundstrom I, Sitnicka E, Sasaki Y, Jacobsen SEW (2001) Upregulation of flt3 expression within the bone marrow $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ stem cell compartment is accompanied by loss of self-renewal capacity. Immunity 15:659–669
44. McKenna HJ, Stocking KL, Miller RE, Brasel K, Smedt TD, Maraskovsky E, Maliszewski CR, Lynch DH, Smith J, Pulendran B, Roux ER, Lyman SD, Peschon JJ (2000) Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. Blood 95:3489–3497
45. Kimura T, Asada R, Wang J, Kimura T, Morioka M, Matsui K, Kobayashi K, Henmi K, Imai S, Kita M, Tsuji T, Sasaki Y, Ikehara S, Sonoda Y (2007) Identification of long-term repopulating potential of human cord blood-derived $CD34^{-}flt3^{-}$ severe combined immunodeficiency-repopulating cells by intra-bone marrow injection. Stem Cells 25:1348–1355
46. Sitnicka E, Buza-Vidas N, Larsson S, Nygren JM, Liuba K, Jacobsen SEW (2003) Human $CD34^{+}$hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express flt3: distinct flt3 and c-kit expression and response patterns on mouse and candidate human hematopoietic stem cell. Blood 102:881–886
47. Ebihara Y, Wada M, Ueda T et al (2002) Reconstitution of human haematopoiesis in non-obese diabetic/severe combined immunodeficient mice by clonal cells expanded from single $CD34^{+}CD38^{-}$ cells expressing flk2/flt3. Br J Haematol 119:525–534
48. Adolfsson J, Mansson R, Buza-Vidas N, Hultquist A, Liuba K, Jensen CT, Bryder D, Yang L, Borge O-J, Thoren LAM, Anderson K, Sitnicka E, Sasaki Y, Sigvardsson M, Jacobsen SEW (2005) Identification of $flt3^{+}$ lympho-myeloid stem cells lacking erythro-megakaryocytic potential: a revised road map for adult blood lineage commitment. Cell 121:295–306
49. Dao MA, Arevalo J, Nolta JA (2003) Reversibility of CD34 expression on human hematopoietic stem cells that retain the capacity for secondary reconstitution. Blood 101:112–118
50. Boxall SA, Cook GP, Pearce D, Bonnet D, El-Sherbiny YM, Blundell MP, Howe SJ, Leek JP, Markham AF, de Wynter EA (2009) Haematopoietic repopulating activity in human cord blood $CD133^{+}$ quiescent cells. Bone Marrow Transplant 43:627–635
51. Takahashi M, Matsuoka Y, Sumide K, Nakatsuka R, Fujioka T, Kohno H, Sasaki Y, Matsui K, Asano H, Kaneko K, Sonoda Y (2014) CD133 is a positive marker for a distinct class of primitive human cord blood-derived CD34-negative hematopoietic stem cells. Leukemia 28:1308–1315
52. Petrenko O, Beavis A, Klaine M, Kittappa R, Godin I, Lemischka IR (1999) The molecular characterization of the fetal stem cell marker AA4. Immunity 10:691–700
53. Danet G, Luogo JL, Butler G, Lu MM, Tenner AJ, Simon MC, Bonnet DA (2002) C1qRp define a new human stem cell population with hematopoietic and hepatic potential. Proc Natl Acad Sci U S A 99:10441–10445
54. Anjos-Alfonso F, Currie E, Palmer HG, Foster KE, Taussig DC, Bonnet D (2013) CD34-cells at the apex of the human hematopoietic stem cell hierarchy have distinctive cellular and molecular signatures. Cell Stem Cell 13:161–174
55. Ikewaki N, Yamato H, Kulski JK, Inoko H (2010) Flow cytometric identification of CD93 expression on naïve T lymphocytes ($CD4^{+}CD45RA^{+}$ cells) in human neonatal umbilical cord blood. J Clin Immunol 30:723–733
56. Mccune JM, Namikawa R, Kaneshima H, Shultz LD, Lieberman M, Weissman IL (1998) The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. Science 241:1632–1639
57. Baum CM, Weissman IL, Tsukamoto AS, Buckle A-M, Peault B (1992) Isolation of a candidate human hematopoietic stem-cell population. Proc Natl Acad Sci U S A 89:2804–2808
58. Craig W, Kay R, Cutler RL, Lansdorp PM (1993) Expression of Thy-1 on human hematopoietic progenitor cells. J Exp Med 177:1331–1342

59. Majeti R, Park CY, Weissman IL (2007) Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell 1:635–645
60. Notta F, Doulatov S, Laurenti E, Poeppl A, Jurisica I, Dick JE (2011) Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science 333:218–221
61. Qian H, Buza-Vidas N, Hyland CD, Jensen CT, Antonchuk J, Mansson R, Thoren LA, Ekblom M, Alexander WS, Jacobsen SEW (2007) Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell Stem Cell 1:671–684
62. Yoshihara H, Arai F, Hosokawa K, Hagiwara T, Takubo K, Nakamura Y, Gomei Y, Iwasaki H, Matsuoka S, Miyamoto K, Miyazaki H, Takahashi T, Suda T (2007) Thrombopoitin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell Stem Cell 1:685–697
63. Takahashi M, Matsuoka Y, Iwaki R, Nakatsuka R, Fujioka T, Kohno H, Matsui K, Asano H, Sasaki Y, Kaneko K, Sonoda Y (2012) Functional significance of MPL expression in the human primitive hematopoietic stem cell compartment. The 54th annual meeting of the American society of hematology, Dec 8, Atlanta, USA (Abstr)
64. Doulatov S, Notta F, Eppert K, Nguyen L, Ohashi PS, Dick JE (2010) Revised map of the human progenitor hierarchy shows the origin of macrophage and dendritic cells in early lymphoid development. Nat Immunol 11:585–593
65. Doulatov S, Notta F, Laurenti E, Dick JE (2012) Hematopoiesis: a human perspective. Cell Stem Cell 10:120–136
66. Wilson A, Oser GM, Jaworski M, Blanco-Bose WE, Laurenti E, Adolphe C, Essers MA, Macdonald HR, Trump A (2007) Dormant and self-renewing hematopoietic stem cells and their niche. Ann N Y Acad Sci 1106:64–75
67. Smith JNP, Calvi LM (2013) Current concepts in bone marrow microenvironmental regulation of hematopoietic stem and progenitor cells. Stem Cells 31:1044–1050
68. Matsuoka Y, Sasaki Y, Takahashi M, Nakatsuka R, Uemura Y, Inoue M, Ogawa H, Takahashi T, Ishikawa J, Hino H, Sonoda Y (2010) Prospective isolation and functional characterization of human bone marrow-derived hematopoietic stem cell-supportive mesenchymal stromal cells. Blood 116:1575–1576 (Abstr)
69. Brunstein GG, Wagner JE (2006) Umbilical cord blood transplantation and banking. Annu Rev Med 57:403–417
70. Frassoni F, Gualandi F, Podesta M, Raiola AM, Ibatici A, Piaggio G, Sessarego M, Sessarengo N, Gobbi M, Sacchi N, Labopin M, Bacigalupo A (2008) Direct intrabone transplant of unrelated cord-blood cells in acute leukemia: a phase I/II study. Lancet Oncol 9:831–839
71. Brunstein CG, Barker JN, Weisdorf DJ, DeFor TE, McKenna D, Chong SY, Miller JS, McGlave PB, Wagner JE (2009) Intra-BM injection to enhance engraftment after myeloablative umbilical cord blood transplantation with two partially HLA-matched units. Bone Marrow Transplant 43:935–940
72. Okada M, Yoshihara S, Taniguchi K, Kaida K, Ikegami K, Kato R, Tamaki H, Inoue T, Soma T, Kai S, Kato S, Ogawa H (2012) Intrabone marrow transplantation of unwashed cord blood using reduced-intensity conditioning treatment: a phase I study. Biol Blood Marrow Transplant 18:633–639

Chapter 5
Cell Therapies in Cardiology

Wojciech Wojakowski, Agata Czekaj and Michał Tendera

5.1 Introduction

Over the last 12 years, the idea of using stem and progenitor cells (SPC) to regenerate the human heart and vessels underwent rapid translation from basic studies and animal preclinical experiments to clinical application in clinical trials. The pioneering work of Anversa's group in a murine model was published in Nature in 2001, and in the same year, the first patient with acute myocardial infarction (MI) was treated by Strauer and colleagues with autologous bone marrow (BM) cells followed by the publication of the first clinical trial next year [1–4]. Such a fast-track translation was almost unprecedented in cardiovascular (CV) medicine where the clinical use of innovative medical therapies and devices took considerably more time. Since then, the field has undergone the periods of enthusiasm and inflated expectations followed by some degree of disappointment to reach a stage of steady progress and unbiased evaluation under the rules of evidence-based medicine (EBM). Two aspects of SPC therapy are essential for future application: better understanding of basic mechanisms as well as assessment in controlled randomized clinical trials (RCT). So far, multitude of small scale clinical trials with the surrogate end-points were carried out followed by meta-analyses allowing some clinically relevant conclusions for the future progress of the field.

The widely accepted terms "stem cell therapy" and "regenerative therapy" used frequently in scientific papers and media might be an oversimplification because neither true cardiac regeneration nor association of the improved myocardial function with the "stemness" of injected cells have been convincingly demonstrated in humans. In fact, most of clinical trials used heterogenous population of cells including only small fraction of SPC. In addition, functional and sometimes structural improvement of cardiac tissue showed in the trials may be attributed to many other factors than regeneration, which is so far impossible to prove in humans. Better

W. Wojakowski (✉) · A. Czekaj · M. Tendera
Third Division of Cardiology, Medical University of Silesia, Ziolowa 45,
40–635 Katowice, Poland
e-mail: wojtek.wojakowski@gmail.com

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,*
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_5,

understanding of these facts, especially by media and patients seeking help will certainly get the expectations realistic and help to evaluate the effectiveness in a balanced manner as for any other type of innovative therapy. This chapter will discuss some mechanistic aspects of SPC therapy followed by methods of cell procurement and application, as well as a summary of outcomes of the clinical trials.

5.2 Types of SPC for CV Therapies

For practical reasons, such as the limited number of SPC and difficulties with their isolation, the majority of cell therapy studies used unselected BM-derived mononuclear cells (MNC), and so far the clinical experience with these cells is most comprehensive. MNC is a heterogeneous population consisting of hematopoietic stem cells (HSC), endothelial progenitor cells (EPC), mesenchymal stem cells (MSC), and more mature lineages. Figure 5.1 summarized the types and sources of stem cells for CV repair. The separation techniques based on Ficoll-gradient centrifugation, Lymphoprep protocol, or gelatin-polysuccinate density gradient sedimentation were previously published [5–7]. Although the studies used similar population of MNC, the differences in the protocols were implicated as causative factors of different outcomes, in particular between Reinfusion of Enriched Progenitor cells and Infarct Remodeling in Acute MI (REPAIR-AMI) and Autologous Stem Cell Transplantation in Acute Myocardial Infarction (ASTAMI) studies. Direct comparison of the protocols showed that cells isolated according to the REPAIR-AMI protocol had a significant advantage because it produced higher MNC, HSC, and MSC yield with significantly better colony-forming unit, stromal cell-derived factor-1 (SDF-1)-induced migration capacity, expression of CXCR4, and higher angiogenic potential when compared with Lymphoprep protocol. Likewise, storage of BM-MNC had an impact on cell function and possibly on the outcomes [8]. It is unknown if this mechanism is also causative in other RCTs showing lack of Bone Marrow Cells (BMC) effects (e.g., Swiss Multicenter Intracoronary Stem Cells Study in AMI, SWISS-AMI) which did not use the ASTAMI protocol. Another essential factor is the use of heparin during harvesting and infusion of BMC. It was shown that heparin, but not another anticoagulant bivalirudin, in clinically relevant concentrations attenuates SDF-1/CXCR4 signaling leading to impaired migration capacity and homing, possibly by direct binding to SDF-1 and CXCR4. Since the SDF-1/CXCR4 axis is a key factor in SPC trafficking to the heart, this finding might affect the future protocols of BMC isolation [9]. These data, however, remain an assumption since no direct comparison of protocols with and without heparin with respect to the clinical efficacy are available. BOne MarrOw Transfer to Enhance ST-elevation Infarct Regeneration-2 (BOOST-2) will show whether separation of MNC based on gelatin-polysuccinate sedimentation might have an impact on the clinical outcome. The practical message from previous studies has been implemented in ongoing large-scale outcome trial (The Effect of Intracoronary Reinfusion of Bone Marrow-derived Mononuclear Cells on All Cause Mortality in Acute Myocardial

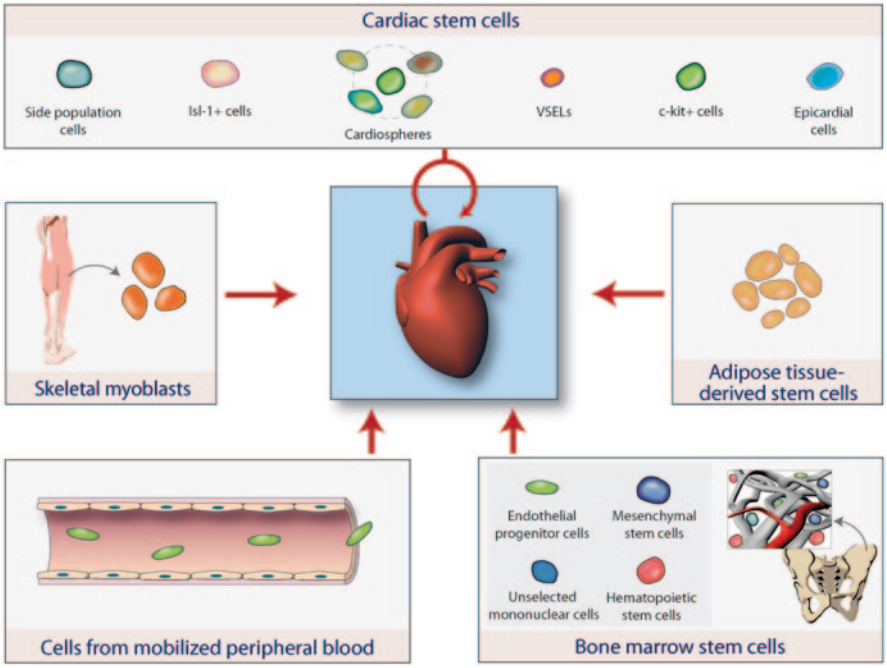

Fig 5.1 Types and sources of stem cells for CV repair

Infarction (BAMI)) in which the most successful protocol of REPAIR-AMI and bivalirudin are used for BMC harvest and transplantation. Other populations of autologous BM cells are selected CD34+, CD133+, and CD34+CXCR4+ cells. Such subpopulations of cells isolated by either immunomagnetic selection or other proprietary protocols were used in a limited number of patients. In Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction Trial (REGENT) trial, CD34+CXCR4+ cells were compared to MNC and standard of care. Interestingly, the recovery of the left ventricle ejection fraction (LVEF) was similar for selected cells and MNC despite the fact that the number of CD34+CXCR4+ cells was almost 100-times smaller than unselected MNC [10]. Theoretically, the use of subpopulation of selected cells might improve the myocardial engraftment possibly because there would be no competition for chemokine receptors with other mature cell [11]. On the other hand, this effect might be offset by depletion of macrophages essential for myocardial repair after MI [12]. So far, there are no data showing convincingly that a particular subtype of autologous cells is superior to MNC. Recent meta-analysis of Delewi et al. included 16 MI trials of which 15 used MNC and only one selected CD34+CXCR4+ cells [13].

More recently, BM-derived MSC emerged as a promising population of nonhematopoietic cells for CV studies. It is mainly due to their capacity for immunomodulatory and anti-inflammatory effects as well as a more primitive phenotype and

broader differentiation capacity than HSC. MSCs are plastic-adherent cells negative for CD45, CD34, CD14, HLA-DR, CD11b, CD79a, or CD19 markers, express CD105, CD90, and Stro-1 and in vitro differentiate to osteoblasts, adipocytes, and chondroblasts [14–16]. Most importantly, due to their immune-privileged status MSC can be implanted in an allogenic approach. This may overcome the problems of cellular senescence and dysfunction observed in elderly patients with comorbidities like diabetes and chronic renal failure. Importantly, MSC can be harvested from a healthy donor, expanded and used as "off-the shelf" therapy, which is particularly useful in MI patients [14]. Not only BM but also adipose tissue is an easy accessible and safe source of MSC. The protocols were published and automated systems are commercially available (Celution, Cytori Therapeutics) for adipose-derived MSC. Data from relatively small studies are encouraging showing safety and efficacy of MSC in chronic myocardial ischemia and MI. One of the most promising pathways of clinical research with MSC is use of cardiopoiesis-guided cells which allowed predifferentiation of autologous MSC using a "cocktail" of growth factors for individual patients prior to implantation. Such approach was evaluated in C-Cure trial with promising results on safety and feasibility and currently ongoing Congestive Heart Failure cArdiopoietic Regenerative Therapy-1 (CHART-1) trial will further evaluate the clinical effectiveness of cardiopoiesis-guided MCS (C3BSCQR-1) [17].

Two recent clinical studies showed safety and feasibility of cardiac stem cells (CSCs) or cardiosphere-derived cells (CDC) in patients with ischemic cardiomyopathy (ICM). These cells were isolated during cardiac surgery or by endocardial biopsy and expanded according to previously published protocols. CSC is a homogeneous population of cells defined as either C-kit+cells, while CDC is heterogeneous populations of cells displaying cardiac, endothelial, and MSC phenotypes. So far, phase I studies (Stem Cell Infusion in Patients with Ischemic CardiOmyopathy (SCIPIO) and CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction (CADUCEUS)) showed promising results in terms of surrogate endpoints although this needs confirmation in larger RCTs [18, 19].

Skeletal myoblasts were isolated from the muscle biopsy and used in early clinical studies. Their use was, however, hampered by proarrhythmic effects, requiring implantation of implantable cardioverter-defibrillator (ICD) in subsequent trials. As shown in preclinical studies, skeletal myoblasts form foci of the striated skeletal muscle without proper electromechanical coupling with the recipients myocardium. Early nonrandomized studies showed promising results, but this was not confirmed in larger RCTs (The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC), Safety and Effects of Implanted (Autologous) Skeletal Myoblasts (MyoCell) Using an Injection Catheter (SEISMIC)) and interest in skeletal myoblasts has waned [20, 21].

Populations of cells with most potent differentiation capacity such as embryonic stem cells (ESCs) and induced-pluripotent stem cells (iPSC) were regarded as an interesting option for CV repair strategies, however, so far, there is no clinical experience due to ethical, legal, and biological (tumor formation, immunogenicity) reasons with the former and unproved safety and risk of carcinogenesis with the

latter population of cells [22, 23]. It is unlikely that ESC will be used for clinical trials. Clinical application of iPSC must be postponed until rigorous experiment proved their long-term safety.

5.3 Mechanisms of SPC-Dependent Tissue Repair

The concept of cell therapy has been based upon the fundamental properties of stem cells (self-renewal, differentiation potential) and initially aimed at the generation of new cardiac myocytes. Subsequent preclinical studies showed that other factors are more likely to be involved because data showing the formation of new cardiomyocytes have been challenged [24, 25]. Also in studies showing transdifferentiation of exogenous cells into cardiac myocytes, this was extremely rare event of unknown clinical significance. So far, several other mechanisms of cardiac repair after cell therapy have been postulated primarily mediated by paracrine effects (antiapoptotic, proangiogenic, activation of resident CSC, antifibrotic) as well as differentiation into blood vessels [26, 27]. Paracrine mechanisms seem very likely because the SPC cells were shown to secrete a variety of cytokines, chemokines and growth factors and more recently microvesicles (MV) containing micro-ribonucleic acids (RNAs). By attenuation of the inflammatory process, activation of resident CSC and induction of angiogenesis the cells or cell-derived MV might trigger innate reparatory mechanisms [28, 29]. Such a mechanism would explain the persistent effects of cell therapy despite their low engraftment rate (2–10%) and rapid clearance [30]. It remains unknown which mechanism contributes the largest extent in the myocardial repair; however, it seems likely that it is dependent on the clinical setting and local environment. After cell therapy in acute MI, the anti-inflammatory and antiapoptotic effects would be most significant contributors to myocardial repair, and in chronic myocardial ischemia the angiogenic properties would be most advantageous [23]. Figure 5.2 shows the theoretical mechanisms of cell therapy.

5.4 Key Modulators of SPC Functional Capacity

Use of autologous cells has very serious limitation, which is their biological quality. Cells isolated from patients with multiple CV risk factors, such as diabetes, lipid disorders, or other comorbidities (renal failure) have impaired migratory potential, differentiation, and colony forming capacity. They also display features of cellular senescence such as telomere attrition [31–33]. Cellular senescence related to aging and other conditions such as diabetes can adversely affect the paracrine function of the SPC [15, 16]. For cells prepared for transplantation also external factors, such as use of heparin, contamination with erythrocytes, storage conditions (pH, duration) can have a profound impact of cell quality. In meta-analyses, younger age is one of the predictive factors for positive effect of cell therapy. Presumably it is related to

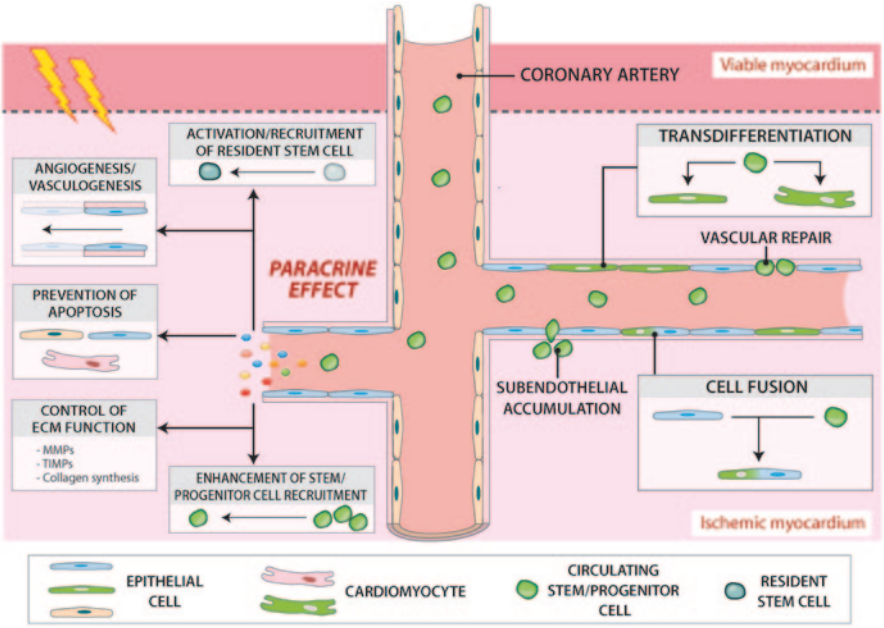

Fig 5.2 Mechanisms of cell therapy

better BMC function in younger patients [13]. In theory, use of allogenic cells, such as MSC from healthy young donors, would overcome this obstacle.

5.5 Methods of Cell Delivery

Detailed description of the methods of percutaneous and surgical cell delivery was published elsewhere and is beyond the scope of the article [34, 35]. In the majority of clinical trials, two routes were used, either intracoronary infusion (stop-flow technique) or intramyocardial delivery (transendocardial or epicardial). The intracoronary cell delivery route was most commonly used in acute MI and heart failure studies, and intramyocardial delivery was preferred in case of refractory angina. In general, the myocardial retention of cells is relatively small, both in acute MI and chronic myocardial ischemia ranging between 2–10% depending on timing and methodology of measurement. Recent head-to-head randomized comparison showed that intramyocardial delivery might significantly improve CD34+ cell retention in nonischemic cardiomyopathy (NICM) [11, 30]. The choice of the delivery method depends on the clinical scenario. The rationale of the intracoronary in acute MI is that the infarct related artery supplies the area of reperfused necrotic scar and stunned, potentially salvageable peri-infarct zone. Another, practical reason is that after primary percutaneous coronary intervention the infarct-related

artery is stented and the occlusion of the artery with a balloon within the stent is safe and does not cause vessel injury [3]. Thirdly, animal studies showed that there is upregulated expression of chemokines and chemokine receptors in the infarct border zone which in turn facilitate the SPC homing and retention [36]. In fact, cells delivered by intracoronary route engraft within infarct border zone and within infarct itself (see Fig. 5.3) [30].

Transendocardial route using the NOGA system (Biologics Delivery Systems, USA) allows targeting of SPC to the viable ischemic myocardium. Such approach is based on the electromechanical maps to access the areas of the myocardium supplied by the diffusely diseased or occluded vessel which will not be accessible by intracoronary route. Such an approach is safe and feasible, and transednocardial cell therapy was used extensively in Europe and the USA. Practical observation from these trials is that the SPC therapy should be targeted to the viable segments of the myocardium rather that scar tissue.

Figure 5.4 shows intracoronary and intramyocardial delivery of cells.

Recently other intramyocardial delivery systems such as C-Cath (Cardio3 Biosciences) and Helix (Biocardia) were developed and are under clinical evaluation. Other delivery routes such as transvenous retrograde delivery through the coronary sinus or noncatheter-based methods were used in a small number of patients and at this time it is difficult to assess their effectiveness. In general, cell delivery procedures are safe, relatively simple, and feasible.

5.6 Summary of Clinical Data

Cell therapy was evaluated in clinical trial in patients with acute MI, chronic myocardial ischemia (no-option angina), and heart failure due to ischemic (ICM) and nonischemic cardiomyopathy (NICM).

5.6.1 Safety

As with any innovative technology at the early stage of development, the verification of safety is the primary goal. So far extended follow-up is available for early trials in patients with acute MI and heart failure treated with BMC. Both nonrandomized small trials and RCT showed excellent safety profile with no excess of adverse events (AE) up to 10 years after the therapy [37]. Long-term follow-up of REPAIR-AMI trial confirmed safety and no increased risk of neoplasm and arrhythmia up to 5 years [38]. Similarly, 5-year follow-up of the REGENT trial showed favorable safety (Tendera et al., AHA 2013). Only safety issues were reported in the early trials with skeletal myoblasts where arrhythmia has been consistently observed in the actively treated patients [23]. Conversely data on the interrogation of ICD devices, that the risk of arrhythmia was in fact reduced in heart failure patients

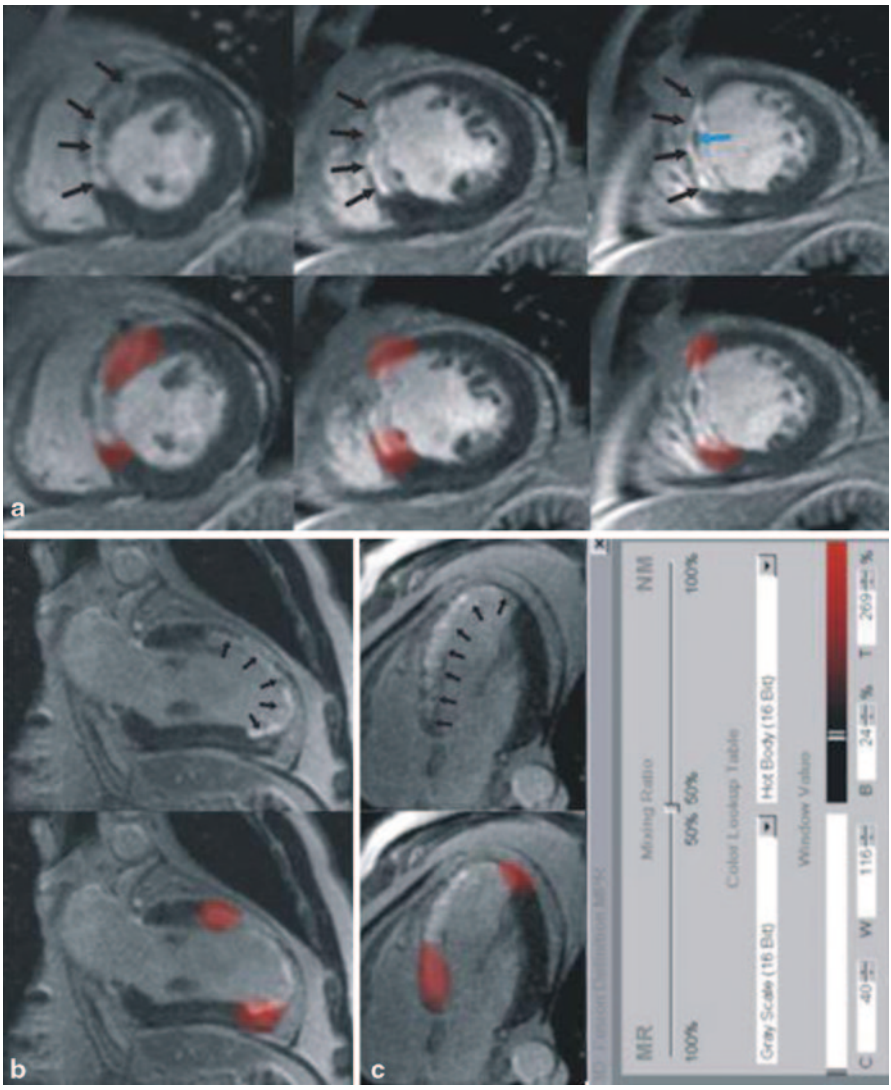

Fig 5.3 Myocardial homing of radiolabelled CD34+ cells after intracoronary injection in patient after acute MI. Fusion of SPECT and magnetic resonance imaging showing that cells engraft within the infarct border zone, but not the infarct area. Myocardial cell uptake is determined by infarct size rather than left ventricle ejection fraction

treated with BMC [39]. In addition, BMC therapy does not increase the risk for progression of coronary atherosclerosis and in-stent restenosis [40]. It is still too early to assess the long-term safety of CSC and CDC, as well as modified stem cells.

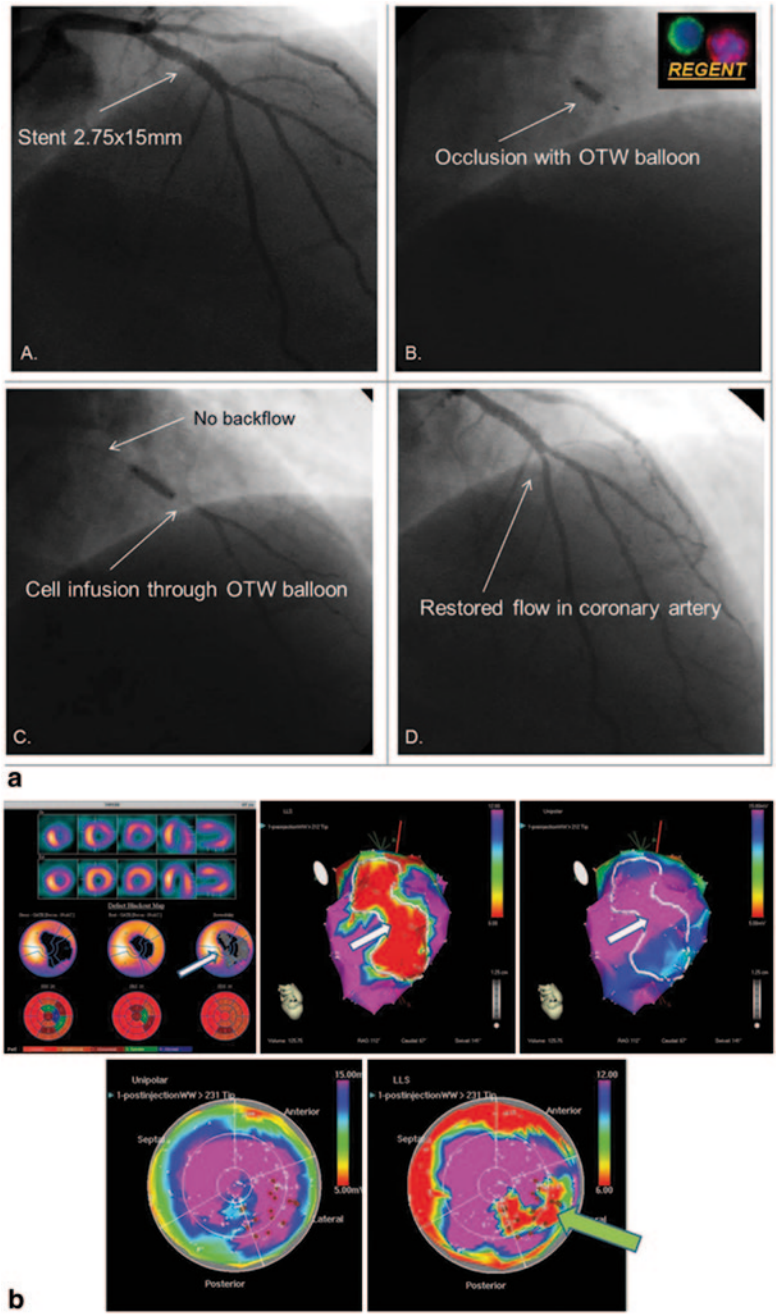

Fig 5.4 Examples of intracoronary and intramyocardial delivery of cells. **a** Intracoronary infusion through the over-the-wire (OTW) angioplasty balloon 6 days after acute MI in patients enrolled in REGENT trial. **b** Transendocardial delivery in areas of hibernating myocardium visualized in NOGA electromechanical map in patients with chronic myocardial ischemia (no-option refractory angina). Hibernating myocardium areas in NOGA map correspond to areas of reversible perfusion deficit in adenosine stress test SPECT (*white arrows*). Injection sites marked by *brown dots* (*green arrow*)

5.6.2 Surrogate End-Points

Effects of BM and peripheral blood-derived SPC cells on LVEF and remodeling has been primary end-point in numerous clinical trials. These parameters were evaluated by left ventricle (LV)-angiography, magnetic resonance imaging (MRI), or scintigraphy (SPECT). Metaanalyses and systematic reviews of nonrandomized studies and RCA show consistently modest improvement of LVEF and reduction of LV remodeling in patients with acute MI [13, 37, 41–45] as well as cardiomyopathy [46] (see Fig. 5.5). Similarly, there was a trend to reduction of the size of the infarct scar although this parameter was reported in the minority of trials [13, 37, 41–43]. The positive results obtained with intracoronary delivery of BMC were not replicated for mobilization of cells with granulocyte colony-stimulating factor (G-CSF) without intracoronary injection [45, 47]. Another surrogate end-points were evaluated in patients with refractory angina [number of heart segments with perfusion deficit, LVEF, angina Canadian Cardiovascular Society (CCS) class, time-to-ischemia in exercise test, quality of life] and cardiomyopathy [LVEF, New York Heart Association (NYHA) class, N-terminal pro-brain natriuretic peptide (NT-proBNP) levels, quality of life]. Data from meta-analyses showed favorable impact of cell therapy in patients with refractory angina, mainly expressed as an increased exercise capacity and reduction of CCS class and angina frequency [46, 48]. Meta-analyses have certain limitations (e.g., heterogeneity of studies included), but are highly regarded in a hierarchy of evidence in the era of EBM. It is reasonable to conclude that intracardiac BMC therapy leads to a moderate improvement of LV function and remodeling without increasing the risk of AE in patients with acute MI. There is also an improvement in functional capacity and symptoms as well as myocardial perfusion and LVEF in patient with chronic myocardial ischemia and refractory angina. In patients with ICM/NICM, cell therapy improves LVEF and symptoms.

5.6.3 Clinical End-Points

An ultimate goal of all innovative therapies is not only to improve symptoms and demonstrate efficacy with respect to surrogate end-points but also to improve the survival. So far, no study with SPC had a statistical power to evaluate the effects of cells on mortality, CV mortality, and MI, which is a common limitation for all small-sized clinical trials. Data from long-term follow-up of RCT investigating the clinical outcome are based on post-hoc analyses and should be interpreted as hypothesis-generating. Long-term follow-up of REPAIR-AMI showed better event free survival in terms of composite end-points of death, MI, or need for revascularization and death, MI, hospitalization for heart failure in patients treated with BMC in comparison to placebo [38, 49]. Recent meta-analysis had consistent results showing reduction of all-cause mortality, CV mortality, recurrent MI, and stent thrombosis in patients treated with SPC [37]. These findings should be confirmed in large RCT, such as The Effect of Intracoronary Reinfusion of Bone Marrow-derived

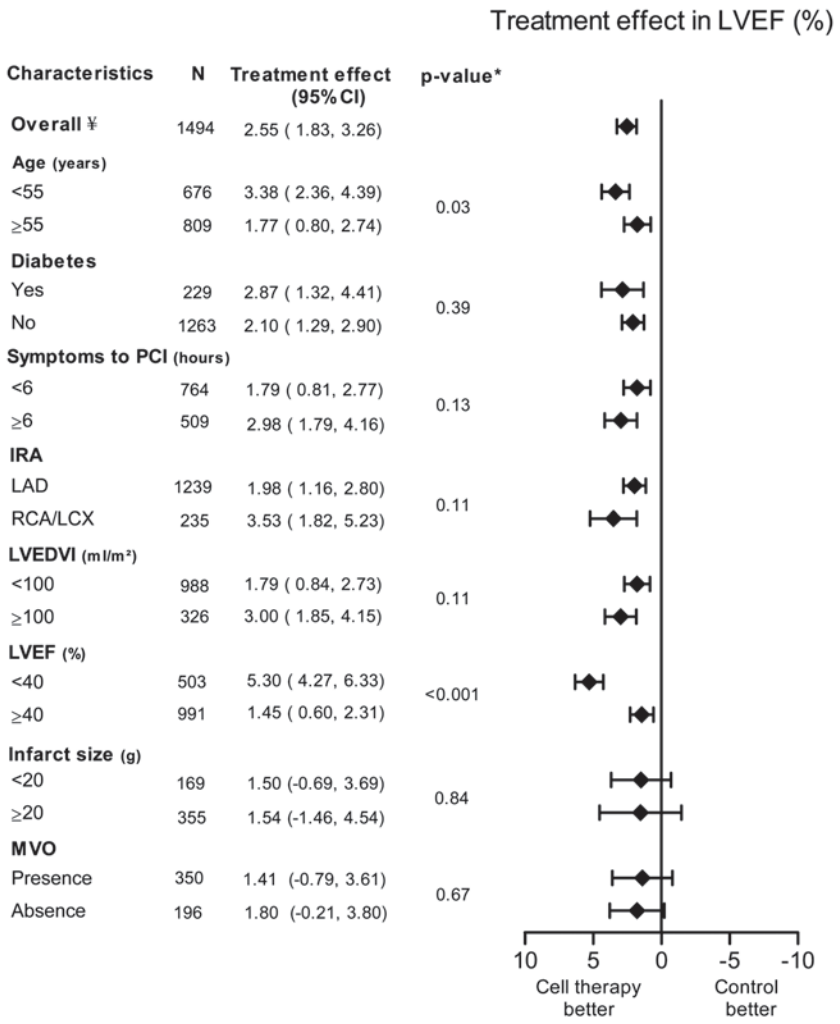

Fig 5.5 Metaanalysis of clinical trials with bone marrow cells in patients with acute myocardial infarction showing improvement of left ventricle ejection fraction (*LVEF*) and subgroup analysis indicating that younger patients and those with LVEF below 40% have better improvement of LVEF. *CI* confidence interval, *IRA* infarct-related artery, *LAD* left anterior descending artery, *LCX* left circumflex artery, *LVEDVI* left ventricular end-diastolic volume index, *MVO* microvascular obstruction, *PCI* percutaneous coronary intervention, *RCA* right coronary artery. *Asterisk* P-value for subgroup differences. ([13] eurheartj.eht372, reprinted with permission)

Mononuclear Cells (BM-MNC) on All-Cause Mortality in Acute Myocardial Infarction (BAMI) which is underway in Europe (www.clinicaltrials.gov). BAMI is a multicenter, randomized open-label, controlled, parallel-group phase III study with primary end-point of all-cause mortality. Three thousand patients with acute MI will be randomly assigned to intracoronary infusion of autologous BM-MNC after

successful reperfusion or to the standard of care. Major inclusion criterion is the reduction of LVEF ≤45 % which is consistent with earlier observations that only patients with baseline impairment of LV function have improved LFEF recovery [10, 50].

Conclusions

Cell therapy has been investigated in the clinical setting for more than a decade and proved to be safe. There are consistent data suggesting its effectiveness with regard to surrogate end-points. Preclinical data showed that paracrine effects rather than bona fide regeneration account for effects of SPC therapy. Cell therapy should be further evaluated according to EBM as any other therapeutic innovation. There is a continuous progress with new SPC technologies and ongoing clinical trials which are likely to have positive impact on the field. Despite the favorable outcomes of early clinical trials, cell therapy should be re-evaluated within the context of improved effectiveness of pharmacological therapy, progress of the interventional cardiology and cardiac surgery and significant reduction of risk in patients with acute MI and heart failure.

Acknowledgments This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 278967 and Operational Programme Innovative Medicine "Innowative use of stem cells in cardiology" POIG 01.01.02.00–109/09.
We are grateful to Dr. Tomasz Jadczyk for his help in preparation of the figures.

References

1. Orlic D, Kajstura J, Chimenti S et al (2001) Bone marrow cells regenerate infarcted myocardium. Nature 410:701–705
2. Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P (2001) Transplanted adult bone marrow cells repair myocardial infarcts in mice. Ann NY Acad Sci 938:221–229 (discussion 229–30)
3. Strauer BE, Brehm M, Zeus T et al (2002) Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 106:1913–1918
4. Strauer BE, Brehm M, Zeus T et al (2001) Intracoronary, human autologous stem cell transplantation for myocardial regeneration following myocardial infarction. Dtsch Med Wochenschr 126:932–938
5. Schachinger V, Assmus B, Britten MB et al (2004) Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI trial. J Am Coll Cardiol 44:1690–1699
6. Lunde K, Solheim S, Aakhus S et al (2006) Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. New Engl J Med 355:1199–1209
7. Wollert KC, Meyer GP, Lotz J et al (2004) Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. Lancet 364:141–148

8. Seeger FH, Tonn T, Krzossok N, Zeiher AM, Dimmeler S (2007) Cell isolation procedures matter: a comparison of different isolation protocols of bone marrow mononuclear cells used for cell therapy in patients with acute myocardial infarction. Eur Heart J 28:766–772
9. Seeger FH, Rasper T, Fischer A et al (2012) Heparin disrupts the CXCR4/SDF-1 axis and impairs the functional capacity of bone marrow-derived mononuclear cells used for cardiovascular repair. Circ Res 111:854–862
10. Tendera M, Wojakowski W, Ruzyllo W et al (2009) Intracoronary infusion of bone marrow-derived selected CD34+CXCR4+cells and non-selected mononuclear cells in patients with acute STEMI and reduced left ventricular ejection fraction: results of randomized, multicentre myocardial regeneration by intracoronary infusion of selected population of stem cells in acute myocardial infarction (REGENT) trial. Eur Heart J 30:1313–1321
11. Vrtovec B, Poglajen G, Lezaic L et al (2013) Comparison of transendocardial and intracoronary CD34+cell transplantation in patients with nonischemic dilated cardiomyopathy. Circulation 128:S42–S49
12. Ben-Mordechai T, Holbova R, Landa-Rouben N et al (2013) Macrophage subpopulations are essential for infarct repair with and without stem cell therapy. J Am Coll Cardiol 62:1890–1901
13. Delewi R, Hirsch A, Tijssen JG et al (2013) Impact of intracoronary bone marrow cell therapy on left ventricular function in the setting of ST-segment elevation myocardial infarction: a collaborative meta-analysis. Eur Heart J 15:989–998
14. Williams AR, Hare JM (2011) Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. Circ Res 109:923–940
15. Beltrami AP, Cesselli D, Beltrami CA (2012) Stem cell senescence and regenerative paradigms. Clin Pharmacol Ther 91:21–29
16. Cesselli D, Beltrami AP, D'Aurizio F et al (2011) Effects of age and heart failure on human cardiac stem cell function. Am J Pathol 179:349–366
17. Bartunek J, Behfar A, Dolatabadi D et al (2013) Cardiopoietic stem cell therapy in heart failure: the C-CURE (Cardiopoietic stem cell therapy in heart failure) multicenter randomized trial with lineage-specified biologics. J Am Coll Cardiol 61:2329–2338
18. Bolli R, Tang XL, Sanganalmath SK et al (2013) Intracoronary delivery of autologous cardiac stem cells improves cardiac function in a porcine model of chronic ischemic cardiomyopathy. Circulation 128:122–131
19. Makkar RR, Smith RR, Cheng K et al (2012) Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. Lancet 379:895–904
20. Menasche P, Alfieri O, Janssens S et al (2008) The myoblast autologous grafting in ischemic cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. Circulation 117:1189–1200
21. Duckers HJ, Houtgraaf J, Hehrlein C et al (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 6:805–812 (In collaboration with the Working Group on Interventional Cardiology of the European Society of Cardiology)
22. Oh Y, Wei H, Ma D, Sun X, Liew R (2012) Clinical applications of patient-specific induced pluripotent stem cells in cardiovascular medicine. Heart 98:443–449
23. Sanganalmath SK, Bolli R (2013) Cell therapy for heart failure: a comprehensive overview of experimental and clinical studies, current challenges, and future directions. Circ Res 113:810–834
24. Murry CE, Soonpaa MH, Reinecke H et al (2004) Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 428:664–668
25. Shi D, Reinecke H, Murry CE, Torok-Storb B (2004) Myogenic fusion of human bone marrow stromal cells, but not hematopoietic cells. Blood 104:290–294
26. Tongers J, Losordo DW, Landmesser U (2011) Stem and progenitor cell-based therapy in ischaemic heart disease: promise, uncertainties, and challenges. Eur Heart J 32:1197–1206

27. Burchfield JS, Dimmeler S (2008) Role of paracrine factors in stem and progenitor cell mediated cardiac repair and tissue fibrosis. Fibrogenesis Tissue Repair 1:4
28. Gnecchi M, Zhang Z, Ni A, Dzau VJ (2008) Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 103:1204–1219
29. Ratajczak MZ, Kucia M, Jadczyk T et al (2012) Pivotal role of paracrine effects in stem cell therapies in regenerative medicine: can we translate stem cell-secreted paracrine factors and microvesicles into better therapeutic strategies? Leukemia 26:1166–1173
30. Musialek P, Tekieli L, Kostkiewicz M et al (2013) Infarct size determines myocardial uptake of CD34+cells in the peri-infarct zone: results from a study of (99 m)Tc-extametazime-labeled cell visualization integrated with cardiac magnetic resonance infarct imaging. Circ Cardiovas Imaging 6:320–328
31. Hill JM, Zalos G, Halcox JP et al (2003) Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. New Engl J Med 348:593–600
32. Werner N, Nickenig G (2006) Influence of cardiovascular risk factors on endothelial progenitor cells: limitations for therapy? Arterioscler Thromb Vasc Biol 26:257–266
33. Vasa M, Fichtlscherer S, Aicher A et al (2001) Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circulation Res 89:E1–E7
34. Campbell NG, Suzuki K (2012) Cell delivery routes for stem cell therapy to the heart: current and future approaches. J Cardiovasc Transl Res 5:713–726
35. Dib N, Menasche P, Bartunek JJ et al (2010) Recommendations for successful training on methods of delivery of biologics for cardiac regeneration: a report of the international society for cardiovascular translational research. JACC Cardiovasc Interv 3:265–27.
36. Kucia M, Dawn B, Hunt G et al (2004) Cells expressing early cardiac markers reside in the bone marrow and are mobilized into the peripheral blood after myocardial infarction. Circ Res 95:1191–1199
37. Jeevanantham V, Butler M, Saad A, Abdel-Latif A, Zuba-Surma EK, Dawn B (2012) Adult bone marrow cell therapy improves survival and induces long-term improvement in cardiac parameters: a systematic review and meta-analysis. Circulation 126:551–568
38. Assmus B, Rolf A, Erbs S et al (2010) Clinical outcome 2 years after intracoronary administration of bone marrow-derived progenitor cells in acute myocardial infarction. Circ Heart Fail 3:89–96
39. Leistner DM, Schmitt J, Palm S et al (2011) Intracoronary administration of bone marrow-derived mononuclear cells and arrhythmic events in patients with chronic heart failure. Eur Heart J 32:485–491
40. Assmus B, Walter DH, Lehmann R et al (2006) Intracoronary infusion of progenitor cells is not associated with aggravated restenosis development or atherosclerotic disease progression in patients with acute myocardial infarction. Eur Heart J 27:2989–2995
41. Abdel-Latif A, Bolli R, Tleyjeh IM et al (2007) Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. Arch Intern Med 167:989–997
42. Lipinski M, Biondi-Zoccai G, Abbate A et al (2007) Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials. J Am Coll Cardiol 50:1761–1767
43. Martin-Rendon E, Brunskill S, Doree C et al (2008) Stem cell treatment for acute myocardial infarction. Cochrane Database Syst Rev Oct 8;(4):CD006536
44. Clifford DM, Fisher SA, Brunskill SJ et al (2012) Stem cell treatment for acute myocardial infarction. Cochrane Database Syst Rev 2:CD006536. doi: 10.1002/14651858.CD006536.pub3
45. Zimmet H, Porapakkham P, Porapakkham P et al (2012) Short- and long-term outcomes of intracoronary and endogenously mobilized bone marrow stem cells in the treatment of ST-segment elevation myocardial infarction: a meta-analysis of randomized control trials. Eur J Heart Fail 14:91–105

46. Fisher SA, Doree C, Brunskill SJ, Mathur A, Martin-Rendon E (2013) Bone marrow stem cell treatment for ischemic heart disease in patients with no option of revascularization: a systematic review and meta-analysis. PloS ONE 8:e64669
47. Moazzami K, Roohi A, Moazzami B (2013) Granulocyte colony stimulating factor therapy for acute myocardial infarction. Cochrane Database Syst Rev 5:CD008844
48. Li N, Yang YJ, Zhang Q, Jin C, Wang H, Qian HY (2013) Stem cell therapy is a promising tool for refractory angina: a meta-analysis of randomized controlled trials. Can J Cardiol 29:908–914
49. Schachinger V, Erbs S, Elsasser A et al (2006) Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial. Eur Heart J 27:2775–2783
50. Schachinger V, Erbs S, Elsasser A et al (2006) Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. New Engl J Med 355:1210–1221

Chapter 6
Mechanisms Regulating Trafficking of Stem Cells in Ischemic Heart Disease

Yuri Klyachkin and Ahmed Abdel-Latif

6.1 Burden of Ischemic Heart Disease

Ischemic heart disease (IHD), which includes heart failure induced by myocardial infarction (MI) is the single most prevalent cause of morbidity and mortality worldwide. Interestingly, US IHD death rates have declined from 1997 to 2007 by 27.8%, yet the burden of IHD or myocardial ischemia remains high. Currently, IHD caused one of every six deaths in the USA, and despite the significant advancements in medical and revascularization therapies, the prognosis of millions of patients with IHD remains poor [1]. Approximately 785,000 Americans will have a new IHD event every year, and up to 470,000 will have a recurrent episode. Alarmingly, every 25 s an American will suffer from a MI brought on by IHD, resulting in one death per minute [1]. IHD is brought on by interruption of oxygenated blood supply to the heart muscle due to an occlusion of a coronary artery. The resulting ischemia causes myocardial cell death which often results in extensive tissue damage. Currently, heart transplantation is the only viable therapy to replace the infarcted myocardium but it has numerous glaring shortcomings such as limited availability of donors, post-procedural complications, side effects of long-term immunosuppressive therapies, and overall suboptimal patient prognosis. Until recently, the notion that MI-damaged myocardium could regenerate was non-existent. However, recent data from animal and human studies demonstrated the capability of adults to renew their cardiac cells with both resident cardiac stem cells and bone marrow (BM)-derived progenitor stem cell populations which propelled a new wave of translational research. The field of understanding the underpinnings of stem cell mobilization to sites of injury is rapidly growing. This chapter will examine breakthroughs in our understanding of the mechanisms of stem cell mobilization in the setting of IHD and future therapeutic strategies.

A. Abdel-Latif (✉) · Y. Klyachkin
Department of Cardiology, University of Kentucky, Lexington, KY, USA
e-mail: abdel-latif@uky.edu

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,*
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_6,

6.2 The Role of Bone Marrow (BM)-Derived Cells in Continuous Renewal of Cardiomyocytes

Up to 10 years ago, it was believed that the human heart is not capable of self-renewal and therefore the MI-damaged myocardium could not be regenerated. However, this notion was challenged and refuted by multiple groundbreaking animal and human studies. Early evidence for myocardial regeneration was presented in a study investigating the chimerism of sex-mismatched transplanted heart in humans by demonstrating active renewal of all three major cell lines in human hearts. The number of recipient-originated cardiomyocytes, vascular smooth muscle cells, and endothelial cells increased significantly in hearts from female donors that were transplanted into male recipients. It was demonstrated that these primitive cells, expressed stem cell antigens including c-kit, multidrug resistance protein 1 (MDR1), and Stem Cell Antigen 1 (Sca-1). A fraction of these cells were Y-chromosome-positive, suggesting that these cells originated from the host. Most importantly, specific and poorly understood pathways resulted in the loss of stem-cell markers, active proliferation, and acquisition of the mature phenotype followed by cell colonization and de novo formation of myocytes, coronary arterioles, and capillaries [2]. However, the authors could not identify the source of these newly formed cardiac cells and to address this question, a follow-up investigation analyzed hearts of patients who have undergone gender-mismatched bone marrow (BM) transplantation. The key findings suggested that BM acts as a source for stem/progenitor cells contributing to cardiomyocyte renewal after daily cell death and wear-and-tear, and is responsible for at least part of the cell chimerism observed in other studies [3]. Together, these data established human BM as a reservoir of BM stem/progenitor cells (BMSPCs) that contribute to de novo cardiomyocyte formation and possibly repair. Further studies suggest that the magnitude of this phenomenon is quite significant demonstrating that BMSPCs are responsible for replacing at least half of the adult cardiomyocytes during normal physiological aging [4]. Further studies demonstrated that the human adult heart is capable of replacing its entire population of cardiomyocytes, endothelial cells, and fibroblasts 6–8 times during normal life span and under physiological conditions; this chimerism is age dependent and is also influenced by pathological conditions such as heart failure [5] and ischemic injury [6].

6.3 BM-Derived Pluripotent Stem Cells are Mobilized in the Peripheral Circulation Following Myocardial Ischemia in Animal Models and Humans

Under steady state conditions, BMSPCs are able to escape from the BM in response to chemotactic gradients and can be readily detected in the peripheral blood (PB, [7]). However, numerous factors have been shown to accelerate BMSPC mobilization including strenuous exercise [8], tissue or organ injury (including ischemic

cardiac events; [9, 10]), and administration of pharmacological agents [11, 12]. The gradient of chemotactic stromal derived factor-1 (SDF-1) was thought to be the major determining factor of BMSPCs' destination [13, 14]. Since many of BMSPCs express the SDF-1 receptor-CXCR4 and SDF-1 is expressed by osteoblasts and fibroblasts in the BM microenvironment, it is conclusive that the SDF-1-CXCR4 interaction confines BMSPCs to the BM niches [14]. BMSPCs also express very late antigen-4 (VLA-4 or $\alpha_4\beta_1$-integrin) which interacts with its ligand (vascular adhesion molecule-1 or CD106), further contributing to BMSPC retention in the BM [15–17].

Myocardial ischemia, particularly large myocardial infarction (MI), produce multiple stimuli including various chemokines, cytokines, kinins, bioactive lipids, and members of the activated complement cascade that lead to the mobilization and subsequent homing of BMSPCs. Indeed, several reports have confirmed that mobilization of stem cells originating from the BM occurs in response to myocardial ischemic injury [18–23] and heart failure [24]. Similar observations were noted in patients with acute neurological ischemia [25] and patients with extensive skin burn [26].

The first evidence for the mobilization of CD34+ mononuclear cells in Acute Myocardial Infarction (AMI) was demonstrated by Shintani et al. [23]. The authors demonstrated successful in vitro differentiation of circulating BMSPCs into endothelial cells that expressed CD31, VE-cadherin and the kinase insert domain receptor (KDR) [23]. Leone et al. demonstrated that the levels of circulating CD34+ cells in the setting of AMI were higher when compared to patients with mild chronic stable angina and healthy controls. The magnitude of CD34+ cell mobilization correlated with the recovery of regional and global left ventricular (LV) function recovery as well as other functional LV parameters [19]. Similarly, Wojakowski et al. demonstrated the mobilization of multiple BMSPC populations in patients with AMI and found significant correlation between the number of circulating CD34+ cells and plasma SDF-1 levels [27]. In their following publication, the authors demonstrated the correlation between circulating BMSPCs and ejection fraction at baseline and lower brain natriuretic peptide (BNP) levels [20]. Furthermore, the successful mobilization of BMSPCs correlates with the recovery of multiple parameters of LV function as assessed by the accurate and reproducible methods such as cardiac magnetic resonance imaging (MRI, CMR; [28]). Interestingly, the mobilization of BMSPCs is reduced by the successful revascularization of the culprit vessel in acute ST-elevation MI (STEMI; [29]). However, the majority of the above-mentioned studies have focused on the mobilization of partially committed stem cells such as hematopoietic stem/progenitor cells (HSPCs) and endothelial progenitor cells (EPCs).

We and others have demonstrated the mobilization of pluripotent stem cells (PSCs) including populations enriched in very small embryonic like stem cells (VSELs) in the setting of myocardial ischemia [30, 31]. The number of circulating VSELs was highest in patients with STEMI, particularly in the early phases following the injury, when compared to patients with lesser degrees of ischemia such as non-STEMI (NSTEMI) and those with chronic ischemic heart disease (IHD; [30]). The mobilization of PSCs appears to be related to the extent of myocardial ischemia

and the degree of myocardial damage. Moreover, the ability of patients to mobilize PSCs in the peripheral circulation in response to AMI decreases with age, reduced global LV ejection fraction (LVEF) and diabetes supporting the notion of an age/comorbidity related decline in the regenerative capacity [30, 31]. Indeed, animal models confirm the reduction of number as well as pluripotent features of BM-derived VSELs with age [32]. Similarly, studies have demonstrated the reduction of number as well as functional capacity of EPCs in diabetic patients [33].

The pluripotent features of mobilized VSELs, including the presence of octamer-binding transcription factor-4 (Oct4) and stage specific embryonic antigen-4 (SSEA-4), were confirmed both on the ribonucleic acid (RNA) and protein levels. Utilizing the capabilities of the ImageStream system, we demonstrated that circulating VSELs during AMI have very similar embryonic features similar to their BM and cord blood (CB) counterparts including the small size (7–8 μm), large nucleus, and high nuclear-to-cytoplasm ratio. Furthermore, circulating VSELs during myocardial ischemia express markers of early cardiac and endothelial progenitors that suggest that the mobilization is rather specific and that circulating VSELs are destined to aid in the myocardial regeneration following injury [18, 30, 31].

Stimuli responsible for the mobilization and homing of BMSPCs in the setting of myocardial ischemia show similarities and differences with those involved in hematopoietic stem cells (HSCs) homing to the BM. The role of SDF-1 and its receptor (CXCR4) axis in the retention HSPCs in BM is undisputed [34, 35]; however, its role in the mobilization and homing of BM-SPCs to a highly proteolytic microenvironment, such as the ischemic/infarcted myocardium, is somewhat less certain. Studies have demonstrated that multiple members of the metalloproteinases (MMP) family, such as MMP2, MMP9, and MMP13, are upreagulated in the myocardium following infarction [36]. The elevated levels of the MMPs contribute to the degradation of chemokines such as SDF-1 and the byproduct of this degradation acts as an inhibitor to the sole SDF-1 receptor, CXCR4 [37, 38]. In support of this hypothesis, Agrawal et al. demonstrated that the conditional deletion of CXCR4 in cardiomyocytes did not influence the recovery of LV function, reduce the scar size, or alter the homing of mesenchymal stem cells (MSCs) to the myocardium following MI [39]. Thus, there is growing evidence that other mechanisms, beside the SDF-1/CXCR4 axis, are contributing to the mobilization and homing of BMSPCs in myocardial ischemia and other tissue injury conditions [40, 41]. These data suggest an important interplay between the complement cascade, the immune system, cathelicidins, low levels of SDF-1, and sphingosine-1 phosphate (S1P) and other bioactive lipids in the mobilization and homing of HSPCs. Our data suggest that these complex interactions might be involved in the mobilization of BMSPCs in acute myocardial ischemia as well [42]. Clinically, pharmacological modulators of S1P receptors are already approved by the Food and Drug Administration (FDA) and can be utilized to enhance BMSPC mobilization in the setting of IHD. Similarly, modulation of the complement cascade can be also utilized in this process similar to their role in the mobilization of HSPCs.

Table 6.1 Diverse involvement of sphingolipids in cellular and cardiovascular function

Name	Cardiovascular function	References
Sphingosine-1 phosphate (S1P)	Cell adhesion	Fyrst et al. [82]
	BMSPC mobilization	Hasegawa et al. [83]
	Survival	Sattler et al. [84]
	Mitogenesis	Morita et al. [85]
	Vascular permeability	
	Immune cell trafficking	
	Angiogenesis	
	Cardioprotection	
Ceramide-1 phosphate (C1P)	Survival/DNA synthesis	Fyrst et al. [82]
	BMSPC mobilization and homing	Levi et al. [86]
	Inhibition of apoptosis	
	Inflammation	
	Eicosanoid synthesis	
	Mast cell degranulation	
	Neutrophil phagocytosis	
Lipoprotein A (LPA)	Dendritic cell chemotaxis	Morris et al. [87]
	Innate immune cell activation	Siess et al. [88]
	Vascular remodeling	
	Atherosclerosis	

BMSPC Bone marrow stem/progenitor cells, *DNA* Deoxyribonucleic acid

6.4 Sphingolipids and Stem Cell Signaling

Sphingolipids are a class of lipids consisting of a backbone composed of sphingoid bases and an amino alcohol sphingosine [43]. Initially, they were believed to be sheathing nerves, and the interest in their research remained confined to a small group of scientists. As the evidence for pathophysiological importance of sphingolipids grew, so did their research field. As of today, sphingolipids are shown to be involved in stimulation of cell proliferation, inhibition of apoptosis, and regulation of cell shape and cell motility (Table 6.1; [44–47]).

Sphingolipids, which are derived from ceramide (Fig. 6.1), are integral structural components of cell membranes. Ceramide can be deacylated to sphingosine which is then phosphorylated by sphingosine kinases (SPHK1 or SPHK2) to yield S1P (Fig. 6.1). Ceramide 1-phosphate (C1P) can be generated by phosphorylation of ceramide (N-acyl sphingosine) by ceramide kinase [48]. Both S1P and C1P have limited half-lives and their levels are kept in check by numerous enzymes. S1P is irreversibly degraded by S1P lyase (SPL), and is also regulated by lipid phosphate phosphatases (LPP1–3) and S1P-specific phosphatases (SPP1 and SPP2; [49–53]), C1P is regulated by LPP1–3 [49, 53]. The major source of plasma S1P are red blood cells, activated platelets, albumin, high-density lipoproteins (HDL), and extracellular SPHK1 derived from vascular endothelial cells [44, 54, 55] while the primary contribution to C1P plasma levels comes from intracellular C1P which has been

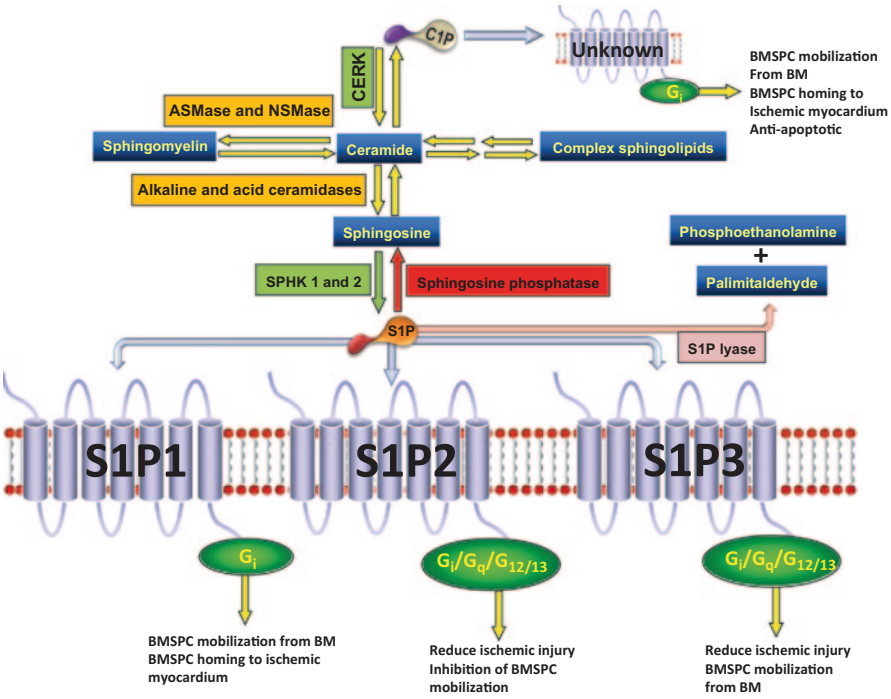

Fig. 6.1 Sphingosine 1-phosphate (S1P) metabolism and signaling in bone marrow stem and progenitor cells (BMSPCs). Interconversion of membrane sphingolipids and final phosphorylation of sphingosine by sphingosine kinases (SPHKs) results in formation of S1P which signals through S1P1, S1P2, and S1P3 receptors in the BMSPCs. These three receptors activate a distinct set of pathways through Gi, Gq, or G12/13 proteins which results in BMSPC mobilization from the bone marrow niches (S1P1 and S1P3); inhibition of BMSPC mobilization from the bone marrow (S1P2); and BMSPC homing to ischemic myocardium (S1P1)

released from damaged cells [56]. Both S1P and C1P interact with a variety of G protein-coupled seven-transmembrane receptors. There are five S1P receptor subtypes ($S1P_{1-5}$) that are widely expressed throughout mammalian tissues. $S1P_4$ and $S1P_5$ are expressed and function in the immune and nervous system, respectively, $S1P_{1-3}$ are most abundant throughout the cardiovascular system and are expressed on BMSPCs. $S1P_1$ is coupled exclusively via G_i to Ras-MAP kinase, phosphoinositide (PI) 3-kinase-Akt pathway and phospholipase C pathway. $S1P_2$ and $S1P_3$ are coupled to multiple G proteins, such as G_q, $G_{12/13}$, and G_i to activate phospholipase C pathway and Rho pathway [45–47, 57]. The signaling cascade activated by S1P binding to either $S1P_1$ or $S1P_3$ is responsible for HSPC migration [58–60]. Activation of $S1P_2$, however, yields an opposite effect—negatively regulating HSPC-mobilization [61]. While the receptor for C1P is yet to be identified, its signaling is sensitive to pertussis toxin, thereby implicating a G_i protein coupled receptor (Fig. 6.1; [62, 63]).

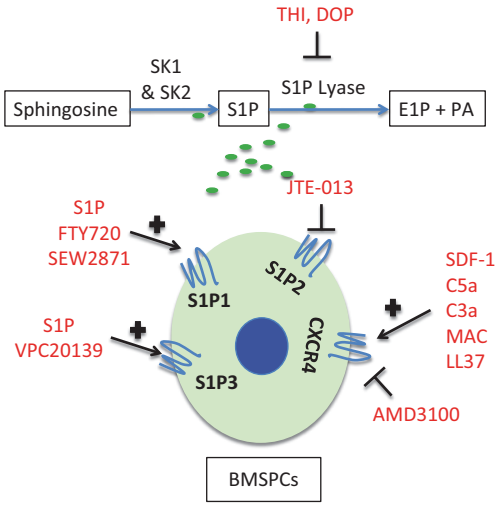

Fig. 6.2 Potential strategies to improve mobilization and homing of bone marrow stem and progenitor cell (*BMSPC*) by targeting bioactive lipids' pathways. Sphingosine 1-phosphate (*S1P*) is irreversibly degraded with *S1P* lyase enzyme into ethanolamine 1-phosphate (*E1P*) and palmitaldehyde (*PA*). Inhibition of the *S1P* lyase enzyme using tetrahydroxybutylimidazole (*THI*) or deoxypyrodixne (*DOP*) will increase the plasma levels of *S1P* and hence improve the mobilization and homing of *BMSPC* in response to acute ischemic myocardial injury. Similarly, blocking the *S1P2* receptor, which is known to reduce stem cell mobilization, or stimulating the *S1P1* receptor, known to enhance this migration, will augment the response of *BMSPC* towards elevated levels of *S1P* following acute ischemic injury

6.5 Sphingosine 1-Phosphate Chemoattracts BMSPCs

Since S1P receptors are G protein-coupled seven-transmembrane receptor they are placed in the same class as chemokine receptors. This observation raised one important question: Can S1P act as a direct chemoattractant for BMSPCs? Figurs 6.2, 6.3, and the subsequent discussion describe factors and pharmacological agents that play a key role in BMSPC mobilization. Early studies demonstrated a dose-dependent chemotactic effect of S1P on human HSPCs in a modified Boyden chamber assay [64]. Subsequent studies established that the gradient of S1P between BM and PB is a major determining factor in HSPCs and differentiated lymphocyte egress. While SDF-1 still has an undisputed role in HSPCs mobilization, it was demonstrated that plasma derived from normal and mobilized PB strongly chemoattracts murine BM HSPCs independent of plasma SDF-1 levels [41]. This was evident when removal of lipids from plasma by charcoal stripping abolished HSPCs chemotaxis but did not affect responsiveness towards SDF-1 [41].

Ratajczak et al. further showed that steady state plasma S1P levels create a gradient favoring HSPCs egress from the BM. While HSPCs are actively retained in BM via SDF-1-CXCR4 and VLA4-V-CAM1 interactions, disruption of these interactions via CXCR4 antagonist AMD3100 or triggering a proteolytic environment in

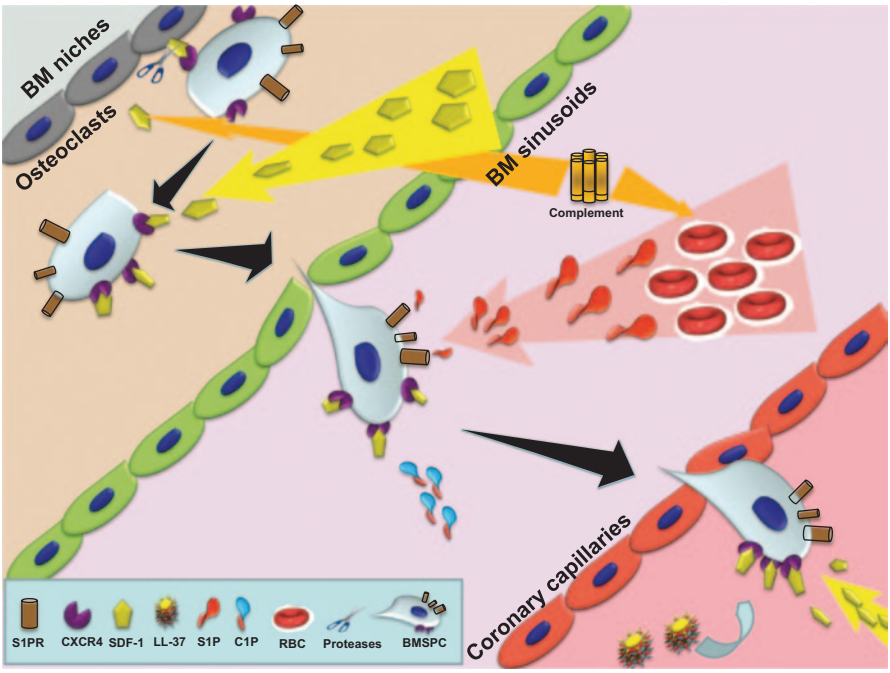

Fig. 6.3 Sequence of events in bone marrow stem and progenitor cell (*BMSPC*) mobilization from the bone marrow (*BM*) towards ischemic myocardium during MI. Acute MI initiates an inflammatory response resulting in release of proteases (by granulocytes and osteoclasts) in the *BM* which proteolytically inactivate the *SDF-1 CXCR4* interaction between *BM* osteoclasts and *BMSPCs*. The now mobilized *BMSPCs* follow an increasing *SDF-1* and bioactive lipid (sphingosine 1-phosphate, *S1P,* and ceramide 1-phosphate, *C1P*) gradient to exit the *BM* niches into the PB. Acute inflammation also promotes release of cathelicidins (LL-37) which facilitate clustering of *CXCR4* into lipid rafts thereby increasing their sensitivity towards lower levels of circulating *SDF-1*. Together, the increased sensitivity towards *SDF-1* and bioactive lipid gradients facilitate *BMSPC* homing towards ischemic myocardium

the BM would release HSPCs form their niches and therefore free them to follow the bioactive lipid gradients to the PB. Furthermore, a robust innate immune response can result in the activation of the complement cascade (CC) and increases plasma S1P levels through activating its release from platelets [65–67], red blood cells [68, 69], and endothelial cells [70]. Granulocyte colony-stimulating factor (G-CSF) is currently the most frequently used mobilizing agent that efficiently mobilizes BMSPCs after a few consecutive daily injections [71]. It has been established that G-CSF triggers complement complex activation which stimulates granulocytes to release proteolytic enzymes, thereby perturbing SDF-1-CXCR4/VLA-4-VCAM1 interactions in BM niches and facilitating HSPCs release [72]. The lasting effect of G-CSF promotes CC activation and formation of the membrane attack complex (MAC) that was shown to interact with erythrocytes [73]. While erythrocytes serve as the major reservoir of S1P in the PB [68, 74], they are highly protected

from MAC by CD59 and decay-accelerating factor (DAF) receptors [75]. However, Ratajczak et al. demonstrated that expression of these receptors on erythrocytes does not give complete protection from activated MAC since G-CSF-induced MAC exposure resulted in plasma S1P levels sufficient for HSPCs egress [41].

While it has been established that S1P is responsible for HSPC trafficking, the mechanism to explain this regulation is still under investigation. Recent evidence suggests that SDF-1 and S1P work synergistically to facilitate migration of primitive murine progenitor cells out of the BM [76]. In vitro studies on immature human CD34+ cells demonstrated that $S1P_1$ upregulation decreases their chemotactic activity towards SDF-1 due to reduced cell surface expression of CXCR4 suggesting a potential interaction between S1P and SDF-1 [77]. Further studies confirmed this notion showing that short-term inhibition of S1P/$S1P_1$ axis during steady state conditions or during CXCR4 inhibition caused reduction of SDF-1 in the plasma [78]. Furthermore, $S1P_1$ signaling resulted in production of reactive oxygen species (ROS), which in turn contributed to SDF-1 secretion, thereby facilitating HSPCs egress [78].

S1P-SDF-1 interaction in HSPCs egress was further demonstrated with the help of FTY720, a potent $S1P_1$ desensitizing agent which causes S1P receptor internalization [79]. Administration of FTY720 for 24 h resulted in increased plasma SDF-1 levels but had no effect on HSPCs egress. FTY720 treatment did reduce BM ROS signaling, due to $S1P_1$ downregulation, again pointing out the requirement of $S1P_1$ signaling in HSPC egress. Furthermore, mice that were treated with BM-specific S1P lyase inhibitor 4-deoxypyrodixne (DOP; [52]) had increased BM ROS levels and decreased HSPC egress [78]. Together these observations suggest that the increased concentrations of S1P and SDF-1 in the BM negatively affect HSPC egress, further highlighting the fact that both S1P and SDF-1 levels must be tightly regulated for balanced HSPCs mobilization.

While bioactive lipids such as S1P and C1P are powerful mobilizers of BMSPCs, their role in BMSPCs' mobilization and homing to ischemic myocardium is not well understood. The role of other chemoattractants in BMSPC homing to a hostile environment such as the infarcted myocardium is also unclear. However, recent evidence suggests that bioactive lipids, complement, and anti-microbial peptides are involved in BMSPC homing during MI. At the onset of MI, patients had significantly elevated plasma levels of S1P and C1P, which was correlated to elevated numbers of circulating BMSPCs, suggesting a role of bioactive lipids in BMSPC mobilization post-MI [80]. These observations were corroborated by a chemotaxis assay where BMSPCs had a significantly increased migration towards plasma isolated from patients at peak BMSPCs mobilization. Moreover, this migration was selectively blocked by VPC23019, a specific $S1P_1$ antagonist, further implicating S1P as a potent BMSPCs chemoattractant during MI [80]. As previously described in this chapter, MI induces a potent proteolytic environment in which numerous proteolytic enzymes irreversibly degrade potent BMSPCs chemoattractants such as SDF-1. Recent evidence suggests a role for antimicrobial protein cathelicidin LL-37 in sensitizing BMSPCs towards significantly lower levels of SDF-1 [81]. LL-37 sensitizes BMSPCs by integrating CXCR4 in close proximity to one another in the

lipid raft microdomains thereby augmenting CXCR4 signaling. Most importantly, LL-37 was overexpressed in cardiac tissues as well as cardiac fibroblasts following MI. The subsequent chemotaxis assay to confirmed that priming BMSPCs with LL-37 from patients with MI increases their mobilization to low, yet physiological, levels of SDF-1 (2 ng/ml; [80]). Taken together these findings highlight the importance of bioactive lipids and innate immunity in the mobilization and homing of BMSPCs to the ischemic myocardium.

6.6 Fate of Adults Stem Cells Post-MI: Biology of Infarcted Myocardium

However, the question still remains—what is the fate of BMSPCs once they reach the ischemic myocardium? The fate of BMSPCs is ultimately determined by the nature of the myocardial microenvironment. The onset of ischemic injury and subsequent reperfusion results in a robust pro-inflammatory state with elevated levels of locally activated complement [89–91] and ROS [92]. The induction of ROS and subsequent cytokine cascade contributes to rapid neutrophil infiltration of the infarct region, with neutrophil levels peaking between 24–72 h after MI [90, 93, 94]. Neutrophils attracted to the ischemic myocardium release proteolytic enzymes and additional ROS which may cause collateral damage to the infiltrating stem cells. It is clear that the above described mechanisms as well as the lack of a good blood supply to the infarct region make the myocardial microenvironment early on post-MI unsuitable for stem cell arrival. However, as soon as 5 days after MI the acute inflammatory response subsides, and angiogenesis, the major factor in infarct healing, begins to take place [95, 96]. Thus, around this time, when the acute inflammatory response has decreased and the infarct site is being vascularized, stem cells might find the right environment to attach and proliferate. This window of opportunity is limited, however, due to evidence of extensive scar formation as soon as 2 weeks after MI, which would hinder stem cell nesting [96–98]. Clinical studies' findings are in agreement with these pathological findings and temporal trends. Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI trial) study demonstrated that the beneficial effects of BM derived mononuclear cells were most evident when the cells were transplanted >5 days after the acute myocardial injury [99].

6.7 Therapeutic Mobilization of BM-Derived Stem Cells in Myocardial Regeneration

The concept of BM-derived stem cell mobilization using pharmacological agents such as G-CSF has been utilized for a long time. Based on the available clinical experience and safety profile of these therapies, pharmacological stem cell mobilization in the setting of IHD has gained increasing enthusiasm. Mobilized BMSPCs can

be harvested for further transplantation through the intracoronary/intramyocardial routes or allowed for spontaneous homing to the infarcted myocardium and have demonstrated various degrees of success [100–102]. The heterogeneous methodologies of the G-CSF studies and the wider patient selection diluted the effect. The overall lack of efficacy with G-CSF BMSPC mobilization in the setting of acute MI is somewhat incongruent with the salutary effects of BMCs transplantation in humans or stem cell mobilization in animal models for myocardial regeneration.

The largest study utilizing G-CSF in the setting of acute MI was the Regenerate Vital Myocardium by Vigorous Activation of Bone Marrow Stem Cells (REVIVAL-2 trial) trial that included 114 patients [102]. The study randomized AMI patients to 10 μg/kg of G-CSF vs. placebo and LV functional parameters were assessed using CMR. The study demonstrated no significant difference in the tested parameters between patients treated with G-CSF or placebo. However, baseline characteristics in the study population showed normal or near normal LV function and therefore the expected benefit is minimal. Patient selection was a methodological flaw that plagued some of the studies that utilized G-CSF. Indeed, with careful examination of the available literature, patients with reduced LV function at baseline as well as those treated within the first 36 h following AMI benefited the most [103, 104]. On the other hand, safety concerns regarding a potentially increasing evidence of instent restenosis [105] and recurrent ischemia [106] have halted subsequent clinical trials. However, it is important to note that these safety concerns were not confirmed in large studies [102] or in the cumulative meta-analyses [103].

Beyond the methodological flaws encountered in human trials, this lack of efficacy can be explained by multiple factors. While G-CSF and similar therapies mobilize a wide array of BMSPCs in the PB, homing factors may not be sufficient to guide them to the myocardial infarct zone. Indeed, the homing of c-Kit+ cells to the infarcted myocardium improved when G-CSF therapy was combined with local administration of SDF-1 [107]. The myocardial levels of chemoattractants peaks within 24–72 h following injury [108–110] and therefore delayed therapy in some human trials may have missed the homing window to the infarct zone. Similarly, the addition of Flt-3 to G-CSF therapy improved outcomes in animal models [111]. Moreover, different cytokines are known to preferentially mobilize somewhat different subsets of BMCs [112, 113].Future studies investigating the characteristics of G-CSF-mobilized cells will be necessary to glean additional mechanistic insights in this regard.

Recently, a combined approach with stem cell mobilization and enhanced homing using therapies known to increase local SDF-1 or CXCR4 antagonists have been proposed [114, 115]. Going forward, the beneficial effects of BM-derived stem cell mobilization may be augmented by selective mobilization of undifferentiated BMSPCs rather than differentiated inflammatory cells. It is also important to remember that some of the G-CSF arbitrated effects can be mediated by its direct effect on cardiomyocytes which are known to express G-CSF receptor [116]. G-CSF therapy may be inducing the proliferation of cardiomyocytes or the differentiation of resident cardiac stem cells. On a similar note, G-CSF therapy upregulates Akt [117] and may result in reducing apoptosis of ischemic cardiomyocytes if utilized early following the acute event.

6.8 Therapeutic Effects of Adult Stem Cells Post-MI

While it is conclusive that the stem cells arrive to the infarcted myocardium post-MI, their ultimate contribution to myocardial repair is still unclear. Since adult myocardium has very limited potential for self-regeneration, the stem cells may contribute to ischemic myocardium repair via various paracrine mechanisms or differentiation into endothelium and/or cardiomyocytes. There is ample evidence supporting the hypothesis that paracrine mechanisms mediated by factors released by the adult stem cells play an essential role in myocardial repair after stem cell mobilization following MI [111, 118]. Numerous groups have shown that adult stem cells, and especially MSCs, secrete a broad range of chemokines, anti-inflammatory, and anti-apoptotic cytokines and growth factors that are potentially involved in cardiac repair [119]. Interestingly, hypoxia at the site of injury in conjunction with stem cell administration further stimulates production of these factors which includes hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-I, basic fibroblast growth factor (bFGF), and adrenomedullin [120, 121]. The paracrine benefits were further corroborated by administering conditioned medium (CM) from adult stem cells and comparing those effects to actual stem cell therapy [122–124]. These paracrine effects of BM derived cells extend to other populations such as cKit+cells and VSELs, thereby contributing to regeneration of ischemic myocardium [118]. Interestingly, improvement in cardiac functions was disproportionate to the rate of differentiation of BM derived cells suggesting that various factors secreted by these cells can explain the majority of beneficial effects. We observed similar findings with VSELs in an animal model of acute ischemic injury [111].

Differentiation of adult stem cells into cardiomyocytes and their subsequent contribution to post-MI repair has been an area of great debate for the last decade. Initial in vitro studies were able to isolate adult stem cells from either BM or adipose tissue and through various culture conditions, induce their differentiation into beating cells exhibiting cardiomyocyte morphology and physiology [125–134]. Although recent in vivo studies have revealed that heart cells are generated in adult mammals during normal homeostasis as well as post MI, the frequency of generation and the source of new heart cells remain unclear. Some studies suggest a high rate of stem cell differentiation into cardiomyocytes [135]. Other studies suggest that new cardiomyocytes are derived from the division of pre-existing cardiomyocytes at a very slow rate [4, 136, 137]. In summary, the evidence presented in this chapter accents the fact that adult stem cells contribute to myocardial repair by a wide array of effects including paracrine mechanisms, differentiating into functional tissues (cardiac or endothelial) as well as other diverse therapeutic features to preserve yet undamaged cells and contribute to endogenous creation of new functional tissue.

Conclusions

IHD is approaching epidemic levels in the USA and the Western world. Unfortunately, available therapies are largely symptomatic with no strategies to repair the damaged myocardium, which has initiated increasing interest in regenerative therapies particularly those utilizing BM derived stem cells. However, studies examining the therapeutic utility of BMSPC infusion [138] or their mobilization [139] after MI achieved limited success due to the lack of focused and mechanism-based approaches. The review of literature presented herein support the theory that multiple agents, beyond traditional chemokines, including bioactive lipids such as S1P and C1P contribute to the myocardial ischemia induced BMSPC mobilization and homing. Multiple new therapies that modulate plasma levels of S1P or its receptor expression are approved by the FDA and can be utilized in improving the mobilization of BMSPCs in myocardial ischemia in future myocardial regenerative studies (Fig. 6.2). Similarly, priming BM-derived cells with LL-37 can be used to improve their homing to the ischemic myocardium and thus overcome a major hurdle in stem cell regenerative myocardial therapies (Fig. 6.3). Most importantly, the success of stem cell therapy depends on the timing, dosing and the route of administration. The limited window of opportunity for successful mobilization of BMSCs is bound by the intense pro-inflammatory response in the first 4 days post MI and fibrosis as soon as 10–14 days post-MI. Therefore, development of successful therapies aimed at selective mobilization of BMSPCs depends on timing and restricting inflammatory cell egress. Nonetheless, further research is clearly required in the optimization of timing protocols and the method and route of delivery of BMSCs.

References

1. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM et al (2011) Heart disease and stroke statistics-2011 update: a report from the American Heart Association. Circulation 123(4):e18-e209
2. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B et al (2002) Chimerism of the transplanted heart. N Engl J Med 346(1):5–15
3. Deb A, Wang S, Skelding KA, Miller D, Simper D, Caplice NM (2003) Bone marrow-derived cardiomyocytes are present in adult human heart: A study of gender-mismatched bone marrow transplantation patients. Circulation 107(9):1247–1249
4. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S et al (2009) Evidence for cardiomyocyte renewal in humans. Science 324(5923):98–102
5. Kajstura J, Rota M, Cappetta D, Ogorek B, Arranto C, Bai Y et al (2012) Cardiomyogenesis in the aging and failing human heart. Circulation 126(15):1869–1881 [Research Support, N.I.H., Extramural Research Support, U.S. Gov't, Non-P.H.S.]
6. Hsieh PC, Segers VF, Davis ME, MacGillivray C, Gannon J, Molkentin JD et al (2007) Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. Nature Med 13(8):970–974

7. Bonig H, Priestley GV, Oehler V, Papayannopoulou T (2007) Hematopoietic progenitor cells (HPC) from mobilized peripheral blood display enhanced migration and marrow homing compared to steady-state bone marrow HPC. Exp Hematol 35(2):326–334 [Comparative Study Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]
8. Mobius-Winkler S, Hilberg T, Menzel K, Golla E, Burman A, Schuler G et al (2009) Time-dependent mobilization of circulating progenitor cells during strenuous exercise in healthy individuals. J Appl Physiol 107(6):1943–1950
9. Wojakowski W, Tendera M (2005) Mobilization of bone marrow-derived progenitor cells in acute coronary syndromes. Folia Histochem Cytobiol 43(4):229–232
10. Wojakowski W, Landmesser U, Bachowski R, Jadczyk T, Tendera M (2012) Mobilization of stem and progenitor cells in cardiovascular diseases. Leukemia 26(1):23–33 (official journal of the Leukemia Society of America, Leukemia Research Fund, UK. [Research Support, Non-U.S. Gov't Review])
11. Paczkowska E, Kucia M, Koziarska D, Halasa M, Safranow K, Masiuk M et al (2009) Clinical evidence that very small embryonic-like stem cells are mobilized into peripheral blood in patients after stroke. Stroke 40(4):1237–1244 (a journal of cerebral circulation)
12. Lee HM, Wysoczynski M, Liu R, Shin DM, Kucia M, Botto M et al (2010) Mobilization studies in complement-deficient mice reveal that optimal AMD3100 mobilization of hematopoietic stem cells depends on complement cascade activation by AMD3100-stimulated granulocytes. Leukemia 24(3):573–582 (official journal of the Leukemia Society of America, Leukemia Research Fund, UK. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't])
13. Wright DE, Bowman EP, Wagers AJ, Butcher EC, Weissman IL (2002) Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. J Exp Med 195(9):1145–1154 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]
14. Lapidot T, Dar A, Kollet O (2005) How do stem cells find their way home? Blood 106(6):1901–1910
15. Peled A, Grabovsky V, Habler L, Sandbank J, Arenzana-Seisdedos F, Petit I et al (1999) The chemokine SDF-1 stimulates integrin-mediated arrest of CD34(+) cells on vascular endothelium under shear flow. J Clin Invest 104(9):1199–1211 [Research Support, Non-U.S. Gov't]
16. Levesque JP, Takamatsu Y, Nilsson SK, Haylock DN, Simmons PJ (2001) Vascular cell adhesion molecule-1 (CD106) is cleaved by neutrophil proteases in the bone marrow following hematopoietic progenitor cell mobilization by granulocyte colony-stimulating factor. Blood 98(5):1289–1297 [Comparative Study Research Support, Non-U.S. Gov't]
17. Levesque JP, Hendy J, Takamatsu Y, Simmons PJ, Bendall LJ (2003) Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. J Clin Invest 111(2):187–196 [Research Support, Non-U.S. Gov't]
18. Kucia M, Dawn B, Hunt G, Guo Y, Wysoczynski M, Majka M et al (2004) Cells expressing early cardiac markers reside in the bone marrow and are mobilized into the peripheral blood following myocardial infarction. Circ Res 95(12):1191–1199
19. Leone A, Rutella S, Bonanno G, Abbate A, Rebuzzi A, Giovannini S et al (2005) Mobilization of bone marrow-derived stem cells after myocardial infarction and left ventricular function. Eur Heart J 26(12):1196–1204
20. Wojakowski W, Tendera M, Zebzda A, Michalowska A, Majka M, Kucia M et al (2006) Mobilization of CD34(+), CD117(+), CXCR4(+), c-met(+) stem cells is correlated with left ventricular ejection fraction and plasma NT-proBNP levels in patients with acute myocardial infarction. Eur Heart J 27(3):283–289
21. Grundmann F, Scheid C, Braun D, Zobel C, Reuter H, Schwinger R et al (2007) Differential increase of CD34, KDR/CD34, CD133/CD34 and CD117/CD34 positive cells in peripheral blood of patients with acute myocardial infarction. Clin Res Cardiol 96(9):621–627

22. Massa M, Rosti V, Ferrario M, Campanelli R, Ramajoli I, Rosso R et al (2005) Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction. Blood 105(1):199–206
23. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A et al (2001) Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation 103(23):2776–2779
24. Valgimigli M, Rigolin G, Fucili A, Porta M, Soukhomovskaia O, Malagutti P et al (2004) CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. Circulation 110(10):1209–1212
25. Paczkowska E, Larysz B, Rzeuski R, Karbicka A, Jałowiński R, Kornacewicz-Jach Z et al (2005) Human hematopoietic stem/progenitor-enriched CD34(+) cells are mobilized into peripheral blood during stress related to ischemic stroke or acute myocardial infarction. Eur J Haematol 75(6):461–467
26. Drukała J, Paczkowska E, Kucia M, Młyńska E, Krajewski A, Machaliński B et al (2012) Stem cells, including a population of very small embryonic-like stem cells, are mobilized into peripheral blood in patients after skin burn injury. Stem Cell Rev Mar;8(1):184–94. doi: 10.1007/s12015-011-9272-4
27. Wojakowski W, Tendera M, Michałowska A, Majka M, Kucia M, Maślankiewicz K et al (2004) Mobilization of CD34/CXCR4+, CD34/CD117+, c-met+ stem cells, and mononuclear cells expressing early cardiac, muscle, and endothelial markers into peripheral blood in patients with acute myocardial infarction. Circulation 110(20):3213–3220
28. Wyderka R, Wojakowski W, Jadczyk T, Maslankiewicz K, Parma Z, Pawlowski T et al (2012) Mobilization of CD34+CXCR4+ stem/progenitor cells and the parameters of left ventricular function and remodeling in 1-year follow-up of patients with acute myocardial infarction. Mediators Inflamm 2012:564027 [Research Support, Non-U.S. Gov't]
29. Müller-Ehmsen J, Scheid C, Grundmann F, Hirsch I, Turan G, Tossios P et al (2005) The mobilization of CD34 positive mononuclear cells after myocardial infarction is abolished by revascularization of the culprit vessel. Int J Cardiol 103(1):7–11
30. Abdel-Latif A, Zuba-Surma EK, Ziada KM, Kucia M, Cohen DA, Kaplan AM et al (2010) Evidence of mobilization of pluripotent stem cells into peripheral blood of patients with myocardial ischemia. Exp Hematol Dec;38(12):1131–1142.e1. doi: 10.1016/j.exphem.2010.08.003. Epub 2010 Aug 26
31. Wojakowski W, Tendera M, Kucia M, Zuba-Surma E, Paczkowska E, Ciosek J et al (2009) Mobilization of bone marrow-derived Oct-4+SSEA-4+very small embryonic-like stem cells in patients with acute myocardial infarction. J Am Coll Cardiol 53(1):1–9
32. Zuba-Surma EK, Kucia M, Abdel-Latif A, Dawn B, Hall B, Singh R et al (2008) Morphological characterization of very small embryonic-like stem cells (VSELs) by ImageStream system analysis. J Cell Mol Med 12(1):292–303
33. Fadini GP, Sartore S, Schiavon M, Albiero M, Baesso I, Cabrelle A et al (2006) Diabetes impairs progenitor cell mobilisation after hindlimb ischaemia-reperfusion injury in rats. Diabetologia 49(12):3075–3084 [Research Support, Non-U.S. Gov't]
34. Kucia M, Reca R, Miekus K, Wanzeck J, Wojakowski W, Janowska-Wieczorek A et al (2005) Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis. Stem Cells 23(7):879–894
35. Lapidot T, Kollet O (2002) The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2 m(null) mice. Leukemia 16(10):1992–2003 (official journal of the Leukemia Society of America, Leukemia Research Fund, UK. [Review])
36. Peterson JT, Li H, Dillon L, Bryant JW (2000) Evolution of matrix metalloprotease and tissue inhibitor expression during heart failure progression in the infarcted rat. Cardiovasc Res 46(2):307–315

37. McQuibban GA, Butler GS, Gong JH, Bendall L, Power C, Clark-Lewis I et al (2001) Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. J Biol Chem 276(47):43503–43508 [Research Support, Non-U.S. Gov't]
38. McQuibban GA, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, Overall CM (2002) Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. Blood 100(4):1160–1167
39. Agarwal U, Ghalayini W, Dong F, Weber K, Zou YR, Rabbany SY et al (2010) Role of cardiac myocyte CXCR4 expression in development and left ventricular remodeling after acute myocardial infarction. Circ Res 107(5):667–676
40. Jalili A, Shirvaikar N, Marquez-Curtis L, Qiu Y, Korol C, Lee H et al (2010) Fifth complement cascade protein (C5) cleavage fragments disrupt the SDF-1/CXCR4 axis: further evidence that innate immunity orchestrates the mobilization of hematopoietic stem/progenitor cells. Exp Hematol 38(4):321–332
41. Ratajczak MZ, Lee H, Wysoczynski M, Wan W, Marlicz W, Laughlin MJ et al (2010) Novel insight into stem cell mobilization-plasma sphingosine-1-phosphate is a major chemoattractant that directs the egress of hematopoietic stem progenitor cells from the bone marrow and its level in peripheral blood increases during mobilization due to activation of complement cascade/membrane attack complex. Leukemia 24(5):976–985 (official journal of the Leukemia Society of America, Leukemia Research Fund, UK. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't])
42. Karapetyan AV, Klyachkin YM, Selim S, Sunkara M, Ziada KM, Cohen DA et al (2013) Bioactive lipids and cationic antimicrobial peptides as new potential regulators for trafficking of bone marrow-derived stem cells in patients with acute myocardial infarction. Stem Cells Dev 22(11):1645–1656 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]
43. Merrill AH Jr, Schmelz EM, Dillehay DL, Spiegel S, Shayman JA, Schroeder JJ et al (1997) Sphingolipids-the enigmatic lipid class: biochemistry, physiology, and pathophysiology. Toxicol Appl Pharmacol 142(1):208–225 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S. Review]
44. Yatomi Y, Ozaki Y, Ohmori T, Igarashi Y (2001) Sphingosine 1-phosphate: synthesis and release. Prostaglandins 64(1–4):107–122
45. Takuwa Y (2002) Subtype-specific differential regulation of Rho family G proteins and cell migration by the Edg family sphingosine-1-phosphate receptors. Biochim Biophys Acta 1582(1–3):112–120 [Research Support, Non-U.S. Gov't Review]
46. Spiegel S, Milstien S (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat Rev Mol Cell Biol 4(5):397–407 [Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S. Review]
47. Hla T (2004) Physiological and pathological actions of sphingosine 1-phosphate. Seminars Cell Dev Biol 15(5):513–520 [Research Support, U.S. Gov't, P.H.S. Review]
48. Boath A, Graf C, Lidome E, Ullrich T, Nussbaumer P, Bornancin F (2008) Regulation and traffic of ceramide 1-phosphate produced by ceramide kinase: comparative analysis to glucosylceramide and sphingomyelin. J Biol Chem 283(13):8517–8526 [Comparative Study Research Support, Non-U.S. Gov't]
49. Sciorra VA, Morris AJ (2002) Roles for lipid phosphate phosphatases in regulation of cellular signaling. Biochim Biophys Acta 1582(1–3):45–51 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]
50. Long J, Darroch P, Wan KF, Kong KC, Ktistakis N, Pyne NJ et al (2005) Regulation of cell survival by lipid phosphate phosphatases involves the modulation of intracellular phosphatidic acid and sphingosine 1-phosphate pools. Biochem J 391(Pt 1):25–32 [Research Support, Non-U.S. Gov't]
51. Mechtcheriakova D, Wlachos A, Sobanov J, Kopp T, Reuschel R, Bornancin F et al (2007) Sphingosine 1-phosphate phosphatase 2 is induced during inflammatory responses. Cell Signal 19(4):748–760

52. Schwab SR, Pereira JP, Matloubian M, Xu Y, Huang Y, Cyster JG (2005) Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science 309(5741):1735–1739 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]
53. Brindley DN, English D, Pilquil C, Buri K, Ling ZC (2002) Lipid phosphate phosphatases regulate signal transduction through glycerolipids and sphingolipids. Biochim Biophys Acta 1582(1–3):33–44 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]
54. Kihara A, Igarashi Y (2008) Production and release of sphingosine 1-phosphate and the phosphorylated form of the immunomodulator FTY720. Biochim Biophys Acta 1781(9):496–502 [Research Support, Non-U.S. Gov't Review]
55. Venkataraman K, Thangada S, Michaud J, Oo ML, Ai Y, Lee YM et al (2006) Extracellular export of sphingosine kinase-1a contributes to the vascular S1P gradient. Biochem J 397(3):461–471 [Research Support, N.I.H., Extramural]
56. Lamour NF, Stahelin RV, Wijesinghe DS, Maceyka M, Wang E, Allegood JC et al (2007) Ceramide kinase uses ceramide provided by ceramide transport protein: localization to organelles of eicosanoid synthesis. J Lipid Res 48(6):1293–1304 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S.]
57. Ishii I, Fukushima N, Ye X, Chun J (2004) Lysophospholipid receptors: signaling and biology. Ann Rev Biochem 73:321–354 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]
58. Massberg S, Schaerli P, Knezevic-Maramica I, Kollnberger M, Tubo N, Moseman EA et al (2007) Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. Cell 131(5):994–1008 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]
59. Jo E, Sanna MG, Gonzalez-Cabrera PJ, Thangada S, Tigyi G, Osborne DA et al (2005) S1P1-selective in vivo-active agonists from high-throughput screening: off-the-shelf chemical probes of receptor interactions, signaling, and fate. Chem Biol 12(6):703–715 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]
60. Walter DH, Rochwalsky U, Reinhold J, Seeger F, Aicher A, Urbich C et al (2007) Sphingosine-1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway via the S1P3 receptor. Arterioscler thromb vasc Biol 27(2):275–282 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]
61. Michaud J, Im DS, Hla T (2010) Inhibitory role of sphingosine 1-phosphate receptor 2 in macrophage recruitment during inflammation. J Immunol 184(3):1475–1483 [Research Support, N.I.H., Extramural]
62. Gomez-Munoz A, Kong JY, Salh B, Steinbrecher UP (2004) Ceramide-1-phosphate blocks apoptosis through inhibition of acid sphingomyelinase in macrophages. J Lipid Res 45(1):99–105 [Research Support, Non-U.S. Gov't]
63. Granado MH, Gangoiti P, Ouro A, Arana L, Gonzalez M, Trueba M et al (2009) Ceramide 1-phosphate (C1P) promotes cell migration Involvement of a specific C1P receptor. Cell signal 21(3):405–412 [Research Support, Non-U.S. Gov't]
64. Seitz G, Boehmler AM, Kanz L, Mohle R (2005) The role of sphingosine 1-phosphate receptors in the trafficking of hematopoietic progenitor cells. Ann N Y Acad Sci 1044:84–89
65. Schaphorst KL, Chiang E, Jacobs KN, Zaiman A, Natarajan V, Wigley F et al (2003) Role of sphingosine-1 phosphate in the enhancement of endothelial barrier integrity by platelet-released products. Am J Physiol Lung Cell Mol Physiol 285(1):L258–L267
66. Yatomi Y, Ohmori T, Rile G, Kazama F, Okamoto H, Sano T et al (2000) Sphingosine 1-phosphate as a major bioactive lysophospholipid that is released from platelets and interacts with endothelial cells. Blood 96(10):3431–3438
67. Yatomi Y, Ruan F, Hakomori S, Igarashi Y (1995) Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. Blood 86(1):193–202

68. Hanel P, Andreani P, Graler MH (2007) Erythrocytes store and release sphingosine 1-phosphate in blood. FASEB J 21(4):1202–1209 (official publication of the Federation of American Societies for Experimental Biology)
69. Pappu R, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y et al (2007) Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. Science 316(5822):295–298
70. Venkataraman K, Lee YM, Michaud J, Thangada S, Ai Y, Bonkovsky HL et al (2008) Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. Circ Res 102(6):669–676
71. Ryan MA, Nattamai KJ, Xing E, Schleimer D, Daria D, Sengupta A et al (2010) Pharmacological inhibition of EGFR signaling enhances G-CSF-induced hematopoietic stem cell mobilization. Nat Med 16(10):1141–1146 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]
72. Greenbaum AM, Link DC (2011) Mechanisms of G-CSF-mediated hematopoietic stem and progenitor mobilization. Leukemia 25(2):211–217 (official journal of the Leukemia Society of America, Leukemia Research Fund, UK. [Review])
73. Lindorfer MA, Pawluczkowycz AW, Peek EM, Hickman K, Taylor RP, Parker CJ (2010) A novel approach to preventing the hemolysis of paroxysmal nocturnal hemoglobinuria: both complement-mediated cytolysis and C3 deposition are blocked by a monoclonal antibody specific for the alternative pathway of complement. Blood 115(11):2283–2291
74. Ohkawa R, Nakamura K, Okubo S, Hosogaya S, Ozaki Y, Tozuka M et al (2008) Plasma sphingosine-1-phosphate measurement in healthy subjects: close correlation with red blood cell parameters. Ann Clin Biochem 45(Pt 4):356–363 [Research Support, Non-U.S. Gov't]
75. Bessler M, Hiken J (2008) The pathophysiology of disease in patients with paroxysmal nocturnal hemoglobinuria. Hematology Am Soc Hematol Educ Program 2008:104–110 (the Education Program of the American Society of Hematology American Society of Hematology Education Program. [Research Support, N.I.H., Extramural])
76. Whetton AD, Lu Y, Pierce A, Carney L, Spooncer E (2003) Lysophospholipids synergistically promote primitive hematopoietic cell chemotaxis via a mechanism involving Vav 1. Blood 102(8):2798–2802 [Research Support, Non-U.S. Gov't]
77. Ryser MF, Ugarte F, Lehmann R, Bornhauser M, Brenner S (2008) S1P(1) overexpression stimulates S1P-dependent chemotaxis of human CD34+ hematopoietic progenitor cells but strongly inhibits SDF-1/CXCR4-dependent migration and in vivo homing. Mol Immunol 46(1):166–171
78. Golan K, Vagima Y, Ludin A, Itkin T, Cohen-Gur S, Kalinkovich A et al (2012) S1P promotes murine progenitor cell egress and mobilization via S1P1-mediated ROS signaling and SDF-1 release. Blood 119(11):2478–2488 [Research Support, Non-U.S. Gov't]
79. Brinkmann V (2004) FTY720: mechanism of action and potential benefit in organ transplantation. Yonsei Med J [Review] 45(6):991–997
80. Karapetyan AV, Klyachkin YM, Selim S, Sunkara M, Ziada KM, Cohen DA et al (2013) Bioactive lipids and cationic antimicrobial peptides as new potential regulators for trafficking of bone marrow-derived stem cells in patients with acute myocardial infarction. Stem Cells Dev 2013 Jun 1;22(11):1645–56. doi: 10.1089/scd.2012.0488. Epub 2013 Feb 19
81. Wu W, Kim CH, Liu R, Kucia M, Marlicz W, Greco N et al (2012) The bone marrow-expressed antimicrobial cationic peptide LL-37 enhances the responsiveness of hematopoietic stem progenitor cells to an SDF-1 gradient and accelerates their engraftment after transplantation. Leukemia 2011 Sept 20 (official journal of the Leukemia Society of America, Leukemia Research Fund, UK). Leukemia Apr;26(4):736–45. doi: 10.1038/leu.2011.252. Epub 2011 Sep 20
82. Fyrst H, Saba JD (2010) An update on sphingosine-1-phosphate and other sphingolipid mediators. Nat Chem Biol 6(7):489–497 [Research Support, N.I.H., Extramural Review]
83. Hasegawa Y, Suzuki H, Sozen T, Rolland W, Zhang JH (2010) Activation of sphingosine 1-phosphate receptor-1 by FTY720 is neuroprotective after ischemic stroke in rats. Stroke 41(2):368–374 (a journal of cerebral circulation. [Research Support, N.I.H., Extramural])
84. Sattler K, Levkau B (2009) Sphingosine-1-phosphate as a mediator of high-density lipoprotein effects in cardiovascular protection. Cardiovasc Res 82(2):201–211 [Research Support, Non-U.S. Gov't Review]

85. Morita Y, Perez GI, Paris F, Miranda SR, Ehleiter D, Haimovitz-Friedman A et al (2000) Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine-1-phosphate therapy. Nat Med 6(10):1109–1114 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]
86. Levi M, Meijler MM, Gomez-Munoz A, Zor T (2010) Distinct receptor-mediated activities in macrophages for natural ceramide-1-phosphate (C1P) and for phospho-ceramide analogue-1 (PCERA-1). Mol Cell Endocrinol 314(2):248–255 [Research Support, Non-U.S. Gov't Review]
87. Morris AJ, Panchatcharam M, Cheng HY, Federico L, Fulkerson Z, Selim S et al (2009) Regulation of blood and vascular cell function by bioactive lysophospholipids. J Thromb Haemost 7(Suppl 1):38–43 [Research Support, N.I.H., Extramural Research Support, U.S. Gov't, Non-P.H.S. Review]
88. Siess W, Tigyi G (2004) Thrombogenic and atherogenic activities of lysophosphatidic acid. J Cell Biochem [Review] 92(6):1086–1094
89. Wilde WS, Hill JH, Wilson E, Schielke GP (1971) Exchange of free and albumin-bound Evans blue in interstitium of hamster kidney. Am J Physiol 220(6):1991–1999
90. Dreyer WJ, Michael LH, Nguyen T, Smith CW, Anderson DC, Entman ML et al (1992) Kinetics of C5a release in cardiac lymph of dogs experiencing coronary artery ischemia-reperfusion injury. Circ Res 71(6):1518–1524 [Comparative Study Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.]
91. Ivey CL, Williams FM, Collins PD, Jose PJ, Williams TJ (1995) Neutrophil chemoattractants generated in two phases during reperfusion of ischemic myocardium in the rabbit. Evidence for a role for C5a and interleukin-8. J Clin Invest 95(6):2720–2728 [Comment Research Support, Non-U.S. Gov't]
92. Patel KD, Zimmerman GA, Prescott SM, McEver RP, McIntyre TM (1991) Oxygen radicals induce human endothelial cells to express GMP-140 and bind neutrophils. J Cell Biol 112(4):749–759 [In Vitro Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]
93. Frangogiannis NG, Lindsey ML, Michael LH, Youker KA, Bressler RB, Mendoza LH et al (1998) Resident cardiac mast cells degranulate and release preformed TNF-alpha, initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. Circulation 98(7):699–710 [Research Support, U.S. Gov't, P.H.S.]
94. Virag JI, Murry CE (2003) Myofibroblast and endothelial cell proliferation during murine myocardial infarct repair. Am J Pathol 163(6):2433–2440 [Research Support, U.S. Gov't, P.H.S.]
95. Sun Y, Weber KT (2000) Infarct scar: a dynamic tissue. Cardiovasc Res [Review] 46(2):250–256
96. Li RK, Mickle DA, Weisel RD, Rao V, Jia ZQ (2001) Optimal time for cardiomyocyte transplantation to maximize myocardial function after left ventricular injury. Ann Thorac surg 72(6):1957–1963 [Research Support, Non-U.S. Gov't]
97. Hu X, Wang J, Chen J, Luo R, He A, Xie X et al (2007) Optimal temporal delivery of bone marrow mesenchymal stem cells in rats with myocardial infarction. Eur J Cardiothorac surg 31(3):438–443 (official journal of the European Association for Cardio-thoracic Surgery. [Research Support, Non-U.S. Gov't])
98. Jiang CY, Gui C, He AN, Hu XY, Chen J, Jiang Y et al (2008) Optimal time for mesenchymal stem cell transplantation in rats with myocardial infarction. J Zhejiang Univ Sci B 9(8):630–637 [Research Support, Non-U.S. Gov't]
99. Schächinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H et al (2006) Intracoronary bone marrow-derived progenitor cells in acute myocardial in acute myocardial Infarction. N Engl J Med 355(12):1210–1221
100. Engelmann M, Theiss H, Hennig-Theiss C, Huber A, Wintersperger B, Werle-Ruedinger A et al (2006) Autologous bone marrow stem cell mobilization induced by granulocyte colony-stimulating factor after subacute ST-segment elevation myocardial infarction undergoing late revascularization: final results from the G-CSF-STEMI (granulocyte colony-stimulating factor ST-Segment elevation myocardial infarction) trial. J Am Coll Cardiol 48(8):1712–1721

101. Ince H, Petzsch M, Kleine H, Eckard H, Rehders T, Burska D et al (2005) Prevention of left ventricular remodeling with granulocyte colony-stimulating factor after acute myocardial infarction: final 1-year results of the Front-Integrated Revascularization and Stem Cell Liberation in Evolving Acute Myocardial Infarction by Granulocyte Colony-Stimulating Factor (FIRSTLINE-AMI) Trial. Circulation 112(9 Suppl):I73–I80
102. Zohlnhöfer D, Ott I, Mehilli J, Schömig K, Michalk F, Ibrahim T et al (2006) Stem cell mobilization by granulocyte colony-stimulating factor in patients with acute myocardial infarction: a randomized controlled trial. JAMA 295(9):1003–1010
103. Abdel-Latif A, Bolli R, Zuba-Surma EK, Tleyjeh IM, Hornung CA, Dawn B (2008) Granulocyte colony-stimulating factor therapy for cardiac repair after acute myocardial infarction: a systematic review and meta-analysis of randomized controlled trials. Am Heart J 156(2):216–226
104. Achilli F, Malafronte C, Lenatti L, Gentile F, Dadone V, Gibelli G et al (2010) 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 [Multicenter Study Randomized Controlled Trial Research Support, Non-U.S. Gov't]
105. Kang HJ, Kim HS, Zhang SY, Park KW, Cho HJ, Koo BK et al (2004) Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. Lancet 363(9411):751–756 [Clinical Trial Comparative Study Randomized Controlled Trial]
106. Hill JM, Syed MA, Arai AE, Powell TM, Paul JD, Zalos G et al (2005) Outcomes and risks of granulocyte colony-stimulating factor in patients with coronary artery disease. J Am Coll Cardiol 46(9):1643–1648
107. Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M et al (2003) Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 362(9385):697–703 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S.]
108. Wang Y, Haider H, Ahmad N, Zhang D, Ashraf M (2006) Evidence for ischemia induced host-derived bone marrow cell mobilization into cardiac allografts. J Mol Cell Cardiol 41(3):478–487 [Research Support, N.I.H., Extramural]
109. Ma J, Ge J, Zhang S, Sun A, Shen J, Chen L et al (2005) Time course of myocardial stromal cell-derived factor 1 expression and beneficial effects of intravenously administered bone marrow stem cells in rats with experimental myocardial infarction. Basic Res Cardiol 100(3):217–223 [Research Support, Non-U.S. Gov't]
110. Kucia M, Dawn B, Hunt G, Guo Y, Wysoczynski M, Majka M et al (2004) Cells expressing early cardiac markers reside in the bone marrow and are mobilized into the peripheral blood after myocardial infarction. Circ Res 95(12):1191–1199
111. Dawn B, Tiwari S, Kucia MJ, Zuba-Surma EK, Guo Y, Sanganalmath SK et al (2008) Transplantation of bone marrow-derived very small embryonic-like stem cells attenuates left ventricular dysfunction and remodeling after myocardial infarction. Stem Cells 26(6):1646–1655
112. Neipp M, Zorina T, Domenick MA, Exner BG, Ildstad ST (1998) Effect of FLT3 ligand and granulocyte colony-stimulating factor on expansion and mobilization of facilitating cells and hematopoietic stem cells in mice: kinetics and repopulating potential. Blood 92(9):3177–3188 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]
113. Hess DA, Levac KD, Karanu FN, Rosu-Myles M, White MJ, Gallacher L et al (2002) Functional analysis of human hematopoietic repopulating cells mobilized with granulocyte colony-stimulating factor alone versus granulocyte colony-stimulating factor in combination with stem cell factor. Blood 100(3):869–878 [Comparative Study Research Support, Non-U.S. Gov't]
114. Jujo K, Hamada H, Iwakura A, Thorne T, Sekiguchi H, Clarke T et al (2010) CXCR4 blockade augments bone marrow progenitor cell recruitment to the neovasculature and reduces mortality after myocardial infarction. Proc Natl Acad Sci U S A 107(24):11008–11013 [Research Support, N.I.H., Extramural]

115. Zaruba MM, Theiss HD, Vallaster M, Mehl U, Brunner S, David R et al (2009) Synergy between CD26/DPP-IV inhibition and G-CSF improves cardiac function after acute myocardial infarction. Cell Stem Cell 4(4):313–323 [Research Support, Non-U.S. Gov't]
116. Shimoji K, Yuasa S, Onizuka T, Hattori F, Tanaka T, Hara M et al (2010) G-CSF promotes the proliferation of developing cardiomyocytes in vivo and in derivation from ESCs and iPSCs. Cell Stem Cell 6(3):227–237 [Research Support, Non-U.S. Gov't]
117. Ohtsuka M, Takano H, Zou Y, Toko H, Akazawa H, Qin Y et al (2004) Cytokine therapy prevents left ventricular remodeling and dysfunction after myocardial infarction through neovascularization. FASEB J 18(7):851–853 (official publication of the Federation of American Societies for Experimental Biology [Comparative Study])
118. Loffredo FS, Steinhauser ML, Gannon J, Lee RT (2011) Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair. Cell Stem Cell 8(4):389–398 [Research Support, N.I.H., Extramural]
119. Caplan AI, Dennis JE (2006) Mesenchymal stem cells as trophic mediators. J Cell Biochem 98(5):1076–1084 [Research Support, N.I.H., Extramural Review]
120. Yoon YS, Wecker A, Heyd L, Park JS, Tkebuchava T, Kusano K et al (2005) Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. J Clin Invest 115(2):326–338 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]
121. Nagaya N, Kangawa K, Itoh T, Iwase T, Murakami S, Miyahara Y et al (2005) Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 112(8):1128–1135 [Research Support, Non-U.S. Gov't]
122. Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H et al (2005) Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 11(4):367–368 [Letter Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]
123. Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F et al (2006) Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 20(6):661–669 (official publication of the Federation of American Societies for Experimental Biology. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't])
124. Takahashi M, Li TS, Suzuki R, Kobayashi T, Ito H, Ikeda Y et al (2006) Cytokines produced by bone marrow cells can contribute to functional improvement of the infarcted heart by protecting cardiomyocytes from ischemic injury. Am J Physiol Heart Circ physiol 291(2):H886–H893 [In Vitro Research Support, Non-U.S. Gov't]
125. Condorelli G, Borello U, De Angelis L, Latronico M, Sirabella D, Coletta M et al (2001) Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardium regeneration. Proc Natl Acad Sci U S A 98(19):10733–10738 [Research Support, Non-U.S. Gov't]
126. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW et al (2001) Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 107(11):1395–1402 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.]
127. Grounds MD, White JD, Rosenthal N, Bogoyevitch MA (2002) The role of stem cells in skeletal and cardiac muscle repair. J Histochem Cytochem 50(5):589–610 (official journal of the Histochemistry Society [Research Support, Non-U.S. Gov't Review])
128. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J et al (1999) Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103(5):697–705 [Research Support, Non-U.S. Gov't]
129. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B et al (2001) Bone marrow cells regenerate infarcted myocardium. Nature 410(6829):701–705 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]
130. Gepstein L (2002) Derivation and potential applications of human embryonic stem cells. Circ Res 91(10):866–876 [Research Support, Non-U.S. Gov't Review]

131. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ et al (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7(2):211–228 [Research Support, Non-U.S. Gov't]
132. Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H, Guilak F (2002) Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. Biochemical and biophysical research communications 290(2):763–769 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]
133. Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM et al (2002) Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun 294(2):371–379 [Research Support, Non-U.S. Gov't]
134. Cousin B, Andre M, Arnaud E, Penicaud L, Casteilla L (2003) Reconstitution of lethally irradiated mice by cells isolated from adipose tissue. Biochem Biophys Res Commun 301(4):1016–1022 [Research Support, Non-U.S. Gov't]
135. Hosoda T, D'Amario D, Cabral-Da-Silva MC, Zheng H, Padin-Iruegas ME, Ogorek B et al (2009) Clonality of mouse and human cardiomyogenesis in vivo. Proc Natl Acad Sci U S A 106(40):17169–17174 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]
136. Soonpaa MH, Field LJ (1997) Assessment of cardiomyocyte DNA synthesis in normal and injured adult mouse hearts. Am J Physiol 272(1 Pt 2):H220–H226 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]
137. Walsh S, Ponten A, Fleischmann BK, Jovinge S (2010) Cardiomyocyte cell cycle control and growth estimation in vivo-an analysis based on cardiomyocyte nuclei. Cardiovasc Res 86(3):365–373 [Comparative Study Research Support, Non-U.S. Gov't]
138. Abdel-Latif A, Bolli R, Tleyjeh IM, Montori VM, Perin EC, Hornung CA et al (2007) Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. Arch Intern Med 167(10):989–997 [Research Support, N.I.H., Extramural Review]
139. Abdel-Latif A, Bolli R, Zuba-Surma EK, Tleyjeh IM, Hornung CA, Dawn B (2008) Granulocyte colony-stimulating factor therapy for cardiac repair after acute myocardial infarction: a systematic review and meta-analysis of randomized controlled trials. Am Heart J 156(2):216–226.e9 [Meta-Analysis Research Support, N.I.H., Extramural Review]

Chapter 7
Stem Cell Therapies in Neurology

Naoki Tajiri, Meaghan Staples, Sandra Acosta, Mibel Pabon, Travis Dailey, Yuji Kaneko and Cesar V. Borlongan

Abbreviations

CNS	Central nervous system
GDNF	Glial cell line-derived neurotrophic factor
HSC	Hematopoietic stem cell
MSC	Mesenchymal stem cell
NPC	Neural progenitor cell
NSC	Neural stem cell
SNc	Substantia nigra pars compacta
VSELs	Very small embryonic-like stem cells

7.1 Introduction

Until recently, cells of the brain and central nervous system (CNS) were believed to be incapable of regeneration. While mammals, particularly humans, lack the capacity to repair their CNS, some vertebrates, such as the urodele amphibians, show remarkable plasticity of the CNS [17, 18]. Currently, man's shortcoming in neuroregeneration is attributed to limited neurogenesis, the presence of glial scarring, and Nogo A, an active inhibitor of axonal regeneration [103, 130]. In 1998, Eriksson and colleagues found new neurons in the dentate gyrus of the hippocampus of adult humans, suggesting that neurogenesis continues in this region of the brain throughout life [39]. In 2006 a patient who had been in a minimally conscious state for nearly 20 years showed improvement in speech and language. Furthermore, magnetic resonance imaging and computed tomography–positron emission tomography of that patient showed evidence of axonal growth and CNS remodeling [119].

C. V. Borlongan (✉) · N. Tajiri · M. Staples · S. Acosta · M. Pabon · T. Dailey · Y. Kaneko
Center of Excellence for Aging & Brain Repair, Department of Neurosurgery and Brain Repair, Morsani College of Medicine, University of South Florida, Tampa, FL 33612, USA
e-mail: cborlong@health.usf.edu

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,* Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_7,

Despite such discoveries, the CNS as a whole demonstrates limited regeneration and recovery following injury, accounting for the highly destructive nature of neurodegenerative diseases. In mammals, neurogenesis is limited to the subgranular zone of the dentate gyrus of the hippocampus, and the subventricular zone of the olfactory bulb. In man, neurogenesis is limited to the dentate gyrus of the hippocampus. Restriction of neurogenesis to specific zones appears to be caused by the microenvironments within each region. Astrocytes in neurogenic regions secrete factors and cytokines that promote neural differentiation whereas astrocytes in non-neurogenic zones secrete inhibitory factors [6].

While regeneration of the CNS is still limited in man, evidence of neuroregeneration has been shown in mice following focused injury [28, 83]. Following induced apoptosis in level VI corticothalamic neurons of mice, new neurons with projections to the thalamus were generated [83]. Similarly, neuroblasts migrated and entered the cortex where they differentiated into mature pyramidal cells in layer V following induced apoptosis of corticospinal neurons. These studies demonstrate that the mammalian brain is capable of regeneration following focused injury. Furthermore, the generated neurons were capable of extending projections to distant regions of the brain suggesting that in addition to cell replacement therapies, regenerative therapies that stimulate endogenous repair systems may be beneficial.

7.2 Stem Cells and their Phenotypic Characteristics

Stem cells are unspecialized cells found throughout the human body that have the ability to differentiate into a variety of highly specialized cell types (multipotency), and reproduce symmetrically or asymmetrically, resulting in two identical daughter stem cells or a daughter stem cell and a progenitor cell (a stem cell that has begun to differentiate into a specialized cell) [40]. This high potency and proliferative capacity makes stem cells a therapeutic target for neurodegenerative disorders.

Stem cells can be obtained from various sources including the blastocyst (embryonic stem cells), the fetus (fetal stem cells), and the tissue of any organism that has been born (adult stem cells). Stem cells can also be artificially generated from specialized tissue (induced pluripotent stem cells). The capacity for differentiation of stem cells depends largely on the source from which the cells were collected. Adult stem cells are multipotent, and therefore have a limited capacity for differentiation. Adult stem cells can be readily obtained from bone marrow, the umbilical cord and umbilical cord blood, the placenta, adipose tissue, teeth, and menstrual blood [14, 46, 57, 86, 95, 99–101, 111, 118, 131]. Bone marrow yields two distinct types of stem cells including hematopoietic stem cells (HSCs), which have been used to treat hematological disorders [73], and mesenchymal stem cells (MSCs), which have been shown to be beneficial in animal models of traumatic brain injury and stroke [46]. Embryonic stem cells are pluripotent, meaning they can differentiate into any type of cell in the human body. This pluripotency means that embryonic stem cells can more effectively differentiate into neural cells than their multipotent

counterparts, making them the ideal target for neuroregenerative therapies. However, the use of embryonic stem cells remains highly controversial as the collection of embryonic (and fetal) stem cells requires the termination of an embryo. Induced pluripotent stem cells are artificially created by inserting specific genes and proteins into adult tissue cells such as skin cells [72]. These cells are similar to embryonic stem cells, though their initial yield is quite small [81].

An appealing alternative to embryonic stem cells is the availability of very small embryonic-like stem cells (VSELs). These tiny stem cells, which exhibit robust progenitor stem cell markers, can be isolated from a variety of adult organs, including the brain. After tissue and organ injuries, these very small stem cells are mobilized into the peripheral blood (PB). Beyond PB, purified VSELs can be isolated from umbilical cord blood and bone marrow, allowing easy access to these stem cells. Because of their small size and low constitution of PB, their isolation requires special flow cytometric protocols which have been established for identification [66]. Indeed, human VSELs are smaller than an erythrocyte and they are considered to belong to the nonhematopoietic fraction of leukocytes (Lin−/CD45 cells) expressing CD34, CD133, and CXCR4 antigens [66]. In addition to CD34, CD133, and CXCR4 antigens expressed by VSEL, phenotypic markers employed to identify them include positive expression of CD45 (mouse and human), Sca-1 (mouse), CXCR4, and progenitor stem cell markers (i.e., Oct-4, Nanog, and Stage-Specific Embryonic Antigen (SSEA)), altogether reminiscent of epiblast/germ line stem cells [66]. That VSELs display phenotypic features of epiblast/germ stem cells recapitulating the early stages of embryonic development, these stem cells pose as excellent candidate for stem cell regenerative therapies. As noted above, with VSELs harvested from the patients' own mobilized PB, stored umbilical cord blood, and bone marrow aspirates, therapeutic applications of these cells support their use for autologous transplantation. However, technical challenges in the expansion relatively low number of cells in the initial yield of VSELs may limit the target patient population for autologous transplantation, especially for treating acute brain injuries. Indeed, optimal functional benefits of stem cell therapy for acute and subacute stage are achieved with early therapeutic intervention (time 0–1 week after injury). Accordingly, the ready availability of stem cells for immediate treatment initiation in acute brain injuries may require cell amplification of VSELs which may not be feasible with routine multiparameter staining and regular high-speed sorting [135]. To circumvent this tedious amplication process, one can envision that an ample supply of VSELs can be accomplished from mobilized PB, stored umbilical cord blood, and bone marrow aspirates by providing them as frozen cell products that can be immediately thawed at the time of injury. Alternatively, a relatively short and economical three-step method has been established for cell isolation that allowed approximately 60% recovery of the initial number of Lin−/CD45−/CD133+ umbilical cord blood-derived VSELs [98]. This efficient cell isolation process takes 2–3 h per umbilical cord blood unit, which is equally feasible for mobilized PB and bone marrow aspirates, thereby facilitating the harvest of VSELs freshly isolated and with cell fate commitment directed toward a neurological lineage appropriate for treating brain disorders [98].

In general, the robust multipotency and pluripotency of stem cells provide the basis for their therapeutic applications in neurodegenerative disorders, in that the transplanted stem cells can rapidly replace impaired and dead neural cells upon differentiation. However, the pluripotency and highly proliferative capacity of stem cells increase their potential to become cancerous due to rapid growth or improper differentiation. As such, further investigation and careful regulation is needed to develop safe and efficacious stem cell treatments for neurodegenerative conditions.

Recent animal-based research on the use of stem cells for treatment of neurological disorders has shown that the observed benefit frequently results from a mechanism other than cell replacement [92, 100]. Most studies evaluate the transplantation of stem cells which involves harvesting the cells and then amplifying them in culture prior to transplantation. However, this method overlooks the availability of stem cells within specialized niches in the organs of the body. An alternative approach is to activate endogenous peripheral stem cells throughout the body, rather than transplant new ones. Interestingly, stem cell transplantation in animal models has been shown to stimulate such endogenous stem cell populations [5, 93].

7.3 Regenerative Approaches

In the ensuing sections tackling regenerative approaches, we highlight the major brain disease for each type of neurologic disorder considered as a therapeutic target for stem cell therapy.

7.3.1 Acute Brain Injuries: Stroke

Different stem cell-based therapies display varying efficacy in treating differing types of neurological disorders. As such, stem cell therapies can be categorized by the neurological disorders which they most efficaciously treat. The first category encompasses conditions caused by acute injury. Acute injury typically peaks within 24 h of a single injury-causing event, and reaches completion within 48 h. Pertinent acute injuries include stroke, spinal cord injury, neonatal hypoxic-ischemic encephalopathy, and traumatic brain injury; all of which are responsible for major mortality and morbidity. Due to the quick onset and short duration of acute injuries, potential neuroprotective therapies need to be administered early; typically within 3–6 h of onset. This has proven to be challenging in the clinical setting. Any treatment outside of the 48-h window will offer no protection, and will instead be mainly restorative, targeting angiogenesis, neurogenesis, and synaptogenesis. Figure 7.1 shows the impact of therapeutic treatment plotted against time of administration after onset.

The less restrictive time window of neurorestorative treatments makes them well suited for utilization in a clinical setting.

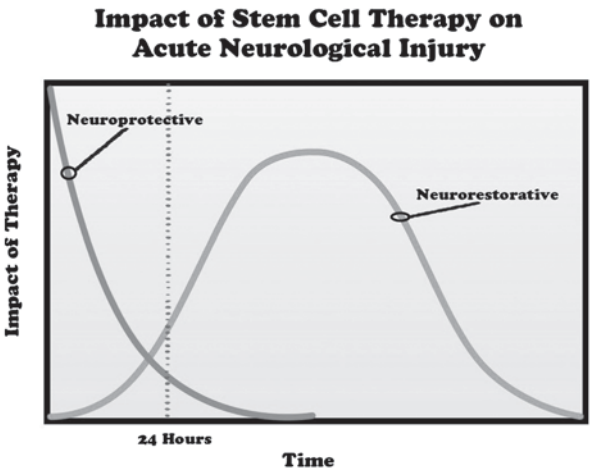

Fig. 7.1 Neuroregenerative capacity of stem cell therapy. In order to elicit a neuroprotective effect following acute neuronal injury, therapy needs to be initiated within 24–48 h of insult. Therapy started more than 48 h after the insult will mainly facilitate neuroregeneration. [44]

Cerebral ischemia is a neurological disorder caused by acute injury, which consists of the destruction of a variety of cell types including neurons, astrocytes, endothelial cells, and pericytes. As such, any pertinent stem cell therapy will need to encompass the regeneration of both neural cells and supporting structures such as blood vessels. Previous studies have suggested that an endogenous repair mechanism, such as the upregulation of nestin in neural stem cells (NSC) and neural progenitor cells (NPC) [35, 75], ensues following cerebral ischemia. Surprisingly, evidence suggests that such NSCs and NPCs in the brain and spinal cord are actually astroglial cells [2, 32, 33]. While neurogenesis in humans is largely restricted to the dentate gyrus of the hippocampus, NSC and NPC can be harvested from other non-hippocampal regions of the human brain [107, 108, 117]. In rodents, following middle cerebral artery occlusion (MCAO), the subventricular progenitor cells proliferate and migrate to the striatum where they contribute to the formation of striatal spiny neurons and glial cells [4]. Although the majority of these neuroblasts undergo apoptosis, leaving no evidence of neuron formation in the cortex, these subventricular progenitor cells continue to migrate to the striatum for at least 4 months following injury. This characteristic "homing" of sorts is mediated by CXCR4, found on NSCs and NPCs, and stromal cell-derived factor-1 (SDF-1) which is upregulated in the astrocytes and endothelial cells of injured tissue [48, 56, 112]. Similarly, SDF-1 upregulation, which occurs for a minimum of one moth post-ischemia, has also been shown to drive the migration of bone-marrow-derived stem cells involved in repair [45]. While these studies demonstrate the existence of an endogenous repair system that is activated by an ischemic event, it alone is largely insufficient for significant recovery.

Stimulation of such endogenous repair systems through the utilization of trophic factors is a potential therapy that is likely to have an early impact on cerebral ischemia-induced tissue damage. As such, hematopoietic cytokines (cytokines with trophic factors on bone marrow cells) are already in clinical stroke trials [7].

Granulocyte colony-stimulating factor (G-CSF), an Food and Drug Administration (FDA)-approved drug used to mobilize CD34 cells for bone marrow transplant and treatment of neutropenia after chemotherapy, has been shown to be effective in rodent stroke models, and has a therapeutic treatment window extending 24 h [105]. During cerebral ischemia, the G-CSF receptor is upregulated on neurons [102], and serves to reduce apoptosis and activate endogenous NPCs [102]. Furthermore, following an incident of stroke, G-CSF mobilizes bone-marrow-derived stem cells which participate in neurogenesis and angiogenesis [59]. Similarly, erythropoietin, a cytokine involved in red blood cell production, demonstrates neuroprotection in rodent models of cerebral ischemia [16], and displays neurorestorative effects, such as stimulating angiogenesis and neurogenesis, when administered 24 h post-stroke [121]. Although one small clinical trial showed erythropoietin to be safe and efficacious [36], more recent studies reveal no significant benefit, and an increased mortality following administration of erythropoietin [37]. One original safety concern regarding the utilization of erythropoietin was the resulting rise in hematocrit; however, such concerns have been addressed through the development of a carbamylated erythropoietin, which retains neuroprotective capacity while eliminating the concerning erythropoietic effects [34].

The first cell therapy for stroke consisted of intracerebral transplantation of human neuroteratocarcinoma (hNT) cells. Human hNT cells are derived from a teratocarcinoma line (NT2-N) exposed to retinoic acid [12, 62]. Twelve patients with a basal ganglia stroke were transplanted between 6 months and 6 years post-stroke. No tumor formation occurred, and positron emission tomography (PET) scans revealed increased cell uptake in the majority of patients. A subsequent phase II study demonstrated the safety of the transplant but revealed no efficacy [63].

One hindrance to neural transplantation therapy for stroke is the development of a cystic cavity in the tissue, which prevents transplanted cells from having the necessary blood flow and anchoring matrix. One study on neonatal hypoxic-ischemia evaluated the transplantation of a biodegradable scaffold on which NSCs had been seeded prior to being transplanted into the large cavity [91]. The scaffold degraded, leaving transplanted neurons which were capable of directed, target-appropriate neurite outgrowth. The use of a scaffold is an attractive option for ischemic therapy when large cavities are present.

Human umbilical cord blood stem cells have also been reported to be beneficial in animal models of cerebral ischemia [13, 25, 109, 116], particularly through intravenous administration [122]. The time window for intravenous delivery extends up to 48 h [87]. However, several studies have found human umbilical cord blood cells to be ineffective in improving stroke outcomes in animal models, and few human umbilical cord bloods (HUCBs) are found in the brain following transplantation [84]. Cord blood derived CD34 cells have improved functional outcome and increased angiogenesis, when delivered 48 h post-stroke [109]. The beneficial effects elicited by these cells appeared to be from their ability to promote angiogenesis and immunosuppression. Furthermore, entry of the HUCB cells into the brain does not appear to be required to elicit such benefits [13].

Bone-marrow-derived cells are also promising therapeutic agents for stroke. Bone-marrow-derived stem cells and progenitor cells are easily obtained without ethical complications, are highly proliferative, and are likely to have an early impact on neurological diseases. Additionally, the well-established practice of blood banking could be readily applied to the collection and storage of bone-marrow-derived stem cells. Bone marrow contains two populations of cells including HSC and MSCs. MSCs can be collected from bone marrow and other tissues, and are capable of differentiating into cartilage, bone, and adipose tissue. The local environment and extracellular matrix influences MSC differentiation, and when grown under certain conditions, such as on a soft matrix, MSCs can be driven to adopt a neuronal phenotype [15, 29, 38, 42, 123, 124]. MSCs are also known to support other cell types through the secretion of cytokines and trophic factors [23], and have been shown to be beneficial in animal models of stroke [31]. Furthermore, modification by growth-factor-encoding genes such as brain-derived neurotrophic factors and placental growth factor has been shown to enhance the therapeutic effect of human MSCs in rodent models of cerebral ischemia [80, 89]. MSCs are also anti-inflammatory, immunomodulatory, and immune privileged, and therefore have the potential to be used in allogenic transplantations [1, 10] from healthy donors, and allow MSCs to be used as an "off the shelf" treatment.

In an middle cerebral artery occlusion (MCAO) rodent model, MSCs applied intracerebrally, intravenously, or intra-arterially improved functional outcome in a dose-dependent manner [24, 25, 76]. Indeed, functional improvement was observed even when intravenous transplantation was administered up to 1 month post-insult [104]. While no reduction in infarct size was observed, an increase in neuroregeneration, angiogenesis, and synaptogenesis was noted following MSC transplantation [26, 27]. The observed effectiveness of intravenous administration makes MSC transplantation an appealing therapy.

Isolated by Verfaillie and colleagues, multipotent adult progenitor cells (MAPC) are a highly proliferative subset of MSCs [50–52]. MAPCs are pluripotent cells capable of differentiating into cell types of all three germ layers, and can be isolated from bone marrow, the brain, and muscle tissue [51]. MAPCs are capable of generating all tissues when injected into blastocysts, without the formation of teratomas. MAPCs have been shown to be effective in animal models of cerebral ischemia and neonatal hypoxic-ischemic encephalopathy [127, 128]. MAPCs intracerebrally transplanted 1 week after cortical stroke expressed neuronal markers and improved sensorimotor function. Despite the noted improvement, only a few transplanted cells expressed neuronal markers, suggesting that the observed enhancement was from a trophic effect elicited by the transplanted cells [134]. This strongly supports the notion that the underlying mechanism of action of MSCs is the ability to act as a trophic factor, rather than direct cell replacement [31].

Currently, MSC and HUCB appear as the most promising as donor cells for cell therapies with regards to stroke. Both transplanted cell types are effective when delivered intravenously, highly proliferative, and have extended time windows, making them optimal for a clinical setting. The extended time window is of interest as the current treatment, tissue plasminogen activator, has only a 4.5-h window for

administration and, as a result, is only employed in approximately 3 % of ischemic stroke incidents in the USA. The minimally invasive intravenous administration, in combination with an extended time window, makes MSC and HUCB therapies pertinent and fitting treatments for clinical stroke.

7.3.2 Chronic Neurodegenerative Diseases: Parkinson's Disease

Chronic neurodegenerative diseases encompass the second category of conditions which benefit from stem cell therapies. In chronic neurodegenerative diseases, the degeneration can occur without apparent clinical symptoms. As a result, the time of onset is unknown. While cell death and injury in these diseases is slow, it is also inexorable. As such, an ideal therapeutic treatment should be both neuroprotective and neurorestorative. This category of diseases includes amyotrophic lateral sclerosis, Huntington's disease, Alzheimer's disease, and Parkinson's disease. Some of these diseases, such as Parkinson's disease, are characterized by cell loss within a specific cell population. Parkinson's disease is characterized by extensive loss of dopaminergic neurons in the pars compacta of the substantia nigra. Early studies reported that motor disability in patients was improved through the use of fetal dopaminergic neurons from aborted fetuses [19, 43, 79]. While several of these grafts survived in the brains of transplanted patients [43], this approach was discontinued after two randomized, controlled studies showed little benefit, and incidence of dyskinesia [41, 90].

Due to a lack of supporting data, and ethical concerns surrounding the use of cells from aborted fetuses, investigators began pursuing the use of embryonic stem cells. Embryonic cells are able to differentiate into dopaminergic neurons in culture. Furthermore, undifferentiated embryonic stem cells have been shown to differentiate into dopaminergic neurons and attenuate motor deficits when transplanted into the striatum of a rodent model of Parkinson's disease [11]. However, transplantation of such undifferentiated embryonic stem cells is concerning due to the risk of teratoma formation. Partial behavioral recovery was observed in a rodent model of Parkinson's disease following transplantation of human embryonic stem cell-derived neural progenitors into the rodent striatum [9, 107]. However, in the study conducted by Ben-Hur and colleagues [9], the progenitor cells did not achieve a full dopaminergic phenotype, suggesting that differentiation into midbrain dopaminergic neurons should be completed before transplantation. In the 1-methyl-4-phenyl1,2,3,6-tetrahydropyridine (MPTP) primate model of Parkinson's disease, transplantation of embryonic stem cell-derived enriched dopaminergic neurons attenuated functional deficit despite exceptionally low graft survival rates; less than 1 % 14 weeks post-transplant [110].

Dopaminergic neurons derived from adult stem cells present an attractive alternative to ethically hindered embryonic and fetal stem cells. In culture, adult MSC can be induced to differentiate into cells that express many of the same characteristics and genes as dopaminergic neurons [42, 65]. Furthermore, it has been demonstrated

that MSCs transplanted intrastriatally in a mouse MPTP model are able to survive, express tyrosine hydroxylase activity, and improve function [77].

Glial cell line-derived neurotrophic factor (GDNF) has been shown to be involved in the preservation and differentiation of dopaminergic neurons in culture [78]. Potential methods of GDNF gene delivery into the substantia nigra include the use of adenoviral and lentiviral vectors. In a 6 hydroxydopamine rodent model, injection of a replication-defective adenoviral vector encoding human GDNF into the substantia nigra reduced the loss of dopaminergic cells [30]. In an MPTP non-human primate model of Parkinson's disease, lentiviral vector GDNF delivery prevented nigrostriatal degeneration entirely and improved function [64]. The promising results of these two studies led to open-label trials in Parkinson's disease patients, in which GDNF was continuously infused into the striatum with a catheter [94, 106]. The initial findings of these two trials were promising, however, in a randomized controlled study, Parkinson's disease patients receiving bilateral intraputaminal GDNF infusions showed no significant benefit when compared to the control group (Parkinson's disease patients whom did not receive GDNF infusions) [68]. Additionally, several concerns were raised regarding cerebellar toxicity in primates receiving large amounts of GDNF.

An alternative method for GDNF delivery is the utilization of NSCs as cellular delivery vehicles for the large protein which otherwise penetrates the blood brain barrier poorly. As such, human NPCs have been utilized to continuously deliver GDNF following transplantation. Genetically modified human NPCs programmed to release glycosylated GDNF in vitro have been transplanted into the striatum of rats following partial lesion of the dopamine system. These grafted cells survived and secreted GDNF for 8 weeks, partially ameliorating the deficit [8]. Aged primate models demonstrated similar success as human NPCs secreted GDNF for 3 months [8]. Additionally, genetically modified human NPCs transplanted into a rodent model of amyotrophic lateral sclerosis survived, integrated, and released GDNF in the spinal cord of superoxide dismutase 1 (SOD1; G93A) rats [61].

Further investigation regarding potential Parkinson's disease stem cell therapies is needed. Continuous infusion of GDNF via catheter is technically difficult, however genetically modified GDNF-releasing human NPCs with an inducible promoter provide an attractive alternative. Such NPCs have survived and expressed GDNF for significant periods of time in rodent and non-human primate models without evidence of tumor formation [8]. However, unregulated dopamine secretion from grafted dopamine-producing NPCs is likely to cause unwanted dyskinesia and motor control. At this time, activating neuroprotection and neurogenesis via stimulation of endogenous repair mechanisms is most likely to have an immediate impact. Additionally, as Parkinson's disease involves more than just dopaminergic degeneration in the substantia nigra pars compacta (SNc), potential therapies will need to entail more than mesencephalic transplantation.

7.3.3 Inflammation-Mediated Disease: Multiple Sclerosis

The final category of neurological diseases consists of chronic inflammatory and immunologically mediated conditions such as multiple sclerosis. Multiple sclerosis is an immunologically mediated disease often characterized by periods of relapse and remission. The disease begins with inflammation and progresses to axonal degeneration [82, 113] and represents the major cause of neurological disability in the young adults of Western nations. Currently, the most advanced and successful stem cell treatment for multiple sclerosis is autologous bone marrow transplantation. The thinking behind this technique is that it can be used to "reset" and regenerate the immune system using the patient's own HSC [21, 22]. Initial protocols for autologous bone marrow transplantation for multiple sclerosis involved myeloablative conditioning regimens. However, due to the unexpectedly high toxicity of this treatment, protocols have since been amended to utilize non-myeloablative and lymphoablative techniques [22]. Early experience suggested that treatment should be started early in the disease while it still has a largely inflammatory component.

An additional cell therapy with potential to treat multiple sclerosis and neuroinflammatory diseases aims to remyelinate the CNS using NSCs [96]. Multipotent astroglial NPCs have been isolated from various forebrain regions of the human brain and expanded in culture [120]. These genetically modifiable adult NPCs are highly expandable, often with minimal senescence and without evidence of immortalizing mutations; one progenitor cell has the theoretical proliferative capacity to form enough cells for 4×10^7 brains. In a mouse model of multiple sclerosis, intravenous and intraventiculocerebral delivery of syngeneic NPCs inspired remyelination, and reduced astrogliosis and functional deficits [97]. Additionally, several of the grafted NPCs developed neural markers and stimulated endogenous oligodendrocyte progenitor proliferation. In a rodent model of chronic multiple sclerosis, a perivascular accumulation of undifferentiated NPCs occurred in areas of inflammation following intravenous injection of syngeneic neuroshpere-derived multipotent NPCs [97]. The undifferentiated NPCs exhibited anti-inflammatory and immunosuppressive effects which triggered apoptosis in infiltrating T cells, ultimately preventing further disease activity. These studies suggest that in multiple sclerosis patients, stem cells may have an anti-inflammatory effect on pro-inflammatory environments, and a regenerative effect when placed in demyelinated regions [115].

An important characteristic of stem cells is their anti-inflammatory and immunosuppressive capacity in pro-inflammatory neurological diseases. MSC and marrow stromal cells have also been shown to be effective in experimental allergic encephalomyelitis models when delivered intravenously [132, 133]. This data suggests that the anti-inflammatory effects of NSC and MSC could be a promising target for early multiple sclerosis therapy when there is a substantial inflammatory component.

7.4 Translational Gating Items Toward Clinical Applications

7.4.1 Optimum Cell Type

Despite the promising potential of stem cell therapies for neurodegenerative disease, each treatment is bound by several discrepancies which need to be resolved in order to maximize therapeutic benefit. One of the major questions that need to be addressed is that of optimum cell type. The human brain has two endogenous stem cell populations, one within the subventricular zone, and one within the subgranular zone of the hippocampus. These endogenous cells are upregulated in animal models following injury. Although these populations are believed to be involved in repair, this mechanism alone is not sufficient to counter the effects of neurodegenerative diseases. Additionally, due to their location deep within the brain, it is fairly impractical to remove them from an adult patient for culture and re-transplantation. Furthermore, while autologous transplants have been shown to be beneficial [20, 74], the protection/regeneration may not be withstanding as the underlying cause of the degeneration remains unaddressed.

Potential alternatives to using endogenous NSCs include pre-neurally differentiated embryonic stem cell, fetal neural cells, and other undifferentiated stem cells. While these avoid the complications of extracting endogenous neural cells, concerns are raised regarding rejection of the graft. There is evidence suggesting that certain stem cells are immune immature and therefore will not elicit a host response; however, studies have shown that these cells are still cleared by the host fairly rapidly, with only a few surviving more than a month. Numerous studies implementing immunosuppression also show a lack of long-term survival of transplanted cells. In animal studies, cells have been shown to "home" toward the injured tissue as a result of cytokines and inflammatory tissues [49, 92, 125, 126]. Immunosuppression could suppress such signals, ultimately making grafted cells less effective than autologous cells grafted without immunosuppression.

MSCs have also been shown to be beneficial in numerous diseases. Of note, MSCs derived under the standardized methods of the International Society for Cellular Therapy proved detrimental in an animal model of multiple sclerosis, contrary to previous data [47]. In one clinical trial in which autologous MSCs were transplanted intrathecally for chronic spinal cord injury, adverse effects were observed in over half of the participating patients [60]. However, previous studies have shown MSCs to be safe and efficacious in treating spinal cord injury and numerous other disorders [55, 58].

A number of different cell types including MSCs, umbilical cord stem cells, and embryonic stem cells have been shown to differentiate into neural-like cells when prompted under the appropriate conditions in vitro. However, it is unclear if the same conditions, and therefore the same differentiation, would occur in vivo. One benefit of using pre-differentiated stem cells is that there is a reduced likelihood of tumor formation following transplantation. However, pre-differentiated cells are

likely to be immune mature and therefore subject to host-graft rejection, unless the cells are autologous. An advantage of using stem cells derived from bone marrow and adipose tissue is that they can easily be obtained from all patients and can therefore always be autologous.

While each source of stem cells has various advantages and disadvantages, the answer to which cell type is the most beneficial is elusive. In order to determine the optimum cell type for neuroregenerative therapy, further studies are needed regarding the safety and efficacy of each cell type.

7.4.2 Autologous or Allogenic Transplantation and Use of Immunosuppression

As previously discussed, there is a large discrepancy regarding the use of autologous vs. allogenic grafts, and immunosuppression. This topic is especially debated as numerous stem cells have been shown to be immune immature and therefore, may not elicit a host response or prevent certain beneficial effects of stem cells such as a "homing" effect [67]. In a recent study evaluating the safety of allogenic grafts, umbilical cord blood stem cells were transplanted into 114 patients, intravenously and intrathecally, without any adverse side effects [125, 126]. This suggests that umbilical cord blood stem cells may be immune immature and could therefore be used to treat a variety of neurological disorders without the likelihood of an immune response. Umbilical cord blood stem cells, as well as MSCs, have been shown to improve cognition in Alzheimer's animal models [69, 70, 88]. The underlying mechanism of this therapeutic effect is related to the release of anti-inflammatory cytokines and reduced glial activation following transplantation [71, 88].

While many would agree that autologous transplantation would be the most beneficial, recent studies have found that many of the concerns of allogenic transplants, such as host rejection, may not be relevant. At this time, further studies are needed to evaluate the safety, ease, and efficacy of both autologous and allogenic transplants. Additionally, when considering which stem cell type to use, the size and complexity of the dying cell must be taken into consideration. The original size of the cell is determined during development, and it is uncertain as to whether the same developmental cues will be present for the transplanted cells. This is extremely pertinent to potential cell replacement therapies for neurodegenerative diseases.

7.4.3 Route of Administration

The optimal cell type and dosage depends not only on the disease at hand but also on the route of administration. In a clinical setting, the optimal delivery method is always that which is least invasive, and would therefore be oral administration. However, intravenous administration is more likely a suitable option for stem cell transplant, provided the cells can adequately pass through the blood brain barrier.

Of note, several studies of animal models have shown that crossing the blood-brain barrier does not appear to be necessary for the cells to exert beneficial effects [13]. Additionally, since peripheral modulation is involved in certain disorders, intravenous administration may be ideal in these cases.

In certain instances, entry into the brain may be optimal, making intracerebroventrical injection the ideal option. Several comparative studies have evaluated the efficacy of intravenous and intracerebral transplantation of stem cells in an animal model of neonate hypoxic-ischemic injury. The benefits observed by both routes of administration were nearly identical in terms of motor score and hippocampal cell preservation, suggesting that either route is equally beneficial for this disorder [129]. A recent study by Jiang and colleagues [53] showed that intranasal administration may also permit cell entry into the brain, and therefore represent an effective, non-invasive method of administration. In order to advance to a clinical setting, various routes of administration for stem cell therapies need to be evaluated to determine the least invasive and most effective method.

7.4.4 Mode of Action

In addition to the above considerations, one also needs to take into account the mode of action. It is obvious from several studies that replacement of damaged or dead cells is not the primary means of action. Commonly, an ameliorative effect is elicited through the presence of trophic factors and anti-inflammatory molecules, which are provided by the transplanted stem cells. Such molecules make the surrounding pro-inflammatory environment more suitable for cell survival and repair [92]. The secretion of growth factors and ability to regulate pro-inflammatory signals, such as microglial activity, has been demonstrated by numerous stem cell types, suggesting that there are multiple underlying mechanisms. Furthermore, this could mean that a heterogeneous transplantation containing numerous stem cell types could be more beneficial than a homogenous transplant [85].

7.4.5 Tumorigenicity

One potential disadvantage associated with using non-NSCs is their ability to differentiate into other cell types, potentially leading to the formation of cancerous tissue. A recent study demonstrated tumor formation in ataxia telangiectasia patients following transplantation of donated stem cells derived from multiple donors [3]. While the donated cells contained some neural cells, it is unclear as to whether the tumor resulted from those cells or from other "contaminating" cells. There are also numerous studies that show no evidence of cancerous tissue following stem cell transplantation, unless embryonic or induced pluripotent stem cells are used. Although we do not know how commonly tumor formation occurs following the transplantation of embryonic or induced pluripotent cells, the ability of the cells to

generate tumors comprised all three layers is a defining characteristic of embryonic and induced pluripotent stem cells. One study investigated induced pluripotent cells belonging to one of two groups: those which formed tumors, and those which did not form tumors following transplantation in nude mice. When transplanted into the spinal cord of mice, both cell types differentiated into neurons, astrocytes, and oligodendrocytes. However, while both treatments initially showed functional improvement, the tumor-forming induced pluripotent cells led to the development of tumors as the study progressed. Furthermore, the observed improvement was lost over time in the tumor-forming cell line [114]. A study conducted by Joseph and colleagues [54] demonstrated that the knockout of neurofibromin, a component of neurofibromas and malignant peripheral sheath tumors, from NSCs did not alter the occurrence of such tumors, suggesting that NSCs are not involved in the formation of such tumors. While proposals have been made about how to address resulting cancerous formations, each is subject to its own limitations and inherent problems that could potentially add to cancerous tumors or kill healthy cells.

Conclusion

The limited capacity for neurogenesis in the human CNS makes neurological disease especially difficult to treat. Stem cell research may play an important role in the treatment of neurological diseases through cell replacement and stimulation of endogenous repair systems. However, there are still numerous questions that remain unanswered with regard to stem cell therapies. Several topics of investigation include optimal cell type and dosage, mechanism of action, and route of administration. These answers depend largely on the targeted disease and desired mechanism of action. As discussed, recent studies have also found success in using stem cells as trophic factors. The use of genetically modified stem cells shows promise in correcting degenerative disorders by over expressing and stimulating the secretion of endogenous neurotrophic factors. The efficacy of stem cell therapies will likely be related to the extent of which the cells exhibit trophic factors, and other secreted therapeutic molecules (e.g., anti-inflammatory chemokies and cytokines). While substantial progress has been made in neuroregenerative stem cell therapies, further investigation is needed to optimize them for clinical use.

Acknowledgments The Borlongan Laboratory is supported by NIH NINDS UO15U01NS055914-04, NIH NINDS R01NS071956-01, James and Esther King Foundation for Biomedical Research Program, Department of Defense TATRC Program, USF Signature Program in Interdisciplinary Neuroscience, San Bio Inc., Celgene Cellular Therapeutics, KMPHC, and Neural Stem Inc. CVB has patents and pending patents on cell therapy.

References

1. Aggarwal S, Pittenger MF (2005) Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105:1815–1822
2. Alvarez-Buylla A, Seri B, Doetsch F (2002) Identification of neural stem cells in the adult vertebrate brain. Brain Res Bull 57:751–758
3. Amariglio N, Hirshberg A, Scheithauer BW et al (2009) Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Med 6:e1000029
4. Arvidsson A, Collin T, Kirik D et al (2002) Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 8:963–970
5. Bachstetter AD, Pabon MM, Cole MJ et al (2008) Peripheral injection of human umbilical cord blood stimulates neurogenesis in the aged rat brain. BMC Neurosci 9:22
6. Barkho BZ, Song H, Aimone JB et al (2006) Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation. Stem Cells Dev 15:407–421
7. Bath PM, Sprigg N (2007) Colony stimulating factors (including erythropoietin, granulocyte colony stimulating factor and analogues) for stroke. Cochrane Database Syst Rev 18(2):CD005207
8. Behrstock S, Ebert A, McHugh J et al (2006) Human neural progenitors deliver glial cell line-derived neurotrophic factor to parkinsonian rodents and aged primates. Gene Ther 13:379–388
9. Ben-Hur T, Idelson M, Khaner H et al (2004) Transplantation of human embryonic stem cell-derived neural progenitors improves behavioral deficit in Parkinsonian rats. Stem Cells 22:1246–1255
10. Beyth S, Borovsky Z, Mevorach D et al (2005) Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsivenss. Blood 105:2214–2219
11. Björklund LM, Sánchez-Pernaute R, Chung S et al (2002) Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. Proc Natl Acad Sci U S A 99:2344–2349
12. Borlongan CV, Tajima Y, Trojanowski JQ et al (1998) Transplantation of cryopreserved human embryonal carcinoma-derived neurons (NT2 N cells) promotes functional recovery in ischemic rats. Exp Neurol 149:310–321
13. Borlongan CV, Hadman M, Sanberg CD et al (2004) Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke. Stroke 35:2385–2389
14. Borlongan CV, Kaneko Y, Maki M et al (2010) Menstrual blood cells display stem cell-like phenotypic markers and exert neuroprotection following transplantation in experimental stroke. Stem Cells Dev 19:439–452
15. Bossolasco P, Cova L, Calzarossa C et al (2005) Neuro-glial differentiation of human bone marrow stem cells in vitro. Exp Neurol 193:312–325
16. Brines ML, Ghezzi P, Keenan S et al (2000) Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. Proc Natl Acad Sci U S A 97:10526–10531
17. Brockes JP (1997) Amphibian limb regeneration: rebuilding a complex structure. Science 276:81–87
18. Brockes JP, Kumar A (2005) Appendage regeneration in adult vertebrates and implications for regenerative medicine. Science 310:1919–1923
19. Brundin P, Pogarell O, Hagell P et al (2000) Bilateral caudate and putamen grafts of embryonic mesencephalic tissue treated with lazaroids in Parkinson's disease. Brain 123:1380–1390
20. Brunet JF, Redmond DE Jr, Bloch J (2009) Primate adult brain cell autotransplantation, ba pilot study in asymptomatic MPTP-treated monkeys. Cell Transplant 18:787–799
21. Burt RK (1997) Immune ablation and hematopoietic stem cell rescue for severe autoimmune diseases (SADS). Cancer Treat Res 77:317–332
22. Burt RK, Cohen B, Rose J et al (2005) Hematopoietic stem cell transplantation for multiple sclerosis. Arch Neurol 62:860–864

23. Caplan AI, Dennis JE (2006) Mesenchymal stem cells as trophic mediators. J Cell Biochem 98:1076–1084
24. Chen J, Li Y, Wang L et al (2001a) Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. J Neurol Sci 189:49–57
25. Chen J, Sanberg PR, Li Y et al (2001b) Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. Stroke 32:2682–2688
26. Chen J, Li Y, Katakowski M et al (2003a) Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. J Neurosci Res 73:778–786
27. Chen J, Zhang ZG, Li Y et al (2003b) Intravenous administration of human bone marrow stromal cells induces angiogenesis in the ischemic boundary zone after stroke in rats. Circ Res 92:692–699
28. Chen J, Magavi SS, Macklis JD (2004) Neurogenesis of corticospinal motor neurons extending spinal projections in adult mice. Proc Natl Acad Sci U S A 101:16357–16362
29. Cho KJ, Trzaska KA, Greco SJ et al (2005) Neurons derived from human mesenchymal stem cells show synaptic transmission and can be induced to produce the neurotransmitter substance P by interleukin-1 α. Stem Cells 23:383–391
30. Choi-Lundberg DL, Lin Q, Chang YN et al (1997) Dopaminergic neurons protected from degeneration by GDNF gene therapy. Science 275:838–841
31. Chopp M, Li Y (2002) Treatment of neural injury with marrow stromal cells. Lancet Neurol 1:92–100
32. Doetsch F (2003) The glial identity of neural stem cells. Nat Neurosci 6:1127–1134
33. Doetsch F, Caille I, Lim DA et al (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703–716
34. Doggrell SA (2004) A neuroprotective derivative of erythropoietin that is not erythropoietic. Expert Opin Investig Drugs 13:1517–1519
35. Duggal N, Schmidt-Kastner R, Hakim AM (1997) Nestin expression in reactive astrocytes following focal cerebral ischemia in rats. Brain Res 768:1–9
36. Ehrenreich H, Hasselblatt M, Dembowski C et al (2002) Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med 8:495–505
37. Ehrenreich H, Weissenborn K, Prange H et al (2009) Recombinant human erythropoietin in the treatment of acute ischemic stroke. Stroke 40:e647–e656
38. Engler AJ, Sen S, Sweeney HL et al (2006) Matrix elasticity directs stem cell lineage specification. Cell 126:677–689
39. Eriksson PS, Perfilieva E, Björk-Eriksson T et al (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313–1317
40. Eve DJ, Marty PJ, McDermott RJ et al (2008) Stem cell research and health education. Am J Health Educ 39:167–179
41. Freed CR, Greene PE, Breeze RE et al (2001) Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med 344:710–719
42. Guo L, Yin F, Meng HQ et al (2005) Differentiation of mesenchymal stem cells into dopaminergic neuron-like cells in vitro. Biomed Environ Sci 18:36–42
43. Hauser RA, Freeman TB, Snow BJ et al (1999) Long-term evaluation of bilateral fetal nigral transplantation in Parkinson disease. Arch Neurol 56:179–187
44. Hess DC, Borlongan CV (2008) Stem cells and neurological diseases. Cell Prolif 41:94–114
45. Hill WD, Hess DC, Martin-Studdard A et al (2004) SDF-1 (CXCL12) is upregulated in the ischemic penumbra following stroke: association with bone marrow cell homing to injury. J Neuropathol Exp Neurol 63:84–96
46. Huang H, Chen L, Sanberg P (2010) Cell therapy from bench to bedside translation in CNS neurorestoratology era. Cell Med 1:15–46
47. Hunt DP, Irvine KA, Webber DJ et al (2008) Effects of direct transplantation of multipotent mesenchymal stromal/stem cells into the demyelinated spinal cord. Cell Transplant 17:865–873

48. Imitola J, Raddassi K, Park KI et al (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 101:18117–18122
49. Jackson JS, Golding JP, Chapon C et al (2010) Homing of stem cells to sites of inflammatory brain injury after intracerebral and intravenous administration: a longitudinal imaging study. Stem Cell Res Ther 1:17
50. Jiang Y, Jahagirdar BN, Reinhardt RL et al (2002a) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49
51. Jiang Y, Vaessen B, Lenvik T et al (2002b) Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol 30:896–904
52. Jiang Y, Henderson D, Blackstad M et al (2003) Neuroectodermal differentiation from mouse multipotent adult progenitor cells. Proc Natl Acad Sci U S A 100:11854–11860
53. Jiang Y, Zhu J, Xu G et al (2011) Intranasal delivery of stem cells to the brain. Expert Opin Drug Deliv 8:623–632
54. Joseph NM, Mosher JT, Buchstaller J et al (2008) The loss of Nf1 transiently promotes self-renewal but not tumorigenesis by neural crest stem cells. Cancer Cell 13:129–140
55. Joyce N, Annett G, Wirthlin L et al (2010) Mesenchymal stem cells for the treatment of neurodegenerative disease. Regen Med 5:933–946
56. Kabos P, Ehtesham M, Black KL et al (2003) Neural stem cells as delivery vehicles. Expert Opin Biol Ther 3:759–770
57. Karaoz E, Demircan PC, Saglam O et al (2011) Human dental pulp stem cells demonstrate better neural and epithelial stem cell properties than bone marrow-derived mesenchymal stem cells. Histochem Cell Biol 136:455–473
58. Karussis D, Karageorgiou C, Vaknin-Dembinsky A et al (2010) Safety and immunological effects of mesenchymal stem cell transplantation in patients with multiple sclerosis and amyotrophic lateral sclerosis. Arch Neurol 67:1187–1194
59. Kawada H, Takizawa S, Takanashi T et al (2006) Administration of hematopoietic cytokines in the subacute phase after cerebral infarction is effective for functional recovery facilitating proliferation of intrinsic neural stem/progenitor cells and transition of bone marrow-derived neuronal cells. Circulation 113:701–710
60. Kishk NA, Gabr H, Hamdy S et al (2010) Case control series of intrathecal autologous bone marrow mesenchymal stem cell therapy for chronic spinal cord injury. Neurorehabil Neural Repair 24:702–708
61. Klein SM, Behrstock S, McHugh J et al (2005) GDNF delivery using human neural progenitor cells in a rat model of ALS. Hum Gene Ther 16:509–521
62. Kondziolka D, Wechsler L, Goldstein S et al (2000) Transplantation of cultured human neuronal cells for patients with stroke. Neurology 55:565–569
63. Kondziolka D, Steinberg GK, Wechsler L et al (2005) Neurotransplantation for patients with subcortical motor stroke: a phase 2 randomized trial. J Neurosurg 103:38–45
64. Kordower JH, Emborg ME, Bloch J et al (2000) Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. Science 290:767–773
65. Kramer BC, Woodbury D, Black IB (2006) Adult rat bone marrow stromal cells express genes associated with dopamine neurons. Biochem Biophys Res Commun 343:1045–1052
66. Kucia M et al (2007) Morphological and molecular characterization of novel population of CXCR4+ SSEA-4+ Oct-4+ very small embryonic-like cells purified from human cord blood: preliminary report. Leukemia 21:297–303
67. Kulbatski I (2010) Stem/precursor cell-based CNS therapy: the importance of circumventing immune suppression by transplanting autologous cells. Stem Cell Rev 6:405–410
68. Lang AE, Gill S, Patel NK et al (2006) Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. Ann Neurol 59:459–466
69. Lee JK, Jin HK, Bae JS (2009) Bone marrow-derived mesenchymal stem cells reduce brain amyloidbeta deposition and accelerate the activation of microglia in an acutely induced Alzheimer's disease mouse model. Neurosci Lett 450:136–141

70. Lee HJ, Lee JK, Lee H et al (2010) The therapeutic potential of human umbilical cord blood-derived mesenchymal stem cells in Alzheimer's disease. Neurosci Lett 481:30–35
71. Lee HJ, Lee JK, Lee H et al (2012) Human umbilical cord blood-derived mesenchymal stem cells improve neuropathology and cognitive impairment in an Alzheimer's disease mouse model through modulation of neuroinflammation. Neurobiol Aging 33:588–602
72. Lensch MW (2009) Cellular reprogramming and pluripotency induction. Br Med Bull 90:19–35
73. Leung AY, Kwong YL (2010) Haematopoietic stem cell transplantation: current concepts and novel therapeutic strategies. Br Med Bull 93:85–103
74. Le´vesque MF, Neuman T, Rezak M (2009) Therapeutic microinjection of autologous adult human neural stem cells and differentiated neurons for Parkinson's disease: five-year post-operative outcome. Open Stem Cell J 1:20–29
75. Li Y, Chopp M (1999) Temporal profile of nestin expression after focal cerebral ischemia in adult rat. Brain Res 838:1–10
76. Li Y, Chen J, Wang L et al (2001a) Treatment of stroke in rat with intracarotid administration of marrow stromal cells. Neurology 56:1666–1672
77. Li Y, Chen J, Wang L et al (2001b) Intracerebral transplantation of bone marrow stromal cells in a 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine mouse model of Parkinson's disease. Neurosci Lett 316:67–70
78. Lin LF, Doherty DH, Lile JD et al (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260:1130–1132
79. Lindvall O, Widner H, Rehncrona S et al (1992) Transplantation of fetal dopamine neurons in Parkinson's disease: one - year clinical and neurophysiological observations in two patients with putaminal implants. Ann Neurol 31:155–165
80. Liu H, Honmou O, Harada K et al (2006) Neuroprotection by PlGF gene-modified human mesenchymal stem cells after cerebral ischaemia. Brain 129:2734–2745
81. Liu SP, Fu RH, Huang YC et al (2011) Induced pluripotent stem (iPS) cell research overview. Cell Transplant 20:15–19
82. Ludwin SK (2006) The pathogenesis of multiple sclerosis: relating human pathology to experimental studies. J Neuropathol Exp Neurol 65:305–318
83. Magavi SS, Leavitt BR, Macklis JD (2000) Induction of neurogenesis in the neocortex of adult mice. Nature 405:951–955
84. Mäkinen S, Kekarainen T, Nystedt J et al (2006) Human umbilical cord blood cells do not improve sensorimotor or cognitive outcome following transient middle cerebral artery occlusion in rats. Brain Res 1123:207–215
85. Matsuda R, Yoshikawa M, Kimura H et al (2009) Cotransplantation of mouse embryonic stem cells and bone marrow stromal cells following spinal cord injury suppresses tumor development. Cell Transplant 18:39–54
86. Miura M, Gronthos S, Zhao M et al (2003) SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A 100:5807–5812
87. Newcomb JD, Ajmo J, Craig T et al (2006) Timing of cord blood treatment after experimental stroke determines therapeutic efficacy. Cell Transplant 15:213–223
88. Nikolic WV, Hou H, Town T et al (2008) Peripherally administered human umbilical cord blood cells reduce parenchymal and vascular beta-amyloid deposits in Alzheimer mice. Stem Cells Dev 17:423–439
89. Nomura T, Honmou O, Harada K et al (2005) IV infusion of brain-derived neurotrophic factor gene-modified human mesenchymal stem cells protects against injury in a cerebral ischemia model in adult rat. Neuroscience 136:161–169
90. Olanow CW, Goetz CG, Kordower JH et al (2003) A double□blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. Ann Neurol 54:403–414
91. 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
92. Park DH, Eve DJ, Musso J III et al (2009) Inflammation and stem cell migration to the injured brain in higher organisms. Stem Cells Dev 18:693–702

93. Park DH, Eve DJ, Sanberg PR et al (2010) Increased neuronal proliferation in the dentate gyrus of aged rats following neural stem cell implantation. Stem Cells Dev 19:175–180
94. Patel NK, Bunnage M, Plaha P et al (2005) Intraputamenal infusion of glial cell line- derived neurotrophic factor in PD: a two-year outcome study. Ann Neurol 57:298–302
95. Patel AN, Park E, Kuzman M et al (2008) Multipotent menstrual blood stromal stem cells: isolation, characterization, and differentiation. Cell Transplant 17:303–311
96. Pluchino S, Quattrini A, Brambilla E et al (2003) Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. Nature 422:688–694
97. Pluchino S, Zanotti L, Rossi B et al (2005) Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. Nature 436:266–271
98. Ratajczak J, Zuba-Surma E, Paczkowska E et al (2011) Stem cells for neural regeneration—a potential application of very small embryonic-like stem cells. J Physiol Pharmacol 62:3–12
99. Rodrigues MC, Voltarelli J, Sanberg PR et al (2012) Recent progress in cell therapy for basal ganglia disorders with emphasis on menstrual blood transplantation in stroke. Neurosci Biobehav Rev 36:177–190
100. Sanberg PR, Eve DJ, Willing AE et al (2011) The treatment of neurodegenerative disorders using umbilical cord blood and menstrual blood-derived stem cells. Cell Transplant 20:85–94
101. Santiago LY, Clavijo-Alvarez J, Brayfield C et al (2009) Delivery of adipose-derived precursor cells for peripheral nerve repair. Cell Transplant 18:145–158
102. Schneider A, Kruger C, Steigleder T et al (2005) The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. J Clin Invest 115:2083–2098
103. Schwab ME (2004) Nogo and axon regeneration. Curr Opin Neurobiol 14:118–124
104. Shen LH, Li Y, Chen J et al (2007) Therapeutic benefit of bone marrow stromal cells administered 1 month after stroke. J Cereb Blood Flow Metab 27:6–13
105. Shyu WC, Lin SZ, Yang HI et al (2004) Functional recovery of stroke rats induced by granulocyte colony-stimulating factor-stimulated stem cells. Circulation 110:1847–1854
106. Slevin JT, Gerhardt GA, Smith CD et al (2005) Improvement of bilateral motor functions in patients with Parkinson disease through the unilateral intraputaminal infusion of glial cell line-derived neurotrophic factor. J Neurosurg 102:216–222
107. Svendsen CN, Caldwell MA, Shen J et al (1997) Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. Exp Neurol 148:135–146
108. Svendsen CN, Caldwell MA, Ostenfeld T (1999) Human neural stem cells: isolation, expansion and transplantation. Brain Pathol 9:499–513
109. Taguchi A, Soma T, Tanaka H et al (2004) Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. J Clin Invest 114:330–338
110. Takagi Y, Takahashi J, Saiki H et al (2005) Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model. J Clin Invest 115:102–109
111. Tat PA, Sumer H, Jones KL et al (2010) The efficient generation of induced pluripotent stem (iPS) cells from adult mouse adipose tissue-derived and neural stem cells. Cell Transplant 19:525–536
112. Thored P, Arvidsson A, Cacci E et al (2006) Persistent production of neurons from adult brain stem cells during recovery after stroke. Stem Cells 24:739–747
113. Trapp BD, Ransohoff R, Rudick R (1999) Axonal pathology in multiple sclerosis: relationship to neurologic disability. Curr Opin Neurol 12:295–302
114. Tsuji O, Miura K, Okada Y et al (2010) Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. Proc Natl Acad Sci U S A 107:12704–12709
115. Uccelli A, Zappia E, Benvenuto F et al (2006) Stem cells in inflammatory demyelinating disorders: a dual role for immunosuppression and neuroprotection. Expert Opin Biol Ther 6:17–22

116. Vendrame M, Cassady J, Newcomb J et al (2004) Infusion of human umbilical cord blood cells in a rat model of stroke dose-dependently rescues behavioral deficits and reduces infarct volume. Stroke 35:2390–2395
117. Vescovi AL, Snyder EY (1999) Establishment and properties of neural stem cell clones: plasticity in vitro and in vivo. Brain Pathol 9:569–598
118. Vieira NM, Brandalise V, Zucconi E et al (2010) Isolation, characterization, and differentiation potential of canine adipose-derived stem cells. Cell Transplant 19:279–289
119. Voss HU, Uluc AM, Dyke JP et al (2006) Possible axonal regrowth in late recovery from the minimally conscious state. J Clin Invest 116:2005–2011
120. Walton NM, Sutter BM, Chen HX et al (2006) Derivation and large-scale expansion of multipotent astroglial neural progenitors from adult human brain. Development 133:3671–3681
121. Wang L, Zhang Z, Wang Y et al (2004) Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. Stroke 35:1732–1737
122. Willing AE, Lixian J, Milliken M et al (2003) Intravenous versus intrastriatal cord blood administration in a rodent model of stroke. J Neurosci Res 73:296–307
123. Woodbury D, Schwarz EJ, Prockop DJ et al (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364–370
124. Woodbury D, Reynolds K, Black IB (2002) Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J Neurosci Res 69:908–917
125. Yang J, Yan Y, Ciric B et al (2010a) Evaluation of bone marrow- and brain-derived neural stem cells in therapy of central nervous system autoimmunity. Am J Pathol 177:1989–2001
126. Yang WZ, Zhang Y, Wu F et al (2010b) Safety evaluation of allogeneic umbilical cord blood mononuclear cell therapy for degenerative conditions. J Transl Med 8:75
127. Yasuhara T, Matsukawa N, Yu G et al (2006a) Transplantation of cryopreserved human bone marrow-derived multipotent adult progenitor cells for neonatal hypoxic-ischemic injury: targeting the hippocampus. Rev Neurosci 17:215–225
128. Yasuhara T, Matsukawa N, Yu G et al (2006b) Behavioral and histological characterization of intrahippocampal grafts of human bone marrow-derived multipotent progenitor cells in neonatal rats with hypoxic-ischemic injury. Cell Transplant 15:231–238
129. Yasuhara T, Hara K, Maki M et al (2008) Intravenous grafts recapitulate the neurorestoration afforded by intracerebrally delivered multipotent adult progenitor cells in neonatal hypoxic-ischemic rats. J Cereb Blood Flow Metab 28:1804–1810
130. Yiu G, He Z (2006) Glial inhibition of CNS axon regeneration. Nat Rev Neurosci 7:617–627
131. Yu SJ, Soncini M, Kaneko Y et al (2009) Amnion: a potent graft source for cell therapy in stroke. Cell Transplant 18:111–118
132. Zappia E, Casazza S, Pedemonte E et al (2005) Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood 106:1755–1761
133. Zhang J, Li Y, Chen J et al (2005) Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice. Exp Neurol 195:16–26
134. Zhao LR, Duan WM, Reyes M et al (2002) Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. Exp Neurol 174:11–20
135. Zuba-Surma EK, Klich I, Greco N et al (2010) Optimization of isolation and further characterization of umbilical-cord-bloodderived very small embryonic/epiblast-like stem cells (VSELs). Eur J Haematol 84:34–46

Chapter 8
Stem Cell Compartment in Acute Psychotic Syndromes

Jolanta Kucharska-Mazur and Jerzy Samochowiec

8.1 Introduction

The International Classification of Diseases (ICD)-10 retains the term "psychosis" for diseases in which at least one of the following symptoms occurs: delusions, hallucinations, disorganised behaviour and speech, leading to reality distortion regardless of the aetiology of symptoms [1]. A first psychotic episode was diagnosed in no fewer than 21,000 Polish patients in 2011 alone, of whom 14,000 were diagnosed with first-episode schizophrenia. Bromet et al. [2], in a 10-year follow-up study, found the diagnosis of bipolar disorder and schizophrenia made in patients with first-episode psychosis to be quite stable. A total of 42.7% of patients with other diagnoses at baseline had their diagnoses changed to schizophrenia or affective disorder. The duration of untreated psychosis (DUP), i.e. the time from manifestation of the first psychotic symptom to initiation of adequate treatment, is a crucial factor affecting the course of psychosis [3]. Reducing the duration of untreated psychosis significantly improves prognosis. Therefore, early diagnosis and intervention is crucial in the treatment of psychosis.

8.2 The First Episode of Psychosis

8.2.1 Schizophrenia

The so-called clinical high-risk or ultra-high-risk (UHR) syndromes, including cognitive and perceptive disturbances, attenuated negative and positive signs and

J. Samochowiec (✉) · J. Kucharska-Mazur
Department of Psychiatry, Pomeranian Medical University,
Broniewskiego 26, 71-460 Szczecin, Poland
e-mail: kl_psych@pum.edu.pl

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,*
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_8,

brief self-limited psychotic symptoms, are frequently observed prior to the onset of a first psychotic episode [4]. The new Diagnostic and Statistical Manual of Mental Disorders (DSM-5) classification defines UHR as a full-fledged nosological entity. Unfortunately, as 60% of the syndromes are false positive (no progression from UHR to schizophrenia), the decision whether or not intervention should be introduced remains controversial. Potential markers of early schizophrenia are: neuroimaging, genetical, neurophysiological and biochemical studies, but the position of these results is controversial [4]. "The soft encephalitis", an intensively explored aetiological concept, would involve increased dopaminergic neurotransmission in the limbic system caused by elevated levels of kynurenic acid, which might be linked with the aetiology of schizophrenia-positive symptoms [5]. Microglia-mediated immune responses, while microglial cells account for approximately 20% of the total glial population, are involved in most inflammation processes in the brain. While microglia are activated by cytokine mechanisms, they also produce proinflammatory cytokines [6].

Genetic research has been focused on the model of a multi-gene disease with incomplete gene penetration; 12 chromosomal domains, containing 2181 genes including genes encoding proteins relevant to neurogenesis, are potentially linked to schizophrenia [7]. Typical neurostructural changes being the results of gene-environment interaction in schizophrenia [8–11] include: lateral ventricle volume increased by approximately 26% (80% of studies), increased third ventricle volume (73% of studies), decreased frontal lobe volume (59% of studies), decreased temporal lobe volume particularly in the middle section (74% of studies), subcortical structural abnormalities (42% of studies—in the thalamus; 92% of studies—in the septum pellucidum). Both the neurodegenerative process and disorders of brain development have been implicated in causing the above-mentioned changes which is explicitly laid out in the currently explored neurodegenerative and neurodevelopmental theories [12–14].

Histopathological examination of schizophrenic patients shows changes in frontal and temporal lobes, in the limbic system, reduced number of thalamic neurons, abnormal hippocampal neuron architecture, reduced neuronal size in the hippocampus, as evidenced by a reduction of presynaptic and dendritic markers (Synaptosomal-associated protein 25 (SNAP-25), complexin II, microtubule-associated protein 2 (MAP-2)). Studies on gliosis and/or abnormal cytoarchitecture in the cerebral cortex have produced inconclusive results [15]. Brennand et al. modelled schizophrenia using induced pluripotent stem cell (SC)-derived and fibroblast-induced neurons and performed subsequent neural differentiation. The examined neurons showed diminished neuronal connectivity in conjunction with decreased neurite number [16].

8.2.2 Diagnoses other than Schizophrenia

In a British study, patients with a first psychotic episode were most frequently diagnosed with psychotic depression—19%, schizophrenia—11%, bipolar disorder—7.5% and delusional disorders—7% [17].

The course of delusional disorder is significantly different to that of schizophrenia. Compared to schizophrenia, delusional disorder is characterised by the incidence equal in both sexes, later onset, psychosocial stressors less frequently precipitating the onset, less-varied psychopathological symptoms, lower hospitalisation rate and poorer long-term outcome. Consequently, the disease cannot be considered as a prodromal syndrome of schizophrenia [18].

The first-episode risk factors for affective psychosis include family history of affective disorders, particularly in first-degree relatives, severe psychosocial stressors, substance use (particularly with psychostimulating properties) and insufficient omega-3 acid levels. The prodromal symptoms of psychosis include episodic depressive symptoms, anxiety and hypomania symptoms, irritability, attention and sleep disorders [19].

Although the interaction of genetic and environmental factors has been emphasised in investigations on the pathogenesis of affective disorders [20–22], genome-wide association study (GWAS) analyses yielded ambiguous results [23, 24]. Neuroimaging tests showed increased lateral ventricle volume, increased third ventricle volume, decline in corpus callosum area or volume, increased hiperintensity of the white matter on T2-weighted magnetic resonance imaging (MRI) images. Interestingly, although meta-analysis of neuroimaging data demonstrated that people with the first episode of bipolar disorder have reduced white matter volume, the total brain volume and grey matter volume in bipolar disorder patients are either the same or differ only slightly compared to healthy people [25, 26]. White matter microstructure examinations in patients revealed disorganised structure of brain fibrils which might be due to neurodevelopmental disorders or changes of myelin sheaths [27].

Although premorbid levels of intellectual development in biopolar disorder patients do not deviate from the average in a population, neuropsychological deficits are observed during the first episode of the disease. They become more severe as the disease progresses [28]. The deficits are assessed as less severe [29] or stable in 2 years from the baseline and do not seem to significantly differ [30] from those found in schizophrenia.

Neuropathological data seem to suggest a different pattern of psychosis pathology. However, shared causal pathways cannot be excluded [31].

8.3 The Role of Neurogenesis in Pathogenesis of Mental Diseases

The development of the central nervous system (CNS) is precisely regulated at each stage, starting from cell proliferation. Since a study conducted by Eriksson et al. [32], it has been known that neurogenesis also occurs in the adult human brain. It is now well established that neurogenesis occurs in the hippocampal dentate gyrus in the subgranular zone (SGZ) and in the subventricular zone (SVZ) of the lateral ventricles. Neuroblast cells migrate via the rostral migratory stream (RMS) to reach

the main olfactory bulb (OB) where they form mature interneuron populations. New neurons and glial cells are also thought to originate from undifferentiated cells in other regions of the brain: in the prosencephalon (or forebrain), striatum, amygdala, substantia nigra, white matter and in the hypothalamus [33–36]. New neurons can join the already existing neural networks [37]. Neurogenesis in the adult brain has been indicated to be involved in the aetiopathogenesis of depression [38]. Reif et al. [39] found that cognitive deficits in schizophrenia patients may be linked to decreased proliferation of neural SCs. An association between schizophrenia and disrupted-in-schizophrenia 1 (DISC-1) gene which mediates hippocampal development and regulates adult neurogenesis provides further evidence of roles neurodegenerative disorders might play [40–43].

8.4 Stem Cells and Factors Responsible for Their Migration vs. Psychosis

8.4.1 Stem Cells and Psychosis

SCs are involved in pathophysiological processes indicated in many neurological diseases, including spinal cord injuries and stroke [44]. Disturbances of neurogenesis and glial cell formation in the adult brain are currently being investigated and implicated as possible routes of treating neurodegenerative disorders, such as Parkinson's, Alzheimer's and Huntington's disease, amyotrophic lateral sclerosis and spinal muscular atrophy [45, 46]. Dysfunctions of neurogenesis might be a significant cause of psychosis [47]. Although neural SCs with a tissue differentiation potential are found in the adult brain, neurogenesis in the brain is practically restricted to the dentate gyrus and the periventricular zone and the possibility of neuronal migration is limited. Therefore, new neurons generated in the above-mentioned regions are thought not to play a very significant role in the regeneration of other brain regions and therefore brain tissue lesions induce mobilisation of SCs specifically used for tissue regeneration [48]. Very small embryonic-like (VSEL) SCs could potentially play this role.

SCs involved in neurogenesis are controlled by a number of external factors and by local signalling within specialised cell micro-environments (niches). These factors include chemokines, bioactive lipids and constituent components of the complement system [49]. Fibroblast growth factor (FGF) and wingless-type (Wnt) signalling determine the neuroepithelial pathway at the early stages of brain development. Further growth of the neural tube is mediated by retinoic acid and sonic hedgehog protein (Shh) [50]. Regulation of SC pluripotency and differentiation involves Nanog, OCT4 and SOX2 pluripotency transcription factors [51]. Oct4/Sox2 ratio is responsible for differentiation of cell pluripotency: Oct4 upregulation modulates myoendothelial and Oct4 suppression neuroectodermal differentiation [52].

Although adult neurogenesis also occurs, due to histone acetylation and deoxyribonucleic acid (DNA) methylation, subventicular zone cells lose their potential for differentiation into neuronal populations of various brain regions [53]. Overexpression of Oct4 reverses the process, i.e. differentiated cells can be reprogrammed to a pluripotent state [54, 55]. Neural stem cells (NSCs) have limitless ability to self-renew and differentiate into the three primary cell types (astrocytes, oligodendrocytes and neurons) [56]. The following are commonly used as SC-specific markers [57]; for NSCs: nestin [58], Musashi-1 [59], neural cell adhesion molecule (NCAM) [60, 61], CD133 (prominin), Glial Fibrillary Acidic Protein (GFAP) [62], Sox1, Sox2, Sox3; and for neural progenitor cells: nestin, GFAP, Mash1 (Mammalian Achaete–Scute Homolog) [63], Sox1, Sox2, Sox3 oraz Olig1 and Olig2 [64]. The last two transcription factors play a slightly different role, whereas Olig2 functions as motor neuron and oligodendrocyte-lineage determination factor (much higher expression in oligodendroglial progenitor cells than in mature oligodendrocytes), Olig1 is responsible for the final stage of myelin synthesis [65]. β-tubulin III [66] and GalC are commonly used as immature neuronal markers [67].

8.4.2 Factors Involved in Trafficking of Stem Cells

Stromal cell-derived factor-1 (CXCL12, SDF-1) is a chemokine involved in CNS development as well as neuromodulation, neuroprotection and neurogenesis of the adult brain. CXCR4 receptor cells are highly expressed in neural progenitor cells from the SGZ, neuroblasts and immature granule cells [68]. SDF-1 promotes the proliferation, remyelination and differentiation of precursor cells to oligodendrocytes [69]. It also plays a part of a programmed response to CNS injury acting through CXCR4 and CXCR7 [70]. SDF-1 also plays a critical role in the migration of mesenchymal SCs, endothelial progenitor cells, neuroblasts, monocytes and it mediates axonal growth [71, 72].

Lysophospholipids follow classical signalling pathways via G-protein and extracellular signal-related kinase (ERK). Sphingosine-1-phosphate (S1P) is recognised as neurogenesis mediator which is evidenced by neural tube defects due to an increase in apoptosis and a decrease in mitosis in animals that could not synthesise S1P [73, 74]. S1P receptors are expressed in brain areas with active neurogenesis; messenger ribonucleic acid (mRNA) transcripts for $S1P_{1–3}$ and $S1P_5$ receptors were found in neural progenitor cells prepared from embryonic rat hippocampus. S1P causes transient aggregation of rat hippocampal neural progenitor cells and via G-protein activation it induces ERK phosphorylation, DNA synthesis and proliferation of neural progenitor cells [75]. Lysophosphatidic acid (LPA) and S1P receptors are expressed in a neuroepithelial (NEP) cell line derived from human embryonic SCs. LPA and S1P also induce p44/42 ERK MAP kinase phosphorylation, stimulate cell proliferation via epidermal growth factor receptor (EGFR) and ERK and their transient aggregation [74].

S1P stimulates migration of neural progenitor cells to damaged sites of the spinal cord [76] and affects the growth and differentiation of oligodendrocytes in vivo [77]. On the other hand, S1P-mediated astrocytes, when added to neural progenitors derived from embryonic SC lines or from the developing cerebral cortex induce their differentiation into neurons and axon growth [78]. Ratajczak et al. concluded that S1P is a key chemoattractant for bone marrow (BM)-residing hematopoietic stem and progenitor cells (HSPCs): S1P creates a continuously present gradient, several magnitudes stronger than SDF-1. S1P level increases during HSPC mobilisation due to complement cascade (CC) activation and the interaction of the membrane attack complex (MAC) with erythrocytes that are a major reservoir of S1P [79, 80].

The complement system consists of about 35–40 proteins circulating in blood plasma and also present on cell surfaces. There are three pathways of complement activation: the classical, lectin and alternative pathways which lead to the formation of C3a and C5a anaphylatoxins and the so-called MAC, i.e. C5b-9 which has a substantial influence on neuron and glial cells of the CNS, including on damage response [81]. C3a stimulates SCs to populate BM niches, activates retention of haematopoietic and progenitor cells in BM and chemotaxis [82–84].

Complement proteins can also play a variety of functions that do not strictly correlate with their traditional role in immunological defence. Prolonged activation of the complement system may lead to cell damage, which is linked to the aetiology and pathogenesis of neurodegenerative diseases [85–87]. CC proteins affect the remodelling of synaptic connections. Mice deficient in C1q or C3 exhibited defects in CNS synapse elimination [88]. On the other hand, though, complement proteins may potentially simulate synapse loss in the early stages of Alzheimer's disease [89]. C3 and C5 are synthesised in the brain by microglia. Glial cells activate formation of bioactive C3 and C5 molecules with vasoactive and chemotactic properties for neutrophils [90]. C5 has neuroprotective influence on mature cells although it has been found to be mitogenic for human neuroblastoma cells [91]. C5a receptors are found in the CNS neurons and there is neuronal generation of C5a in response to ischemic stress [92]. To sum up, C3a and C5a, the components of the complement system, affect neurogenesis, chemotaxis and phagocytic activity of glial cells. MAC mediates, on the one hand, necrosis (lysis of neurons) while on the other hand, it stimulates Schwann cell proliferation. At low concentration levels, MAC inhibits apoptosis of oligodendrocytes [81] and participates in the mobilisation of SCs from BM into the peripheral blood [79].

Research on the complement system in schizophrenia is focused mainly on the total haemolytic complement activity and findings are inconclusive [87]. Mover et al. [93] suggested a role for C3 and C4 levels in the serum as biological markers of negative symptoms of schizophrenia. Genetic studies on polymorphisms of the complement component genes have yielded inconclusive results [94, 95]. According to Mayilyan et al. [87] the complement system could have a dual role in schizophrenia: neuroprotective in aetiology and neurodegenerative in pathogenesis.

8.5 Investigation of Stem Cells in the First Episode of Psychosis

8.5.1 Stem Cells in Drug-Naïve Patients

A study conducted at the Pomeranian Medical University in Szczecin examined the hypothesis that SCs are involved in the pathogenesis of the first episode of psychosis. We explored the role of VSEL, haematopoietic stem cells (HSC), SDF-1, S1P, selected proteins of the complement system in the aetiopathogenesis of the first psychotic episode and the influence of neuroleptics on the mobilisation of SCs from BM into the peripheral blood and on the level of factors promoting SC migration. Based on our findings, we sought to identify new markers of the first episode of psychosis. The study was conducted on 30 unrelated, drug-naive subjects divided into two groups: first-episode schizophrenic patients ($n=22$, diagnosed with F20, F23.1, F23.2 according to ICD-10) and first-episode non-schizophrenic patients ($n=8$, diagnosed with F 23.0, F 22, F 31). The exclusion criteria for the study were: lack of written informed consent, organic mental disorders and axis I (ICD-10) mental disorders other than those listed above, comorbidity of current mental disorders with substance use, including alcohol, severe somatic diseases, particularly those involving administration of medication, glucose intolerance, currently active medical conditions with inflammatory aetiology—patients were excluded on the basis of laboratory test results and physical examination. The control group consisted of 35 healthy volunteers, matched by age, gender, socio-demographic and clinical factors. Blood samples were collected twice in the psychotic group: before neuroleptic treatment and after clinical improvement, and only once in the controls. Cytometric assay in the peripheral blood was performed including the levels of VSEL (Lin−/CD45−/CD34+), VSEL (Lin−/CD45−/AC 133+), HSC (Lin−/CD45+/CD34+) and HSC (Lin−/CD45+/AC133+). S1P content was determined by reversed-phase high-performance liquid chromatography (RP-HPLC). The immunoenzymatic assay (ELISA) was performed on C3a, C5a, C5b-9 and SDF-1. The expression of Oct4, Nanog, Sox2, Olig1, Olig2, nestin, GFAP, Musashi and β3-tubulin was quantified by the real-time reverse transcription polymerase chain reaction (RT-PCR). A clonogenic assay was conducted.

No linear correlation was found between the number of VSEL or HSC and age. However, when median age was used as a dichotomous division criterion for the whole sample, older subjects had fewer HSC CD 34+ cells compared to younger subjects ($P<0.05$) which is consistent with the literature data [96, 97]. No statistically significant differences of VSEL number between younger and older subjects were found. This was probably due to a small sample size and, also, we could not compare the changing number of VSELs in ageing subjects (our study was short-term).

Reports on the influence of gender on haematopoietic SC mobilisation are ambiguous. Potentially, male subjects respond better to stimulation leading to HSC mobilisation [98]. In our own study, gender did not affect the number of

haematopoietic SCs in psychotic and control subjects which is consistent with findings on healthy volunteers reported by de la Rubia et al. [96]. Other factors potentially affecting SC number (family history of mental disorders, obstetric complications, parental age) did not turn out to be significant in the study. Diagnosis of a first psychotic episode proved to be the most significant factor affecting the number of SCs. In the group of psychotic subjects, the level of VSELs (Lin −/CD45 −/CD34 +) before treatment was higher compared to controls. It remained at the same level after neuroleptic treatment.

In the group of psychotic subjects before treatment, the levels of C3a and S1P were lower, compared to controls. The concentration of C3a after neuroleptic treatment increased to a level appropriate for the control group, whereas S1P levels remained decreased. Based on our findings, we sought to identify new markers of a first psychotic episode using logistic regression analysis. In the psychotic group, the analysis identified three potential variables: VSELs Lin −/CD45 −/CD34 +, levels of C3a and concentration of S1P.

Numerous correlations between SC concentrations and factors involved in SC trafficking were found. The correlations differed between the psychotic and control groups.

In the group of psychotic subjects before treatment, the expression of Oct4, Nanog, Sox2, Olig1, Olig2, nestin, GFAP, Musashi and β3-tubulin genes was higher than that in the controls. Numerous correlations were found in the expression of the genes which remained unchanged after neuroleptic treatment.

As reported by Prof. Ratajczak and his research team, VSELs are deposited during gastrulation in developing tissues and remain there until they may serve as a reserve mobile pool of pluripotent SCs that can be used in adult organisms. VSELs can differentiate into cells from all germ layers with the potential to regenerate damaged tissue [99]. As no results of VSEL behaviour in patients with psychosis of any aetiology have been published to date, we can only compare our findings with results of studies conducted on other diseases.

In studies performed on animal models and on humans, VSELs were found to be mobilised from the BM into the peripheral blood, following a response to stroke, and SDF-1 chemotactic gradient was a major mobilisation factor [100]. Similar findings on CD 34+ mobilisation following stroke were reported by Dunac et al. [101]. In our study on first psychotic episodes, a similar, statistically significant, increase in the number of circulating VSEL CD34+ was observed in the psychotic group, compared to controls ($P=0.0006$). No increase in the number of VSEL CD 133+ or haematopoietic SCs (including CD 34+ and CD133+) was observed. The increase of VSEL CD34+, compared to controls, was particularly noticeable in the "schizophrenic" group ($P=0.0005$). To sum up, in subjects with the first episode of psychosis the mobilisation of VSEL Lin −/CD45 −/CD 34+ (but not HSC) from BM to the peripheral blood is observed. The difference between VSEL and HSC behaviour is important given the fact that inflammation may trigger SC mobilisation from BM to the peripheral blood [102] and no increase in the number of HSC suggests that undefined, non-specific inflammation was the major factor of SC mobilisation. The importance of the lack of haematopoietic SC mobilisation in patients with a

first psychotic episode was also described in a paper written by Kucharska-Mazur et al. [103].

The potential role of SCs in regeneration processes, including nervous tissue regeneration, has not, as yet, been fully understood. Apart from SC direct influence involving their plastic potential, the role of paracrine effects in regeneration has been emphasised [104, 105]. Non-haematopoietic multipotent SCs CD34+ in an in vivo study, when exposed to retinoic acid, antioxidants, growth factors, demethylating agents, intracellular cyclic adenosine monophosphate (cAMP)-increasing agents and Noggin, a neural inducer, can undergo differentiation to either neurons or glial cells. Transplantation of these cells, conducted on animal models following stroke and Parkinson's disease, resulted in improvement [104, 106, 107]. Eglitis and Mezey transplanted adult female mice in the tail vein with genetically marked donor BM cells and the cells were detected in the all their brain regions and in the extravascular space 3 days after the transplant (including the ependymal lining of the ventricles). The donor-derived cells expressed neural markers for microglia and astrocytes (GFAP). The authors maintained that glial cells and microglia arise from precursors residing in the BM [108]. BM derived SCs were transplanted into the lateral cerebral ventricles of new-born mice. SCs migrated to the prosencephalon and the cerebellum, including regions where neurogenesis occurs in the adult brain and differentiated to astrocytes and probably to neurons [109].

Although Kohyama et al. [110] answered positively whether BM-derived nerve and glial cells are fully functional, the question still remains unresolved. The problem is important in view of currently conducted studies. Disturbances in the proliferation of SCs were observed in post-mortem studies in schizophrenic patients [39]. Moreover, disturbances in the functioning of the DISC-1 gene in schizophrenia lead to an uncontrolled growth of dendrites and to neuronal hypertrophy [111].

Owing to their involvement in the regulation of neuronal activity and information transfer through the synaptic cleft, glial cells are considered to be involved in the pathogenesis of schizophrenia [112]. According to a "tripartite synapse" concept in which a synapse consists of three parts, as neurotransmitters activate receptors of adjacent astrocytes across the synaptic cleft, intracellular calcium levels could be increased. Consequently, astrocytes can release gliotransmitters that modulate neurotransmission which harmonises neuronal activity [113]. Astrocytic dysfunction dysregulates the neural network, leads to poor structuralisation of information and impairs cognition of qualitative differences between objects, whereas in schizophrenia—it causes disturbances of tough processes and hallucinations [112]. Catatonic symptoms and affective rigidity may also result from abnormal astrocyte function. According to Kondziella et al. [114], the role of astrocytes in the pathogenesis of schizophrenia is combined with maintaining glutamate homeostasis.

Williams et al. [115] observed in schizophrenia a significant reduction in oligodendrocyte density in basal ganglia and astrocyte structural disturbances. Neuregulin-1, a candidate gene in schizophrenia and bipolar disorder, is involved in the development of oligodendrocytes and CNS myelination processes [116].

The role of microglia in the aetiopathogenesis of schizophrenia has been investigated [117]. Microglial activation may inhibit disorders of neurogenesis in the adult

brain [118] and reduce the chances of survival of new hippocampal neurons [119]. A statistically significant increase of microglia density, compared to controls, in frontal and temporal brain regions in chronic schizophrenia was reported by Garey et al. [120]. Since immunological processes result in tissue damage, it might be a potential factor of VSEL mobilisation from BM to the peripheral blood in psychosis, and particularly in schizophrenia.

It is noteworthy that our study revealed an increased expression of genes involved in pluripotency (Oct4, Nanog) and early neural marker genes (Musashi, nestin, Sox2, Olig1, Olig2, β III tubulin) in the blood of patients with the first episode of psychosis, compared to controls. In a study of VSEL cell mobilisation from the BM to the peripheral blood following stroke, Paczkowska et al. [100] obtained an identical assortment of pluripotency marker genes (Oct4, Nanog) and early neural marker genes (nestin, Sox, β III tubulin, Olig1, Olig2, Musashi-1).

In patients with myocardial infarction, VSEL mobilisation (Lin−/CD133+/CD45−) was observed. However, an RT-PCR test, apart from typical pluripotency marker genes (Oct4, Nanog) revealed increased expression of myocardial and endothelial marker genes (GATA-4, Nkx2.5/Csx, MEF2C, VE-cadherin) [121]. Physical effort activates the mobilisation of VSELs with increased expression of genes identical with those reported in myocardial infarction—Oct4, Nanog, GATA-4, Nkx2.5/Csx, MEF2C, VE-cadherin [122]. Interestingly, mobilised BM SCs in patients with Crohn's disease revealed expression of genes indicated in intestinal epithelial regeneration [123].

Our analysis of the correlation of gene expression in psychotic patients, and particularly in schizophrenic patients, demonstrated that pluripotency markers (Nanog, Oct4) highly correlate with lineage markers of nervous system cells. What should be particularly emphasised is high correlation between the expression of: Oct4 and Nanog with Olig1 and Olig2, i.e. with transcription factors involved in the differentiation of pluripotent cells into oligodendrocytes and their maturation [124], Musashi, i.e. protein of neural progenitor cells necessary for their maintenance and proliferation [125], β III tubulin (neuronal marker) and lack of correlation with GFAP and nestin. The expression of Olig1 and Olig2 was highly correlated both before and after treatment which is consistent with the current knowledge of the interaction between Olig1 and Olig2 genes. The above data suggest a potential involvement of mobilised cells in the development of oligodendrocytes and neurons which might be linked to the aetiopathogenesis of psychosis. A review of the literature produced by Bernstein et al. [126] confirms a decreased density of oligodendrocytes in schizophrenia and changes in the expression of genes involved in oligodendrocyte creation and myelination causing disorders in transfer of information in the brain. A strong association between Olig2 deletion and schizophrenia was demonstrated [127]. Post-mortem studies showed a significant reduction of Olig2 mRNA in the brain of patients with schizophrenia and bipolar disorder, contrary to Olig1 expression which was reduced in schizophrenic patients but did not differ from controls with bipolar disorder [128].

Since GFAP is an astrocyte marker, our findings seem to be in line with those reported by Garey [120] who observed no changes in astroglia density in schizophrenia.

The opinions on changes in the number of neurons in the brain in schizophrenia are ambiguous. However, changes in the structure of neurons undoubtedly affect signal transmission in neural networks and play a significant role in the aetiology of the disease [129]. In the context of our findings on changes in the number and expression of genes, an interesting theory should, perhaps, be reiterated, according to which remodelling of the white and grey matter in the brain in adults between the ages of 20 and 30 may trigger alterations in brain structure and function leading to the development of mental diseases. The theory seems to be confirmed by the fact that the period at which cerebral cortex becomes myelinated overlaps with the period of increased prevalence of mental disorders [130].

In conclusion, VSELs may potentially be involved in regeneration processes in people with psychosis, and particularly with schizophrenia.

8.5.2 Antipsychotic Treatment and Stem Cells

The study conducted in Szczecin on subjects with the first psychotic episode found that treatment with amisulpride is associated with an increase in the number of HSC Lin−/CD45+/AC 133+ and with a trend to increased levels of HSC Lin−/CD45+/CD34+ in the peripheral blood, which seems to be consistent with findings reported by Löffler et al. [182] who observed a similar effect in HSC Lin−/CD45+/CD 34+ levels following a treatment of drug-naive patients with clozapine, another atypical antipsychotic drug. On the other hand, though, clinical experience and scientific research suggest that prolonged treatment with neuroleptics (e.g. clozapine, olanzapine, quetiapine) has an adverse influence on haematopoietic cells, as neuroleptics may have cytotoxic effects [131, 132]. No literature data about the influence of amisulpride on HSC mobilisation have been found.

In a study of embryonic culture, a neuroleptic (quetiapine) in the presence of growth factors stimulated proliferation of neural progenitor cells, their differentiation into oligodendrocytes, myelination and a decrease in microglia activity [133]. Studies on the influence of neuroleptics on NSCs found that haloperidol stimulates and risperidone inhibits their growth [134]. Although research conducted on animal models of schizophrenia confirmed that neuroleptics have protective properties affecting the white matter in the brain, none of the tested drugs (clozapine, olanzapine, haloperidol, quetiapine) could reverse the existing damage during a 3-week-long observation [127]. Increased synthesis of glia following olanzapine (but not haloperidol) treatment was attributable to the body mass increase of patients treated with olanzapine [135].

We found in our study that despite clinical improvement in subjects with a first psychotic episode, no statistically significant decrease of VSEL Lin−/CD45−/CD 34+ levels, compared to those before treatment, was observed ($P=0.36$). This

applied to both the schizophrenic and non-schizophrenic groups. Compared to controls, the number of VSELs CD34+ after treatment was still increased in the psychotic group ($P=0.001$) and its subgroups of schizophrenic ($P=0.02$) and non-schizophrenic patients ($P=0.003$). The number of VSEL CD133+ after treatment did not differ from that before it or from the number of cells in the control group ($P>0.05$).

The expression of genes after treatment in our study was not significantly different from that before it. No correlation was found between the expression of Oct4 and Nanog genes and the expression of Olig1, Olig2 and β III tubulin. Thus, clinical improvement was due to the qualitative change in the regenerative potential of cells in the peripheral blood.

The lack of statistically significant changes in the number of SCs and the quantitative expression of genes may have been caused by a too short time interval between the two examinations—the average treatment duration until observable improvement was 16.4 days. Further research is needed to produce a conclusive answer to elucidate mechanisms affecting the above parameters.

8.5.3 The Duration of Untreated Psychosis and Stem Cells

The duration of untreated psychosis was assessed on the basis of information received from patients and their families and the obtained data could hardly be classed as precise. Only one correlation was found, between the duration of untreated psychosis and the number of VSELs (Lin−/CD45−/CD133+) in the psychotic group after treatment. However, it is difficult to interpret the finding since before treatment no differences in the number of VSELs (Lin−/CD45−/CD133+) were observed between the psychotic and control groups.

8.6 Factors Responsible for the Trafficking of Stem Cells

8.6.1 Sphingosine-1-Phosphate

It is commonly known that SC movement is regulated by chemotactic factors, mainly by S1P and SDF-1. The Szczecin study found a statistically significant decrease of S1P concentration in the plasma of patients with a first psychotic episode compared to the control group ($P=0.0017$). The treatment did not result in S1P increase to a level appropriate for the controls. The difference between S1P concentration in the psychotic and control groups remained statistically significant ($P=0.0067$). We could hypothesise on the role of S1P in the aetiopathogenesis of psychosis.

S1P is involved in the development of neurons and is a mediator of nerve growth factor (NGF-1) which stimulates the development of axons [78]. The finding, that any disturbances in the process lead to schizophrenia-related increase in ventricular

size and decreased brain volume, was the starting point for a new model of pathophysiology of schizophrenia [136]. Increased density of nerve cells accompanied by decreased density of axons is found in schizophrenia [137].

S1P plays a major role in cell proliferation serving as the second transmitter for Ca^{2+} ion mobilisation from intracellular reservoirs [138]. The active system of calcium signalling in neurons regulates information processing, brain rhythms and synaptic plasticity responsible for memory and learning—all of these processes are dysfunctional in schizophrenia [139]. Poor short-term memory may be caused by disorders in rhythm generation through the interaction of inhibitory Gamma aminobutyric acid (GABA)ergic interneurons and stimulatory glutamatergic pyramidal neurons, i.e. by impaired glutamate response of inhibitory neurons due to a decrease in N-Methyl-D-aspartate (NMDA) receptor activity [139, 140]. The observation that the administration of NMDA inhibitors (phencyclidine or ketamine) may induce psychosis in healthy individuals became a starting point for the so-called glutamate hypothesis of schizophrenia [141]. The role of the glutaminergic system in the aetiology of affective disorders has also been investigated [142]. NMDA receptors (NMDAR) affect GABAergic transmission with calcium signalling causing phosphorylation of cAMP response element-binding protein (CREB) transcription factors. As a result, NMDA receptor hypofunction can lead to reduced GABA synthesis. Calcium ions take part in the regulatory mechanisms of GABA release through $Ca_V2.1$ P/Q calcium channels which respond to excessive inhibition of calcium entry. The activity of calcium channels is buffered by parvalbumin whose level is typically decreased in schizophrenia [139]. Although there is no direct evidence for S1P involvement in these aetiological processes of psychosis, studies on H218/AGR16/Edg-5/LP(B2) S1P receptor, involved in cell development processes, provide data about the role of S1P in neural excitability—a loss of the receptor results in increased activity of pyramidal neurons [143].

S1P is known to inhibit cAMP production in spinal cord neurons [144]. The functioning of the glutaminergic system, which is disturbed in schizophrenia, and the effect of antipsychotic drugs are combined with cAMP signalling. The activation of neuronal Ca(2+) sensor 1 (NCS-1) by calcium ions results in reduced sensitivity of dopamine receptor D2 (DRD2) receptors, cAMP increase and following that, activates membrane excitability. In schizophrenia and in bipolar disorders, the level of NCS-1 is increased in the prefrontal cortex [139, 145].

8.6.2 Stromal Cell-Derived Factor-1

The level of SDF-1 in subjects with a first psychotic episode was investigated by Fernandez-Egea et al. [146]. Reduced SDF-1 levels were observed in patients with the first episode of non-affective psychosis and the researchers speculated that this might be the evidence for dysregulation of regeneration process in schizophrenia. In our study, the level of SDF-1 in the psychotic group (or in the schizophrenic subgroup) did not differ from that of the controls. Treatment did not seem to have

affected SDF-1 levels. This difference might have been caused by the lower age of patients investigated by Fernandez-Egea et al. The Szczecin study found that SDF-1 level in the psychotic group was proportional to the subjects' age. This finding is in line with authors who did not observe markedly elevated SDF-1 levels in the peripheral blood during SC mobilisation [147, 148].

8.6.3 Complement Cascade

In the Szczecin study, the lowered levels of complement component 3a in subjects with a first psychotic episode, compared to controls, were statistically significant. After treatment, C3a levels in the psychotic group did not differ from those in the controls. Ambiguous results were obtained in a study conducted by Mayilyan et al. [87]. C3 levels in schizophrenia were first reduced, then unchanged and finally elevated in clinically different groups of patients. Santos Sória et al. [149] reported increased C3 levels in patients with schizophrenia, compared to controls. C3 levels in bipolar disorder patients in euthymia were identical with those in controls. While comparing treated and untreated patients, divergent results were obtained; lower haemolytic activity of C3 was detected in untreated patients [150] and identical plasma levels of C3 in both groups [151].

The potential role of C3a in the pathogenesis of psychosis might be linked to its involvement in the activation of the complement system and in the stimulation of phagocyte chemotaxis in the brain. The latter process removes apoptotic cells. The smooth operation of these mechanisms is instrumental for brain development and neuroplasticity [152]. The administration of C3a leads to expression of nerve growth factor [153]. Contrary to C5a which acts as a proinflammatory agent, C3a acts in an anti-inflammatory way. A lack of C3a signalling results in reduced neurogenesis in mice [154]. An in vitro study found [155] that in neuron- and astrocyte-containing cultures (but not in cultures consisting solely of neurons) C3a anaphylatoxin dose-dependently mediates neuroprotection—it is selectively protective against NMDA-induced neuronal apoptosis. The role of NMDA is elaborated on in 6.1.

8.6.4 Correlation between the Stem Cell Number and Factors Involved in Trafficking of Stem Cells

We found in our study that both psychosis and antipsychotic treatment significantly affect the interrelations between agents responsible for SC motility. While many correlations were found in the control group—SDF1 and C3a, S1P and C5a, SDF1 and C5a (negative correlation), SDF1 and C5b-9, S1P and SDF1 (negative correlation), few correlations were detected before treatment in the treated group: between S1P and C5b-9 in the schizophrenic group; and after treatment: between S1P and C5a in the psychotic group and schizophrenic subgroup. The results obtained in the control group suggest, in line with the literature data [148], that individual factors

are closely interrelated. The lack of similar correlations in the psychotic group may bear evidence of dysregulation of the system in individuals with a first psychotic episode.

It is to be stressed that blood platelets might be implicated in the irregularities. Pronounced oxidative stress was detected in subjects with schizophrenia and so were the resulting alterations in the structure and function of thrombocyte proteins [156, 157]. C3a improves SDF1-induced thrombocyte production [158]. S1P is transported in the blood by platelets, erythrocytes, albumins and high-density lipoproteins and its release from platelets and erythrocytes is induced by C5b-9. C5b-9 also increases SDF-1 release by BM-derived stromal cells [148]. Dysregulation of blood platelet function may potentially trigger irregularities in the gradient of factors that mediate SC migration and ultimately, an increase of VSELs in the peripheral blood which was observed in our study.

The results of our study indicate that while the number of VSELs in the peripheral blood was inversely correlated with S1P levels, SDF1 levels were negatively correlated with HSC number. Interestingly, none of these correlations was of high statistical significance. Owing to the well-understood role of S1P and SDF1 in SC movement [139, 159], a gradient of their concentration seems to be more important than their respective levels. Moreover, the obtained correlations between S1P, SDF1 and complement components C5a, C3a and C5b-9, as well as direct correlations between complement components and the number of SCs, all seem to confirm the role of the complement components in SC motility [158, 160].

8.7 Recovery Factors in the First Episode of Psychosis

The initial score in Positive and Negative Symptom Scale (PANSS) N subscale seems to be a major recovery factor, according to the literature data [161]. Higher initial scores tend to predict slower recovery.

The rate of recovery primarily depended on the initial levels of complement components C3a and C5a. Higher initial levels predicted faster recovery. These results are consistent with expectations based on the postulated role of low C3a levels as a marker for the first episode of psychosis. Unfortunately, there are no data in the available literature that could be used for comparison purposes with our findings.

8.8 Attempts to Identify Markers for the First Episode of Psychosis and Schizophrenia

The objectification of medical diagnosis in psychosis based on measurable laboratory parameters is a fundamental problem of therapeutic intervention that has not been resolved so far. None of the current diagnostic criteria for mental disorders use objective biological markers. The problems posed while seeking to identify such

markers include a variety of clinical pictures and, following on from that, a potential diversity of genetic factors underlying mental disorders.

The current state of psychiatric knowledge does not allow to speak about one disease called schizophrenia, but rather about a cluster of disorders known as schizophrenia. Investigative attempts in genetics are focused on introducing endophenotypes of mental disorders, i.e. sets of measurable disorder (disease) characteristics which are: stable independent of clinical state, highly heritable, found in both healthy and ill family members (to a larger degree in the ill probands), easy to use in medical examination, combined with the pathophysiology of a disorder (disease) [162]. The same task, in regard to schizophrenia, was undertaken in the USA by *The Consortium on the Genetics of Schizophrenia* (COGS) which took measures for selecting endophenotypic markers, which included: (1) specific diagnostic criteria, (2) findings of higher deficit rates in relatives of probands than in the general population, (3) impairments observed regardless of illness state (stable regardless of duration of illness and treatment), (4) replicable in multisite studies, (5) linked to neurobiology of schizophrenia, (6) rapid and easy to perform and to test genetic hypotheses [163].

The research conducted by COGS produced endophenotypes based on neuropsychological, and particularly neurocognitive, data in regard to executive functions, verbal fluency, attention, verbal and short-term memory [164] and electrophysiological data (latency of P50 evoked potential, N100 amplitude, disorders of sensorimotor gating) [165, 166]. The study once again confirmed earlier findings [167] on the usefulness of eye movement disorders as a potential marker of schizophrenia [168].

Deficits in odour identification are being investigated as a potential risk marker for poor outcome in psychosis, and particularly in cognitive impairments, negative symptoms and disorientation [169]. Other potential endophenotypes of schizophrenia include the so-called soft neurological symptoms (also observed in the first psychotic episode) [170], disorders of pain sensation [171], tongue movement disorders [172], motor activity disorders [173] and neuroimaging changes which, we discussed before.

The analysis of the Szczecin study data using stepwise regression yielded two plasma markers: C3a and S1P. They have been assessed as the best predictors of the first episode of psychosis, with high discriminating ability: area under curve of receiver operating characteristics (ROC) 0.8378, $P=0.00004$, odds ratio (OR) = 9.58, cut-off points: C3a < 550 ng/mL, S1P < 2.33 μg/mL. The number of Lin−/CD45−/CD34+ VSELs per 1 μl of blood and S1P have been assessed as the best predictors of the first "schizophrenic" episode of psychosis (area under curve of ROC 0.77, $P=0.012$, OR = 1.5, cut-off points: Lin−/CD45−/CD34+ VSELs > 0.45 cells per 1 μL of blood, S1P < 2.33 μg/mL).

The markers we postulate as predictors have not been replicated in other research centres. No patients with chronic disorders have been examined. Therefore, we cannot possibly claim to have established a mental disorder endophenotype. We only discuss the issue of first psychotic episode markers.

The following have been suggested as potential plasma markers of psychosis: hormones, interleukins, haptoglobin, apolipoproteins, tumour necrosis factors

(TNF), brain-derived neurotrophic factor (BDNF), betacellulin, ferritin, amyloid P, coefficients of oxidative stress, anti-gliadin antibodies [174–179]. A report published by Schwarz et al. [180] described an assortment of 51 substances which included complement protein C3, as a potential marker for schizophrenia. The authors, contrary to our study, did not determine the products of complement protein C3 decomposition. There is no information on whether or not they intended to investigate the role of sphingosine-1- phosphate as psychosis marker. Le-Niculescu et al. [181] sought to identify markers for affective disorders, including bipolar disorders, by determining expression of genes in the peripheral blood. The genes involved in regeneration processes that were selected as potential markers included: myelination genes, receptor genes of fibroblast growth factor and insulin-like growth factor binding protein (IGFBP)4 and IGFBP6 genes. In the context of general attitude towards aetiology of psychosis, those results are in line with our own findings in the Szczecin study.

It is to be expected that further research will identify new biomarkers of psychosis. It is important since prevention and early detection of psychosis will make it possible to introduce more effective therapeutic interventions. Therefore, markers which can validate diagnosis at the very first psychotic episode are the most valuable ones. Development of new biomarkers will enable application of more efficient and more individualised treatment methods. We hope that the findings presented in the paper will broaden the scope of interest for those who study mental disorders and will shift the focus of their attention into the issues of pluripotent SCs in the peripheral blood.

References

1. ICD-10, Klasyfikacja zaburzeń psychicznych i zaburzeń zachowania w ICD-10, Badawcze kryteria diagnostyczne, Uniwersyteckie Wydawnictwo Medyczne Vesalius, Instytut Psychiatrii i Neurologii, Kraków–Warszawa, 1998
2. Bromet EJ, Kotov R, Fochtmann LJ, Carlson GA, Tanenberg-Karant M, Ruggero C, Chang SW (2011) Diagnostic shifts during the decade following first admission for psychosis. Am J Psychiatry 168(11):1186−1194
3. Dell'osso B, Glick ID, Baldwin DS, Altamura AC (7 Aug 2012) Can long-term outcomes be improved by shortening the duration of untreated illness in psychiatric disorders a conceptual framework. Psychopathology. [Epub ahead of print]
4. Correll CU, Hauser M, Auther AM, Cornblatt BA (2010) Research in people with psychosis risk syndrome: a review of the current evidence and future directions. J Child Psychol Psychiatry 51(4):390–431
5. Karakula H, Flis M, Szymona K, Studenna M (2011) Kwas kynureninowy a objawy pozytywne w schizofrenii. Curr Probl Psychiatry 12(4):516–519
6. Perry VH, Cunningham C, Holmes C (2007) Systemic infections and inflammation affect chronic neurodegeneration. Nat Rev Immunol 7(2):161−167
7. Mura G, Petretto DR, Bhat KM, Carta MG (2012) Schizophrenia: from epidemiology to rehabilitation. Clin Pract Epidemiol Ment Health 8:52–66
8. Shenton ME, Dickey CC, Frumin M, McCarley RW (2001) A review of MRI findings in schizophrenia. Schizophr Res 49(1–2):1–52

9. Rund BR (2009) Is there a degenerative process going on in the brain of people with schizophrenia? Front Hum Neurosci 3:36
10. Weinberger RD, Marenco S (2003) Schizophrenia as a neurodevelopmental disorder. In: Hirsch SR, Weinberger DR (eds) Schizophrenia, 2nd edn. Blackwell, London, pp 326–348. doi:10.1002/9780470987353ch18
11. Niznikiewicz MA, Kubicki M, Shenton ME (2003) Recent structural and functional imaging findings in schizophrenia. Curr Opin Psychiatry 16:123–148
12. Gross G, Huber G (2008) Schizophrenia: neurodevelopmental disorder or degenerative brain process? Fortschr Neurol Psychiatr 76(Suppl 1):S57–S62
13. Weinberger DR (1987) Implications of normal brain development for the pathogenesis of schizophrenia. Arch Gen Psychiatry 44(7):660−669
14. Murray RM, Lewis SW (1987) Is schizophrenia a neurodevelopmental disorder? Br Med J (Clin Res Ed) 295(6600):681−682
15. Wong AH, Van Tol HH (2003) Schizophrenia: from phenomenology to neurobiology. Neurosci Biobehav Rev 27(3):269–306
16. Brennand KJ, Simone A, Jou J, Gelboin-Burkhart C, Tran N, Sangar S, Li Y, Mu Y, Chen G, Yu D, McCarthy S, Sebat J, Gage FH (2011) Modelling schizophrenia using human induced pluripotent stem cells. Nature 473(7346):221−225
17. Proctor SE, Mitford E, Paxton R (2004) First episode psychosis: a novel methodology reveals higher than expected incidence; a reality-based population profile in Northumberland, UK. J Eval Clin Pract 10(4):539−547
18. Marneros A, Pillmann F, Wustmann T (2012) Delusional disorders-are they simply paranoid schizophrenia? Schizophr Bull 38(3):561−568
19. McNamara RK, Nandagopal JJ, Strakowski SM, DelBello MP (2010) Preventative strategies for early-onset bipolar disorder: towards a clinical staging model. CNS Drugs 24(12):983−996
20. Fusar-Poli P, Howes O, Bechdolf A, Borgwardt S (2012) Mapping vulnerability to bipolar disorder: a systematic review and meta-analysis of neuroimaging studies. J Psychiatry Neurosci 37(3):170−184
21. Rutten BP, Mill J (2009) Epigenetic mediation of environmental influences in major psychotic disorders. Schizophr Bull 35(6):1045−1056
22. Labrie V, Pai S, Petronis A (2012) Epigenetics of major psychosis: progress, problems and perspectives. Trends Genet 28(9):427−435
23. Bergen SE, O'Dushlaine CT, Ripke S, Lee PH, Ruderfer DM, Akterin S et al (2012) Genome-wide association study in a Swedish population yields support for greater CNV and MHC involvement in schizophrenia compared with bipolar disorder. Mol Psychiatry 17(9):880−886
24. Sklar P i wsp. (Psychiatric GWAS Consortium Bipolar Disorder Working Group) (2011) Large-scale genome-wide association analysis of bipolar disorder identifies a new susceptibility locus near ODZ4. Nat Genet 43(10):977−983
25. Vita A, De Peri L, Sacchetti E (2009) Gray matter, white matter, brain, and intracranial volumes in first-episode bipolar disorder: a meta-analysis of magnetic resonance imaging studies. Bipolar Disord 11(8):807−814
26. De Peri L, Crescini A, Deste G, Fusar-Poli P, Sacchetti E, Vita A (2012) Brain structural abnormalities at the onset of schizophrenia and bipolar disorder: a meta-analysis of controlled magnetic resonance imaging studies. Curr Pharm Des 18(4):486−494
27. Lu LH, Zhou XJ, Keedy SK, Reilly JL, Sweeney JA (2011) White matter microstructure in untreated first episode bipolar disorder with psychosis: comparison with schizophrenia. Bipolar Disord 13(7–8):604−613
28. Lewandowski KE, Cohen BM, Ongur D (2011) Evolution of neuropsychological dysfunction during the course of schizophrenia and bipolar disorder. Psychol Med 41(2):225−241
29. Demily C, Jacquet P, Marie-Cardine M (2009) How to differentiate schizophrenia from bipolar disorder using cognitive assessment? Encephale 35(2):139−145
30. Bombin I, Mayoral M, Castro-Fornieles J, Gonzalez-Pinto A, de la SE, Rapado-Castro M et al (25 Jul 2012) Neuropsychological evidence for abnormal neurodevelopment associated with early-onset psychoses. Psychol Med 1–12 [Epub ahead of print]

31. Beumer W, Gibney SM, Drexhage RC, Pont-Lezica L, Doorduin J, Klein HC et al (8 Aug 2012) The immune theory of psychiatric diseases: a key role for activated microglia and circulating monocytes. J Leukoc Biol [Epub ahead of print]
32. Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4(11):1313−1317
33. Lee DA, Blackshaw S (31 Jul 2012) Functional implications of hypothalamic neurogenesis in the adult mammalian brain. Int J Dev Neurosci [Epub ahead of print]
34. Nunes MC, Roy NS, Keyoung HM, Goodman RR, McKhann G 2nd, Jiang L, Kang J, Nedergaard M, Goldman SA (2003) Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. Nat Med 9(4):439−447
35. Gould E (2007) How widespread is adult neurogenesis in mammals? Nat Rev Neurosci 8(6):481–488
36. Iarygin KN, Iarygin VN (2012) Neurogenesis in the central nervous system and prospects of regenerative neurology. Zh Nevrol Psikhiatr Im S S Korsakova 112(1):4–13
37. Song HJ, Stevens CF, Gage FH (2002) Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. Nat Neurosci 5(5):438–445
38. Kempermann G, Kronenberg G (2003) Depressed new neurons-adult hippocampal neurogenesis and a cellular plasticity hypothesis of major depression. Biol Psychiatry 54(5):499–503
39. Reif A, Fritzen S, Finger M, Strobel A, Lauer M, Schmitt A, Lesch KP (2006) Neural stem cell proliferation is decreased in schizophrenia, but not in depression. Mol Psychiatry 11(5):514–522
40. Schurov IL, Handford EJ, Brandon NJ, Whiting PJ (2004) Expression of disrupted in schizophrenia 1 (DISC1) protein in the adult and developing mouse brain indicates its role in neurodevelopment. Mol Psychiatry 9(12):1100–1110
41. Schumacher J, Laje G, Abou Jamra R, Becker T, Mühleisen TW, Vasilescu C et al (2009) The DISC locus and schizophrenia: evidence from an association study in a central European sample and from a meta-analysis across different European populations. Hum Mol Genet 18(14):2719–2727
42. Kodama M, Fujioka T, Duman RS (2004) Chronic olanzapine or fluoxetine administration increases cell proliferation in hippocampus and prefrontal cortex of adult rat. Biol Psychiatry 56(8):570–580
43. Nasrallah HA, Hopkins T, Pixley SK (2010) Differential effects of antipsychotic and antidepressant drugs on neurogenic regions in rats. Brain Res 1354:23–29
44. Ratajczak J, Zuba-Surma E, Paczkowska E, Kucia M, Nowacki P, Ratajczak MZ (2011) Stem cells for neural regeneration—a potential application of very small embryonic-like stem cells. J Physiol Pharmacol 62(1):3–12
45. Jung YW, Hysolli E, Kim KY, Tanaka Y, Park IH (2012) Human induced pluripotent stem cells and neurodegenerative disease: prospects for novel therapies. Curr Opin Neurol 25(2):125–130
46. Buffo A, Rolando C, Ceruti S (2010) Astrocytes in the damaged brain: molecular and cellular insights into their reactive response and healing potential. Biochem Pharmacol 79:77–89
47. Genius J, Benninghoff J, Reuter N, Braun I, Giegling I, Hartmann A, Möller HJ, Rujescu D (2012) Dysequilibrium of neuronal proliferation and apoptosis in a pharmacological animal model of psychosis. Methods 56(4):519–527
48. Machaliński B, Machalińska A, Paczkowska E (2010) Rola komórek macierzystych w procesie endogennej regeneracji ośrodkowego układu nerwowego na przykładzie wybranych schorzeń naczyniowych. Post Biol Kom 37(1):241–252
49. Lehtinen MK, Walsh CA (2011) Neurogenesis at the brain-cerebrospinal fluid interface. Annu Rev Cell Dev Biol 27:653–679
50. Huang J, Wah IY, Pooh RK, Choy KW (18 Aug 2012) Molecular genetics in fetal neurology. Semin Fetal Neonatal Med [Epub ahead of print]
51. Wang Z, Oron E, Nelson B, Razis S, Ivanova N (2012) Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. Cell Stem Cell 10(4):440–454

52. Thomson M, Liu SJ, Zou LN, Smith Z, Meissner A, Ramanathan S (2011) Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. Cell 145(6):875–889
53. Lunyak VV, Rosenfeld MG (2008) Epigenetic regulation of stem cell fate. Hum Mol Genet 17(R1):R28–R36
54. Deleidi M, Cooper O, Hargus G, Levy A, Isacson O (2011) Oct4-induced reprogramming is required for adult brain neural stem cell differentiation into midbrain dopaminergic neurons. PLoS ONE 6(5):e19926
55. Kim JB, Sebastiano V, Wu G, Araúzo-Bravo MJ, Sasse P, Gentile L et al (2009) Oct4-induced pluripotency in adult neural stem cells. Cell 136(3):411–419
56. Valenzuela M, Sidhu K, Dean S, Sachdev P (2007) Neural stem cell therapy for neuropsychiatric disorders. Acta Neuropsychiatr 19:11–26
57. 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(10):801–806
58. Gilyarov AV (2008) Nestin in central nervous system cells. Neurosci Behav Physiol 38(2):165–169
59. Okano H, Kawahara H, Toriya M, Nakao K, Shibata S, Imai T (2005) Function of RNA-binding protein Musashi-1 in stem cells. Exp Cell Res 306(2):349–356
60. Wielgat P, Braszko JJ (2012) Significance of the cell adhesion molecules and sialic acid in neurodegeneration. Adv Med Sci 57(1):23–30
61. Hombach-Klonisch S, Panigrahi S, Rashedi I, Seifert A, Alberti E, Pocar P et al (2008) Adult stem cells and their trans-differentiation potential-perspectives and therapeutic applications. J Mol Med (Berl) 86(12):1301–1314
62. Middeldorp J, Hol EM (2011) GFAP in health and disease. Prog Neurobiol 93(3):421–443
63. Furmanski O, Gajavelli S, Lee JW, Collado ME, Jergova S, Sagen J (2009) Combined extrinsic and intrinsic manipulations exert complementary neuronal enrichment in embryonic rat neural precursor cultures: an in vitro and in vivo analysis. J Comp Neurol 515(1):56–71
64. Kim HM, Hwang DH, Choi JY, Park CH, Suh-Kim H, Kim SU, Kim BG (2011) Differential and cooperative actions of Olig1 and Olig2 transcription factors on immature proliferating cells after contusive spinal cord injury. Glia 59(7):1094–1106
65. Othman A, Frim DM, Polak P, Vujicic S, Arnason BG, Boullerne AI (2011) Olig1 is expressed in human oligodendrocytes during maturation and regeneration. Glia 59(6):914–926
66. Liu JS (2011) Molecular genetics of neuronal migration disorders. Curr Neurol Neurosci Rep 11(2):171–178
67. Boggs JM, Gao W, Zhao J, Park HJ, Liu Y, Basu A (2010) Participation of galactosylceramide and sulfatide in glycosynapses between oligodendrocyte or myelin membranes. FEBS Lett 584(9):1771–1778
68. Bhattacharyya BJ, Banisadr G, Jung H, Ren D, Cronshaw DG, Zou Y, Miller RJ (2008) The chemokine stromal cell-derived factor-1 regulates GABAergic inputs to neural progenitors in the postnatal dentate gyrus. J Neurosci 28(26):6720–6730
69. Göttle P, Kremer D, Jander S, Odemis V, Engele J, Hartung HP, Küry P (2010) Activation of CXCR7 receptor promotes oligodendroglial cell maturation. Ann Neurol 68(6):915–924
70. Jaerve A, Müller HW (2012) Chemokines in CNS injury and repair. Cell Tissue Res 349(1):229–248
71. Fan Y, Shen F, Frenzel T, Zhu W, Ye J, Liu J, Chen Y, Su H, Young WL, Yang GY (2010) Endothelial progenitor cell transplantation improves long-term stroke outcome in mice. Ann Neurol 67(4):488–497
72. Li M, Yu J, Li Y, Li D, Yan D, Qu Z, Ruan Q (2010) CXCR4 positive bone mesenchymal stem cells migrate to human endothelial cell stimulated by ox-LDL via SDF-1alpha/CXCR4 signaling axis. Exp Mol Pathol 88(2):250–255
73. Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL (2005) Essential role for sphingosine kinases in neural and vascular development. Mol Cell Biol 25:11113–11121

74. Hurst JH, Mumaw J, Machacek DW, Sturkie C, Callihan P, Stice SL, Hooks SB (2008) Human neural progenitors express functional lysophospholipid receptors that regulate cell growth and morphology. BMC Neurosci 9:118
75. Harada J, Foley M, Moskowitz MA, Waeber C (2004) Sphingosine-1-phosphate induces proliferation and morphological changes of neural progenitor cells. J Neurochem 88(4):1026–1039
76. Kimura A, Ohmori T, Ohkawa R, Madoiwa S, Mimuro J, Murakami T, Kobayashi E, Hoshino Y, Yatomi Y, Sakata Y (2007) Essential roles of sphingosine 1-phosphate/S1P1 receptor axis in the migration of neural stem cells toward a site of spinal cord injury. Stem Cells 25(1):115–124
77. Chun J (2005) Lysophospholipids in the nervous system. Prostaglandins Other Lipid Mediat 77(1–4):46–51
78. Spohr TC, Dezonne RS, Nones J, Dos Santos SC, Einicker-Lamas M, Gomes FC, Rehen SK (2012) Sphingosine 1-phosphate-primed astrocytes enhance differentiation of neuronal progenitor cells. J Neurosci Res 90(10):1892–1902
79. Ratajczak MZ, Lee H, Wysoczynski M, Wan W, Marlicz W, Laughlin MJ, Kucia M, Janowska-Wieczorek A, Ratajczak J (2010) Novel insight into stem cell mobilization-plasma sphingosine-1-phosphate is a major chemoattractant that directs the egress of hematopoietic stem progenitor cells from the bone marrow and its level in peripheral blood increases during mobilization due to activation of complement cascade/membrane attack complex. Leukemia 24(5):976–985
80. Ratajczak MZ, Borkowska S, Ratajczak J (3 Sept 2012) An emerging link in stem cell mobilization between activation of the complement cascade and the chemotactic gradient of sphingosine-1-phosphate. Prostaglandins Other Lipid Mediat. doi:10.1016/j.prostaglandins.2012.07.003, pii:S1098-8823(12)00101-3 [Epub ahead of print]
81. Lettiero B, Andersen AJ, Hunter AC, Moghimi SM (2012) Complement system and the brain: selected pathologies and avenues toward engineering of neurological nanomedicines. J Control Release 161(2):283–289
82. Reca R, Mastellos D, Majka M, Marquez L, Ratajczak J, Franchini S, Glodek A, Honczarenko M, Spruce LA, Janowska-Wieczorek A, Lambris JD, Ratajczak MZ (2003) Functional receptor for C3a anaphylatoxin is expressed by normal hematopoietic stem/progenitor cells, and C3a enhances their homing related responses to SDF-1. Blood 101:3784–3793
83. Honczarenko M, Ratajczak MZ, Nicholson-Weller A, Silberstein LE (2005) Complement C3a enhances CXCL12 (SDF-1)-mediated chemotaxis of bone marrow hematopoietic cells independently of C3a receptor. J Immunol 175(6):3698–3706
84. Ratajczak J, Reca R, Kucia M, Majka M, Allendorf DJ, Baran JT, Janowska-Wieczorek A, Wetsel RA, Ross GD, Ratajczak MZ (2004) Mobilization studies in mice deficient in either C3 or C3a receptor (C3aR) reveal a novel role for complement in retention of hematopoietic stem/progenitor cells in bone marrow. Blood 103(6):2071–2078
85. Rubio-Perez JM, Morillas-Ruiz JM (2012) A review: inflammatory process in Alzheimer's disease, role of cytokines. ScientificWorldJournal 2012:756357
86. Panaro MA, Cianciulli A (2012) Current opinions and perspectives on the role of immune system in the pathogenesis of Parkinson's disease. Curr Pharm Des 18(2):200–208
87. Mayilyan KR, Weinberger DR, Sim RB (2008) The complement system in schizophrenia. Drug News Perspect 21(4):200–210
88. Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B, Sher A, Litke AM, Lambris JD, Smith SJ, John SW, Barres BA (2007) The classical complement cascade mediates CNS synapse elimination. Cell 131(6):1164–1178
89. Miller G (2007) Neurobiology. Immune molecules prune synapses in developing brain. Science 318(5857):1710–1711
90. Bitzer-Quintero OK, González-Burgos I (2012) Immune system in the brain: a modulatory role on dendritic spine morphophysiology? Neural Plast 2012:348642

91. O'Barr SA, Caguioa J, Gruol D, Perkins G, Ember JA, Hugli T, Cooper NR (2001) Neuronal expression of a functional receptor for the C5a complement activation fragment. J Immunol 166(6):4154–4162
92. Pavlovski D, Thundyil J, Monk PN, Wetsel RA, Taylor SM, Woodruff TM (2012) Generation of complement component C5a by ischemic neurons promotes neuronal apoptosis. FASEB J (e-pub ahead of print 31 May 2012)
93. Movers AL, Henry M, García-Hemández A, Femández-López L (2007) Acute phase proteins as biological markers of negative psychopathology in paranoid schizophrenia. Actas Esp Psiquiatr 35(4):249–252
94. Rudduck C, Beckman L, Franzén G Lindström L (1985) C3 and C6 complement types in schizophrenia. Hum Hered 35(4):255–258
95. Fañanás L, Moral P, Panadero MA, Bertranpetit J (1992) Complement genetic markers in schizophrenia: C3, B F and C6 polymorphisms. Hum Hered 42(3):162–167
96. de la Rubia JA, Zamora C et al (2002) Spanish group of allogeneic peripheral blood stem cell transplantation. Analysis of factors associated with low peripheral blood progenitor cell collection in normal donors. Transfusion 42(1):4–9
97. Arslan O, Moog R (2007) Mobilization of peripheral blood stem cells. Transfus Apher Sci 37(2):179–185
98. Moog R (2008) Management strategies for poor peripheral blood stem cell mobilization. Transfus Apher Sci 38(3):229–236
99. Ratajczak MZ, Shin DM, Liu R, Mierzejewska K, Ratajczak J, Kucia M, Zuba-Surma EK (2012) Very small embryonic/epiblast-like stem cells (VSELs) and their potential role in aging and organ rejuvenation-an update and comparison to other primitive small stem cells isolated from adult tissues. Aging (Albany NY) 4(4):235–246
100. Paczkowska E, Kucia M, Koziarska D, Halasa M, Safranow K, Masiuk M, Karbicka A, Nowik M, Nowacki P, Ratajczak MZ, Machalinski B (2009) Clinical evidence that very small embryonic-like stem cells are mobilized into peripheral blood in patients after stroke. Stroke 40(4):1237–1244
101. Dunac A, Frelin C, Popolo-Blondeau M, Chatel M, Mahagne MH, Philip PJ (2007) Neurological and functional recovery in human stroke are associated with peripheral blood CD34 + cell mobilization. J Neurol 254(3):327–332
102. Schulz C, von Andrian UH, Massberg S (2009) Hematopoietic stem and progenitor cells: their mobilization and homing to bone marrow and peripheral tissue. Immunol Res 44(1–3):160–168
103. Kucharska-Mazur J, Pędziwiatr D, Poniewierska A, Tkacz M, Suszyńska M, Tarnowski M, Samochowiec J, Ratajczak MZ (4 Dec 2012) A lack of positive effect of enhanced vegetative nervous system tonus on mobilization of hematopoietic stem and progenitor cells in patients suffering from acute psychotic syndromes. Leukemia. doi:10.1038/leu.2012.349. [Epub ahead of print]
104. Sanchez-Ramos JR (2002) Neural cells derived from adult bone marrow and umbilical cord blood. J Neurosci Res 69(6):880–893
105. Ratajczak J, Kucia M, Mierzejewska K, Marlicz W, Pietrzkowski Z, Wojakowski W, Greco NJ, Tendera M, Ratajczak MZ (5 Nov 2012) Paracrine proangiopoietic effects of human umbilical cord blood-derived purified CD133(+) cells-implications for stem cell therapies in regenerative medicine. Stem Cells Dev [Epub ahead of print]
106. Nakano K, Migita M, Mochizuki H, Shimada T (2001) Differentiation of transplanted bone marrow cells in the adult mouse brain. Transplantation 71(12):1735–1740
107. Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61(4):364–370
108. Eglitis MA, Mezey E (1997) Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. Proc Natl Acad Sci U S A 94:4080–4085
109. Kopen GC, Prockop DJ, Phinney DG (1999) Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci U S A 96(19):10711–10716

110. Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, Gojo S et al (2001) Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. Differentiation 68(4–5):235–244
111. Duan X, Chang JH, Ge S, Faulkner RL, Kim JY, Kitabatake Y, Liu XB, Yang CH, Jordan JD, Ma DK, Liu CY, Ganesan S, Cheng HJ, Ming GL, Lu B, Song H (2007) Disrupted-In-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. Cell 130(6):1146–1158
112. Mitterauer BJ (2011) Possible role of glia in cognitive impairment in schizophrenia. CNS Neurosci Ther 17(5):333–344
113. Araque A, Parpura V, Sanzgiri RP, Haydon PG (1999) Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci 22(5):208–215
114. Kondziella D, Brenner E, Eyjolfsson EM, Sonnewald U (2007) How do glial-neuronal interactions fit into current neurotransmitter hypotheses of schizophrenia? Neurochem Int 50(2):291–301
115. Williams MR, Marsh R, Macdonald CD, Jain J, Pearce RK, Hirsch SR, Ansorge O, Gentleman SM, Maier M (11 Dec 2012) Neuropathological changes in the nucleus basalis in schizophrenia. Eur Arch Psychiatry Clin Neurosci [Epub ahead of print]
116. Taveggia C, Thaker P, Petrylak A, Caporaso GL, Toews A, Falls DL, Einheber S, Salzer JL (2008) Type III neuregulin-1 promotes oligodendrocyte myelination. Glia 56(3):284–293
117. Na KS, Jung HY, Kim YK (2012) The role of pro-inflammatory cytokines in the neuroinflammation and neurogenesis of schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry. doi:10.1016/j.pnpbp. 2012.10.022, pii:S0278-5846(12)00278-3, [Epub ahead of print]
118. Monje ML, Toda H, Palmer TD (2003) Inflammatory blockade restores adult hippocampal neurogenesis. Science 302(5651):1760–1765
119. Ekdahl CT, Kokaia Z, Lindvall O (2009) Brain inflammation and adult neurogenesis: the dual role of microglia. Neuroscience 158(3):1021–1029
120. Garey L (2010) When cortical development goes wrong: schizophrenia as a neurodevelopmental disease of microcircuits. J Anat 217(4):324–333
121. Wojakowski W, Tendera M, Kucia M, Zuba-Surma E, Paczkowska E, Ciosek J, Hałasa M, Król M, Kazmierski M, Buszman P, Ochała A, Ratajczak J, Machaliński B, Ratajczak MZ (2009) Mobilization of bone marrow-derived Oct-4+SSEA-4+ very small embryonic-like stem cells in patients with acute myocardial infarction. J Am Coll Cardiol 53(1):1–9
122. Wojakowski W, Kucia M, Liu R, Zuba-Surma E, Jadczyk T, Bachowski R, Nabiałek E, Kaźmierski M, Ratajczak MZ, Tendera M (2011) Circulating very small embryonic-like stem cells in cardiovascular disease. J Cardiovasc Transl Res 4(2):138–144
123. Marlicz W, Zuba-Surma E, Kucia M, Blogowski W, Starzynska T, Ratajczak MZ (2012) Various types of stem cells, including a population of very small embryonic-like stem cells, are mobilized into peripheral blood in patients with Crohn's disease. Inflamm Bowel Dis 18(9):1711–1722
124. Liu Y, Jiang P, Deng W (2011) OLIG gene targeting in human pluripotent stem cells for motor neuron and oligodendrocyte differentiation. Nat Protoc 6(5):640–655
125. McKeown CR, Sharma P, Sharipov HE, Shen W, Cline HT (14 Dec 2012) Neurogenesis is required for behavioral recovery after injury in the visual system of *Xenopus laevis*. J Comp Neurol doi:10.1002/cne.23283 [Epub ahead of print]
126. Bernstein HG, Steiner J, Bogerts B (2009) Glial cells in schizophrenia: pathophysiological significance and possible consequences for therapy. Expert Rev Neurother 9(7):1059–1071
127. Xu H, Li XM (2011) White matter abnormalities and animal models examining a putative role of altered white matter in schizophrenia. Schizophr Res Treatment 2011:826976. doi:10.1155/2011/826976. (Epub 2011 Aug 11)
128. Tkachev D, Mimmack ML, Ryan MM, Wayland M, Freeman T, Jones PB, Starkey M, Webster MJ, Yolken RH, Bahn S (2003) Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. Lancet 362(9386):798–805

129. Höistad M, Segal D, Takahashi N, Sakurai T, Buxbaum JD, Hof PR (2009) Linking white and grey matter in schizophrenia: oligodendrocyte and neuron pathology in the prefrontal cortex. Front Neuroanat 3:9
130. Paus T, Keshavan M, Giedd JN (2008) Why do many psychiatric disorders emerge during adolescence? Nat Rev Neurosci 9(12):947–957
131. Löffler S, Klimke A, Kronenwett R, Kobbe G, Haas R, Fehsel K (2010) Clozapine mobilizes CD34+ hematopoietic stem and progenitor cells and increases plasma concentration of interleukin 6 in patients with schizophrenia. J Clin Psychopharmacol 30(5):591–595
132. Pessina A, Turlizzi E, Bonomi A, Guizzardi F, Cavicchini L, Croera C, Bareggi S (2006) In vitro toxicity of clozapine, olanzapine, and quetiapine on granulocyte-macrophage progenitors (GM-CFU). Pharmacopsychiatry 39(1):20–22
133. Xiao L, Xu H, Zhang Y, Wei Z, He J, Jiang W, Li X, Dyck LE, Devon RM, Deng Y, Li XM (2008) Quetiapine facilitates oligodendrocyte development and prevents mice from myelin breakdown and behavioral changes. Mol Psychiatry 13(7):697–708
134. Ahmed EU, Ahmed S, Ukai W, Matsumoto I, Kemp A, McGregor IS, Kashem MA (2012) Antipsychotic induced alteration of growth and proteome of rat neural stem cells. Neurochem Res 37(8):1649–1659
135. Yamauchi T, Tatsumi K, Makinodan M, Kimoto S, Toritsuka M, Okuda H, Kishimoto T, Wanaka A (2010) Olanzapine increases cell mitotic activity and oligodendrocyte-lineage cells in the hypothalamus. Neurochem Int 57(5):565–571
136. Bellon A, Krebs MO, Jay TM (2011) Factoring neurotrophins into a neurite-based pathophysiological model of schizophrenia. Prog Neurobiol 94(1):77–90
137. Selemon LD, Mrzljak J, Kleinman JE, Herman MM, Goldman-Rakic PS (2003) Regional specificity in the neuropathologic substrates of schizophrenia: a morphometric analysis of Broca's area 44 and area 9. Arch Gen Psychiatry 60(1):69–77
138. Mattie M, Brooker G, Spiegel S (1994) Sphingosine-1-phosphate, a putative second messenger, mobilizes calcium from internal stores via an inositol trisphosphate-independent pathway. J Biol Chem 269:3181–3188
139. Berridge MJ (2012) Calcium signalling remodelling and disease. Biochem Soc Trans 40(2):297–309
140. Lee KH, Williams LM, Breakspear M, Gordon E (2003) Synchronous gamma activity: a review and contribution to an integrative neuroscience model of schizophrenia. Brain Res Brain Res Rev 41(1):57–78
141. Kantrowitz JT, Javitt DC (2010) N-methyl-d-aspartate (NMDA) receptor dysfunction or dysregulation: the final common pathway on the road to schizophrenia? Brain Res Bull 83(3–4):108–121
142. Permoda-Osip A, Rybakowski J (2011) Koncepcja glutaminergiczna chorób afektywnych. Psych Pol 45(6):875–888
143. MacLennan AJ, Carney PR, Zhu WJ, Chaves AH, Garcia J, Grimes JR et al (2001) An essential role for the H218/AGR16/Edg-5/LP(B2) sphingosine 1-phosphate receptor in neuronal excitability. Eur J Neurosci 14(2):203–209
144. Coste O, Brenneis C, Linke B, Pierre S, Maeurer C, Becker W, Schmidt H, Gao W, Geisslinger G, Scholich K (2008) Sphingosine 1-phosphate modulates spinal nociceptive processing. J Biol Chem 283(47):32442–32451
145. Koh PO, Undie AS, Kabbani N, Levenson R, Goldman-Rakic PS, Lidow MS (2003) Up-regulation of neuronal calcium sensor-1 (NCS-1) in the prefrontal cortex of schizophrenic and bipolar patients. Proc Natl Acad Sci U S A 100(1):313–317
146. Fernandez-Egea E, Bruna A, Garcia-Rizo C, Bernardo M, Kirkpatrick B (2009) Stem cell signaling in newly diagnosed, antipsychotic-naive subjects with nonaffective psychosis. Mol Psychiatry 14(11):989–991
147. Massberg S, Schaerli P, Knezevic-Maramica I, Köllnberger M, Tubo N, Moseman EA, Huff IV, Junt T, Wagers AJ, Mazo IB, von Andrian UH (2007) Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. Cell 131(5):994–1008

148. Ratajczak MZ, Kim CH, Abdel-Latif A, Schneider G, Kucia M, Morris AJ, Laughlin MJ, Ratajczak J (2012) A novel perspective on stem cell homing and mobilization: review on bioactive lipids as potent chemoattractants and cationic peptides as underappreciated modulators of responsiveness to SDF-1 gradients. Leukemia 26(1):63–72
149. Santos SL, Moura Gubert C, Ceresér KM, Gama CS, Kapczinski F (2012) Increased serum levels of C3 and C4 in patients with schizophrenia compared to euthymic patients with bipolar disorder and healthy. Rev Bras Psiquiatr 34(1):119–120
150. Hakobyan S, Boyajyan A, Sim RB (2005) Classical pathway complement activity in schizophrenia. Neurosci Lett 374(1):35–37
151. Maes M, Delange J, Ranjan R, Meltzer AY, Desnyder R, Cooremans W, Scharpé S (1997) Acute phase proteins in schizophrenia mania and major depression: Modulation by psychotmpic drugs. Psychiatry Res 66(1):1–11
152. Bonifati DM, Kishore U (2007) Role of complement in neurodegeneration and neuroinflammation. Mol Immunol 44(5):999–1010
153. Heese K, Hock C, Otten U (1998) Inflammatory signals induce neurotrophin expression in human microglial cells. J Neurochem 70(2):699–707
154. Rahpeymai Y, Hietala MA, Wilhelmsson U, Fotheringham A, Davies I, Nilsson AK, Zwirner J, Wetsel RA, Gerard C, Pekny M, Pekna M (2006) Complement: a novel factor in basal and ischemia-induced neurogenesis. EMBO J 25(6):1364–1374
155. van Beek J, Nicole O, Ali C, Ischenko A, MacKenzie ET, Buisson A, Fontaine M (2001) Complement anaphylatoxin C3a is selectively protective against NMDA-induced neuronal cell death. Neuroreport 12(2):289–293
156. Dietrich-Muszalska A, Olas B (2009) Modifications of blood platelet proteins of patients with schizophrenia. Platelets 20(2):90–96
157. Dietrich-Muszalska A, Malinowska J, Olas B, Głowacki R, Bald E, Wachowicz B, Rabe-Jabłońska J (2012) The oxidative stress may be induced by the elevated homocysteine in schizophrenic patients. Neurochem Res 37(5):1057–1062
158. Wysoczynski M, Reca R, Lee H, Wu W, Ratajczak J, Ratajczak MZ (2009) Defective engraftment of C3aR-/- hematopoietic stem progenitor cells shows a novel role of the C3a-C3aR axis in bone marrow homing. Leukemia 23:1455–1461
159. Seitz G, Boehmler AM, Kanz L, Möhle R (2005) The role of sphingosine 1-phosphate receptors in the trafficking of hematopoietic progenitor cells. Ann N Y Acad Sci 1044:84–89
160. Ratajczak MZ, Reca R, Wysoczynski M, Yan J, Ratajczak J (2006) Modulation of the SDF-1-CXCR4 axis by the third complement component (C3)-implications for trafficking of CXCR4+ stem cells. Exp Hematol 34:986–995
161. Lambert M, Karow A, Leucht S, Schimmelmann BG, Naber D (2010) Remission in schizophrenia: validity, frequency, predictors, and patients' perspective 5 years later. Dialogues Clin Neurosci 12(3):393–407
162. Turetsky BI, Calkins ME, Light GA, Olincy A, Radant AD, Swerdlow NR (2007) Neurophysiological endophenotypes of schizophrenia: the viability of selected candidate measures. Schizophr Bull 33(1):69–94
163. Calkins ME, Dobie DJ, Cadenhead KS, Olincy A, Freedman R, Green MF et al (2007) The consortium on the genetics of endophenotypes in schizophrenia: model recruitment, assessment, and endophenotyping methods for a multisite collaboration. Schizophr Bull 33(1):33–48
164. Gur RE, Calkins ME, Gur RC, Horan WP, Nuechterlein KH, Seidman LJ, Stone WS (2007) The consortium on the genetics of schizophrenia: neurocognitive endophenotypes. Schizophr Bull 33(1):49–68
165. Greenwood TA, Braff DL, Light GA, Cadenhead KS, Calkins ME, Dobie DJ et al (2007) Initial heritability analyses of endophenotypic measures for schizophrenia: the consortium on the genetics of schizophrenia. Arch Gen Psychiatry 64(11):1242–1250
166. Turetsky BI, Greenwood TA, Olincy A, Radant AD, Braff DL, Cadenhead KS et al (2008) Abnormal auditory N100 amplitude: a heritable endophenotype in first-degree relatives of schizophrenia probands. Biol Psychiatry 64(12):1051–1059

167. Borkowska A, Rybakowski JK (1997) Conjugate lateral eye movements during exacerbation of paranoid schizophrenia and the acute episode of major depression. Biol Psychiatry 41(10):1065–1067
168. Radant AD, Dobie DJ, Calkins ME, Olincy A, Braff DL, Cadenhead KS et al (2007) Successful multi-site measurement of antisaccade performance deficits in schizophrenia. Schizophr Res 89(1–3):320–329
169. Urban M, Rabe-Jabłońska J (2007) Dysfunkcje węchowe u chorych na schizofrenię. Psychiatr Pol 41(4):503–512
170. Mayoral M, Bombín I, Castro-Fornieles J, González-Pinto A, Otero S, Parellada M, Moreno D, Baeza I, Graell M, Rapado M, Arango C (2012) Longitudinal study of neurological soft signs in first-episode early-onset psychosis. J Child Psychol Psychiatry 53(3):323–331
171. Jochum T, Letzsch A, Greiner W, Wagner G, Sauer H, Bär KJ (2006) Influence of antipsychotic medication on pain perception in schizophrenia. Psychiatry Res 142(2–3):151–156
172. Covington MA, Lunden SL, Cristofaro SL, Wan CR, Bailey CT, Broussard B, Fogarty R, Johnson S, Zhang S, Compton MT (2012) Phonetic measures of reduced tongue movement correlate with negative symptom severity in hospitalized patients with first-episode schizophrenia-spectrum disorders. Schizophr Res 142(1–3):93–95
173. Peralta V, Campos MS, De Jalón EG, Cuesta MJ (2010) Motor behavior abnormalities in drug-naïve patients with schizophrenia spectrum disorders. Mov Disord 25(8):1068–1076
174. Schwarz E, Guest PC, Rahmoune H, Harris LW, Wang L, Leweke FM et al (2012) Identification of a biological signature for schizophrenia in serum. Mol Psychiatry 17(5):494–502
175. Borovcanin M, Jovanovic I, Radosavljevic G, Djukic Dejanovic S, Bankovic D, Arsenijevic N, Lukic ML (2012) Elevated serum level of type-2 cytokine and low IL-17 in first episode psychosis and schizophrenia in relapse. J Psychiatr Res 46(11):1421–1426
176. Pedrini M, Massuda R, Fries GR, de Bittencourt PMA, Schnorr CE, Moreira JC et al (2012) Similarities in serum oxidative stress markers and inflammatory cytokines in patients with overt schizophrenia at early and late stages of chronicity. J Psychiatr Res 46(6):819–824
177. Aiello G, Horowitz M, Hepgul N, Pariante CM, Mondelli V (2012) Stress abnormalities in individuals at risk for psychosis: a review of studies in subjects with familial risk or with "at risk" mental state. Psychoneuroendocrinology 37(10):1600–1613
178. Dickerson F, Stallings C, Origoni A, Vaughan C, Khushalani S, Yolken R (2012) Markers of gluten sensitivity in acute mania: a longitudinal study. Psychiatry Res 196(1):68–71
179. Barbosa IG, Rocha NP, Huguet RB, Ferreira RA, Salgado JV, Carvalho LA, Pariante CM, Teixeira AL (2012) Executive dysfunction in euthymic bipolar disorder patients and its association with plasma biomarkers. J Affect Disord 137(1–3):151–155
180. Schwarz E, Izmailov R, Spain M, Barnes A, Mapes JP, Guest PC, Rahmoune H, Pietsch S, Leweke FM, Rothermundt M, Steiner J, Koethe D, Kranaster L, Ohrmann P, Suslow T, Levin Y, Bogerts B, van Beveren NJ, McAllister G, Weber N, Niebuhr D, Cowan D, Yolken RH, Bahn S (2010) Validation of a blood-based laboratory test to aid in the confirmation of a diagnosis of schizophrenia. Biomark Insights 5:39–47
181. Le-Niculescu H, Kurian SM, Yehyawi N, Dike C, Patel SD, Edenberg HJ, Tsuang MT, Salomon DR, Nurnberger JI Jr, Niculescu AB (2009) Identifying blood biomarkers for mood disorders using convergent functional genomics. Mol Psychiatry 14(2):156–174
182. Löffler S, Klimke A, Kronenwett R, Kobbe G, Haas R, Fehsel K (2010) Clozapine mobilizes CD34+ hematopoietic stem and progenitor cells and increases plasma concentration of interleukin 6 in patients with schizophrenia. J Clin Psychopharmacol Oct;30(5):591–595

Chapter 9
Skin Regeneration and Circulating Stem Cells

Julia Borowczyk and Justyna Drukała

9.1 Introduction

The skin serves as a barrier that separates the internal organs of our body from the external environment. It protects the organism from many physical, chemical, and biological factors from the outside world, such as heat, radiation, trauma, and pathogens [1]. The structure of the outermost layer of epidermis keeps the skin and entire organism hydrated by preventing water loss. These critical functions are fulfilled by accessory epidermal appendages that range from hair follicles (HFs), nails, sebaceous glands (SGs), and sweat glands in mammals to scales and feathers in other vertebrates [2]. Mammalian skin is composed of two primary layers, the epidermis and dermis. About 20 different cell types with different embryonic origins compose the adult skin. The epidermis is mainly made up of keratinocytes that create a stratified epithelium and together with all its appendages are derived from the neuroectoderm. The dermis is the second significant element of the skin that lies beneath the epidermis and is separated from it by the basement membrane—a thin layer rich in extracellular matrix (ECM) proteins and growth factors. It is definitely thicker and responsible for the mechanical strength of the skin due to the presence of proteins and glucosaminoglycans organised in a precise net. The dermis is composed of an array of cells that originate from mesoderm, i.e., fibroblasts producing ECM proteins, blood vessel cells that supply nutrients to the skin, arrector pili muscle cells that attach to each HF, the subcutaneous fat cells, and immune cells. The melanocytes, involved in skin pigmentation and sensory nerve endings, are neural crest-derived cells. All these cell types reside in the skin [3]. However, the presence of stem cells (SCs) in the epidermis is crucial because they enable skin renewal and therefore maintain homeostasis and hair regeneration, and also induce a quick and

J. Drukała (✉) · J. Borowczyk
Laboratory of Cell & Tissue Engineering, Department of Cell Biology,
Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University,
Gronostajowa 7, 30–387 Krakow, Poland
e-mail: justyna.drukala@uj.edu.pl

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,*
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_9,

efficient reaction in the case of injury. These cells reside in the basal layer of the epidermis and in the adult HFs and SGs. The SCs possess two essential features: they are able to self-renew for extended periods of time and they demonstrate the ability of multi-lineage differentiation [4].

9.2 Skin Regeneration and Wound Healing

Skin repair and regeneration are two independent processes occurring in damaged skin. The final result of wound closure depends on a balance between these processes. Proper cutaneous wound repair requires a well-coordinated response of inflammation, neovascularisation, ECM formation and re-epithelialisation. Skin wound healing is a multistep process finally resulting in scar formation. Scar tissue is not identical to the skin which it replaced and is usually of inferior functional quality, i.e., it lacks sweat glands and HFs. The inflammatory response after injury results in activation of neutrophils and macrophages which release cytokines regulating granular tissue formation, namely, angiogenesis in primitive connective tissue. Activated fibroblasts become the dominant cell type producing the collagen that forms a scar. At the same time keratinocytes proliferate and migrate across granular tissue to close the wound in the re-epithelialisation process. On the contrary, skin regeneration is strictly connected with the presence of SCs which give rise to a population of proliferating cells rebuilding damaged tissue in the shape of pre-injury. Moreover, scarless tissue re-creation is related to a notably reduced inflammatory process and proper ECM components deposition. In the case of deep wounds, SC niches resident in the skin are damaged and the tissue repair process dominates. Despite a high regenerative potential of epidermal tissue, other populations of SCs may also participate in skin re-creation after wounding [5, 6].

9.3 Homeostasis of Skin Epithelium

The mammalian epidermis is a stratified squamous epithelium. The epidermis consists of a proliferative basal layer and three layers of differentiating cells: the spinous layer, the granular layer and the outermost stratum corneum. The inner (basal) layer is comprised of proliferating cells that produce, secrete and assemble the ECM and express characteristic markers, including keratins and transcription factors [7]. The basal cells adhere to the underlying basement membrane that serves as a physical barrier from the connective tissue and is composed of several ECM components, involving collagens, proteoglycans and laminins. Especially important is laminin-5, having a major impact on the way the cell interacts with the matrix [8]. Laminin-5 acts as a ligand for α4β1 integrins in structures called hemidesmosomes and α3β1 integrins, abundant in focal adhesions (Fas) [9]. As cells withdraw from the cell cycle, leave the basal layer and move outward, they switch off integrin expres-

sion and execute their programme of terminal differentiation. Cells go from basal through spinous and granular layers where they stop being transcriptionally active and end up as dead enucleated cells of the cornified layer that is continuously shed from the skin surface and replaced by inner cells moving outward in a columnar fashion [3]. The most abundant structural proteins of the epidermis are keratins. The basal to spinous/suprabasal transition, a very first step in the terminal differentiation programme, is strictly connected with a specific switch in keratin expression. Keratinocytes in the basal layer express mainly keratins K5 and K14 which mark cells that possess proliferative potential, whereas the intermediate spinous layer is characterised by expression of K1 and K10. Also, great changes in cell morphology and function take place at the granular to stratum corneum transition, where the cornification process occurs. At this time keratinocytes are transformed into enucleated flattened cells (squames, corneocytes) packed with keratin intermediate filaments, which generate a rigid scaffold of structural proteins (the cornified envelope) [10]. When the process of terminal differentiation is complete, the squames exist as dead cells covered by lipids on the outside and filled with an indestructible mass of keratins that is encased by the cornified envelope [2]. Together, the cell envelope and extracellular lipids provide the vital mechanical and water-permeability barrier of stratum corneum that is essential for the survival of mammals in the terrestrial environment [11]. Although the molecular mechanisms underlying the process of epidermal stratification are still unclear, the prominent signal engaged in the basal to suprabasal switch is Notch. The ligands for Notch reside in the basal layer, whereas the spinous cells exhibit Notch receptor expression. Excessively high activity of Notch triggers expression of K1/K10 and fate of suprabasal cells, while low activity maintains an undifferentiated state [12]. Additionally, evidence suggests that the transcription factor p63, a member of the p53 family of proto-oncogenes, might also be involved. It has been found that p63 is likely to play a key role in regulating the self-renewal and long-term proliferative capacity of the SC and is necessary for initiating the skin stratification programme [7]. It has been reported that suprabasal signalling through Notch transmembrane receptors leads to suprabasal repression of p63 and inhibition of proliferation [13]. Interestingly, the involvement of micro-ribonucleic acids (miRNAs) in transcriptional regulatory switches has been indicated. The suprabasal expression of miR-203, an evolutionarily conserved miRNA, coincides with cell differentiation and stratification. It was shown that precocious expression of miR-203 in basal cells triggers their premature differentiation, whereas the absence of this factor enhances cell proliferation [14]. Although further studies will be needed to elucidate the exact mechanism by which basal cells commit to terminal differentiation, much of the action takes place at the basal to first suprabasal cell interface. In homeostasis, the number of SCs in the epidermis remains constant. This can be achieved by two distinct types of cell division: symmetric and asymmetric. Symmetrical cell divisions result in both daughters adopting the same fate, which for SCs would be the generation of two SCs or two differentiated cells [2]. When asymmetric division takes place, one daughter cell remains a SC throughout self-renewal and the other daughter enters the programme of terminal differentiation. Two types of asymmetric divisions can be distinguished in skin epidermis:

in one the plane of cell division is oriented parallel to the underlying basement membrane and the other places the mitotic spindle perpendicular to it [15]. In the first scenario, both daughter cells are positioned at least transiently inside the basal layer. One cell might receive a signal and progressively reduces its adhesiveness to the underlying basement membrane and commits itself to differentiation. In contrast, the perpendicular positioning of the mitotic spindle ensures a simple mechanism of partitioning the two daughters. One of them maintains contact with the basement membrane, and thus receives signals that promote a stemness state. The other daughter cell deprived of basement membrane attachment begins to terminally differentiate [16]. More studies are needed to determine the role of symmetrical and asymmetrical cell division during skin homeostasis and wound healing.

9.4 Epidermal Stem Cells

It has been recognised decades ago that the epidermis contains SCs that replace the differentiated cells of the interfollicular epidermis (IFE), HFs and SGs. However, the existence of multiple resident populations of stem or progenitor cells has recently been discovered.

9.4.1 Interfollicular Epidermis

Some of the earliest studies revealed that there is heterogeneity in cell cycle time in the basal layer of the epidermis. It was hypothesised that SCs divide very rarely (less frequently) whereas their progeny, so called transit amplifying (TA) cells, undergo a few rounds of division before terminal differentiation in the suprabasal layers [17]. Histological analysis has shown that mouse epidermis is organised in stacks of cells with a hexagonal surface area lying on a bed of ten basal cells. This structure was called an epidermal proliferative unit (EPU) [18]. However, it has not yet been elucidated how many SCs are present in each EPU and where the SCs reside within the unit. The results of in vitro studies indicated that cells with the highest expression of β1-integrin have the highest proliferative potential [19]. It was found that β1-enriched human keratinocytes form characteristic clusters that reside in the basal layer of the epidermis. The structure of human skin is definitely thicker when compared to mouse skin. Additionally, it is undulated to form deep epidermal ridges that extend downward and help to anchor the epidermis to the dermis. Lavker and Sun observed slow-cycling cells located at the best protected site within the IFE—at the base of these ridges [20]. β1-bright cells were also found in ridges in which the dermis comes closest to the skin surface in palmoplantar skin, consistent with previous observations of putative SCs. It was proposed that SCs form clusters that help them maintain residence in unique niches which protect against terminal differentiation stimuli such as Notch activation [21]. However, at different sites

of skin, β1-bright keratinocytes were found outside of these zones, thus in a more exposed position for SCs. Hence, the level of β1-integrin as a stemness marker is insufficient [3].In 2006 Jensen and Watt proposed Lrig1 (Leucine-rich repeats and immunoglobulin-like domain protein 1), the antagonist of epidermal growth factor receptor (EGFR), as another identified human interfollicular epidermal SC marker that helps maintain SC quiescence. Others have shown that Lrig-1 expressing cells can give rise to all the adult mouse epidermal lineages in skin reconstitution assays [22]. Further studies are needed to find additional markers defining the phenotype of IFE SCs with the highest proliferative potential. Such markers should also help to locate the SCs in their functional EPU and verify if less frequent cycling is a determinant for SCs in the IFE population.

9.4.2 Hair Follicles

The bulge located at the bottom of the non-cycling portion of each HF contains SCs that generate the cycling part of HF during the growth phase of the hair cycle. This reservoir of quiescent SCs is placed between the SG duct and the attachment site of the arrector pili muscle (APM) in the most exterior cell layer of the HF called the outer root sheath (ORS) which is contiguous with the IFE (Fig. 9.1) [23, 24]. During the growth phase of hair, SCs give rise to highly proliferative TA cells that move downward along the ORS to the hair bulb where they become matrix cells responsible for producing a new hair [25]. Slow cycling is a characteristic of SCs that reside in the bulge. Although relatively quiescent, bulge SCs are stimulated to exit the SC niche, proliferate and differentiate into various cell types during each hair cycle. It has been posited that migration of bulge SC progeny to the base when they become activated and their involvement in maintaining HF homeostasis is important in sustaining the growth of regenerating follicles [26]. In addition, under conditions of injury in which the resident interfollicular epidermal or SG SCs are damaged, bulge SCs can be recruited to facilitate repair by differentiation along these lineages. In contrast, under normal homeostatic conditions, these cells contribute only minimally, if at all, to the maintenance of IFE [2, 27]. It is now clear that there is more than one SC population both within and outside of the classical SC niche located in the bulge. Mouse bulge cells express a number of markers, including K15, CD34 and Lgr5 (Leucine-rich repeat-containing heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptor). It has been shown that the progeny of K15+ cells, which encompass Lgr5+ and CD34+ cells in the resting phase of HF, contribute to all layers of the HF and occasionally to the SG and IFE. Also, cells with SC properties can be found in other areas of mouse HF, including isthmus with multipotent SC expressing MTS24 and infundibulum, characterised by Lrig1 or/and Lgr6 expression [28]. It can be envisioned that active and quiescent SCs exist in the HF. The presence of active SC populations (e.g., Lgr5+, Lgr6+ and Lrig1+) ensures replenishment of cells in precisely defined regions of the HF. The specific microenvironment of the ‘bulge’ niche keeps quiescent SCs in a non-proliferative

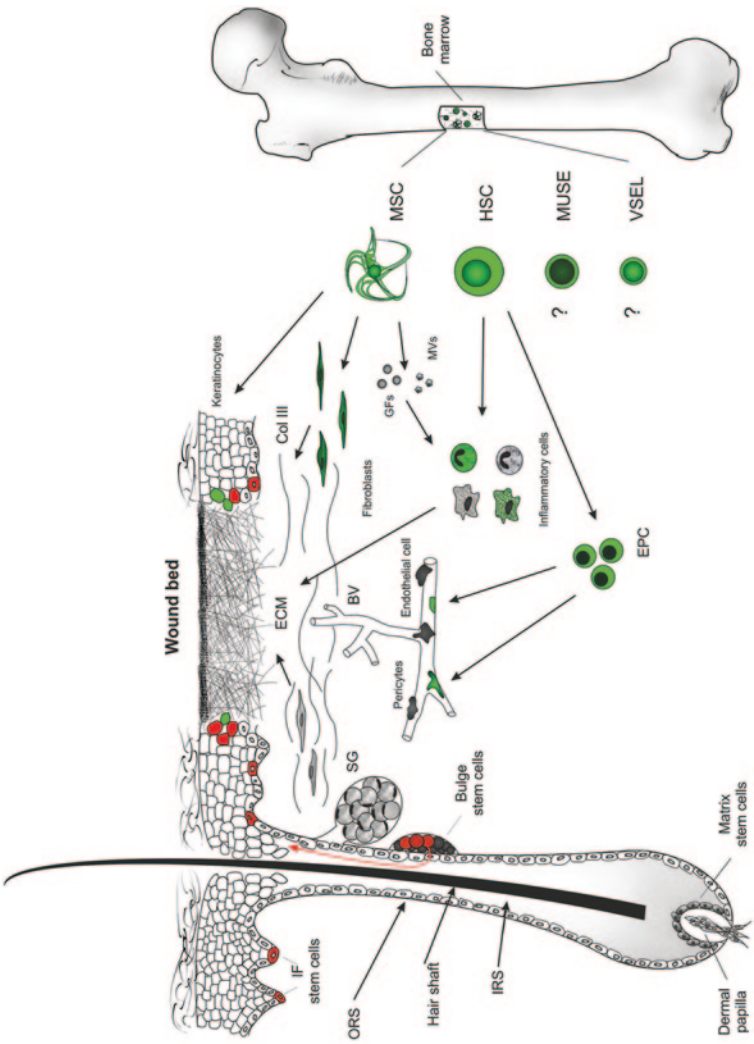

Fig. 9.1 The involvement of stem cells in skin regeneration. The residential skin stem cells play a key role in tissue repair and regeneration. It has been postulated that stem cells mobilised from bone marrow and circulating in peripheral blood are also involved in reconstitution of skin tissue structures. *SG* Sebaceous

state, and thus maintains a reservoir of highly potent SCs capable of successfully managing stress situations such as wound healing [28]. Although several lines of evidence suggest that the human bulge provides a niche for SCs, it is definitely a less distinctive structure than in rodents. Also, in contrast to the bulge of murine follicles, human HFs have a different pattern of biomarkers expression [29, 30]. Positive markers for bulge cells include CD200, pleckstrin homology-like domain, family A, member 1 (PHLDA1), follistatin and frizzled homolog 1 [31]. CD200 is a type-1 transmembrane glycoprotein that delivers a negative immunoregulatory signal through the CD200 receptor (CD200R) [32, 33]. Importantly, Ohyama et al. have demonstrated that CD200+ cells obtained from HF suspensions demonstrated high colony-forming efficiency in clonogenic assays, and thus CD200 is considered as the most specific biomarker for identifying human hair follicular SCs. Further studies are required to assess the SC behaviour of enriched bulge cells and their utility for gene therapy and hair regeneration and whether multiple SC populations in the HF are independent or organised in hierarchy [34].

9.4.3 Sebaceous Glands

In addition to bulge and IFE SCs, evidence for other types of epidermal progenitors has begun to emerge. SG forms at the upper ORS as a terminally differentiating structure that is located just above the bulge of a HF. Differentiated sebocytes produce and secrete lipid-rich sebum into the hair canal that empties out on the skin surface [35, 36]. The results of experiments conducted by Ghazizadeh and Taichman [37] on genetically marked cells have demonstrated the existence of unipotent sebaceous lineage progenitors which are distinct and independent of the HF SC compartment [37]. Recent studies have identified a few resident cells in the SGs that express the transcriptional repressor Blimp1 (B lymphocyte-induced maturation protein 1) and can give rise to the entire gland [36, 38]. These findings suggest that resident progenitors ensure homeostasis, but that bulge SCs are mobilised to migrate upwards and can regenerate SGs when their resident progenitors are lost [34, 36].

9.5 Participation of Bone Marrow-Derived Stem Cells in Wound Healing

Bone marrow (BM) is a well-known reservoir of various SC populations. BM-derived SCs have the capacity for self-renewal and can differentiate into haematopoietic or mesenchymal cell lineages. It is understood that cells recruited from BM are

gland, *MSCs* Mesenchymal stem cells, *HSC* Haematopoietic stem cells, *MUSE* Multilineage differentiating stress enduring cells, *VSELs* Very small embryonic-like stem cells, *EPC* Endothelium progenitor cells, *GFs* Growth factors, *MVs* Microvesicles, *ColIII* Collagen III, *ECM* Extracellular matrix, *IF* Interfollicular, *BV* Blood vessel, *IRS* Inner root sheath, *ORS* Outer root sheath

involved in the inflammatory process of wound healing [39–43]. However, recent studies suggest that the BM contributes non-inflammatory, mesenchymal stem cells (MSCs) to the skin, which are primarily present in the dermis in fibroblast-like morphology or as dendritic cells, and also in the epidermis in a keratinocyte phenotype or in SGs [39, 40, 44]. Moreover, during cutaneous wound healing BM-derived cells differentiate into fibrocytes, i.e., spindle-shaped cells morphologically resembling fibroblasts that express CD45 antigen [45]. It has been shown that a significant percentage of cells in the healed dermis are fibroblast-like cells with BM origin and 30% of them are CD45+ [46]. Furthermore, BM-derived cells were demonstrated to differentiate into CD34+ endothelial progenitor cells, which have been shown to form vascular channels during the first week of wound healing [45]. New vessel formation is a crucial component of wound healing and can occur by two mechanisms: angiogenesis and vasculogenesis. Vasculogenesis involves the formation of de novo blood vessels from circulating vascular progenitor cells (CD34+). Additionally, in vitro studies showed that MSCs are capable of differentiating into vessel forming endothelial cells suggesting that they may contribute to vasculogenesis [39, 47]. On the contrary, other groups demonstrated that MSCs do not participate in the formation of vascular structure [43]. The haematopoietic and mesenchymal populations provide long-term reconstitution of the healed dermis. In addition, MSC progeny produces collagens I and III, whereas the wound-resident cells produce only collagen I. Thus, bone marrow may constitute a rich source of cells that re-establish the healed cutaneous dermis and contribute to collagen III production in the healing skin [46]. Recent studies have shown that bone marrow-derived cells with epithelial phenotype can be found in epithelia and that cutaneous injury leads to increased engraftment of these BM-derived cells as epidermal cells. However, the precise derivations and mechanisms to culture BM-derived keratinocytes are not fully known [43, 46]. Badivas et al. utilised a total bone marrow transplantation model and discovered the presence of bone marrow-derived CD34+ keratinocytes in the hair bulge region, the SC niche for epidermal SCs [39]. In patients that have undergone BM transplantation, donor cells differentiated into keratinocytes and could be detected in the epidermis for at least 3 years [48]. The factor putatively responsible for recruiting mouse BM-derived keratinocyte (BMDK) precursor cells (CD34+, keratin 14+, CD45-) expressing CCR10 receptor into injured skin tissue is cutaneous T-cell attracting chemokine (CTACK/CCL27). This chemokine significantly accelerates wound healing without any influence on either angiogenesis or keratinocyte proliferation at the wound site [49].

9.5.1 Bone Marrow Mesenchymal Stem Cells

Bone marrow MSCs were originally described as plastic adherent, fibroblast-like cells which can differentiate not only into osteoblasts, adipocytes or chondrocytes but also into brain, muscle and kidney tissue [50]. In vivo studies have shown that MSCs can differentiate into tissue specific cells in response to cues provided by different organs [51]. These findings have provided new insights into SC biology

and hold promise for novel restorative techniques. Adult MSCs are believed to be restricted in their ability to produce a wide range of replacement cells, but do nevertheless function as a source of cells for tissue repair and homeostasis. These cells are known to have immunosuppressive effects resulting in evading the allogeneic host immune surveillance system [42, 52]. Some of the properties of MSCs remain obscure. This population of cells is phenotypically poorly characterised. Their differentiation is not tissue-specific, and they are thus qualified as multipotent SCs [53, 54]. It remains under debate whether different subsets of cells are responsible for differentiation into cell types of different lineages [55]. Induction of mechanical stress in skin results in the release of various cytokines which recruit blood-circulating MSCs. It has been shown that these cells can differentiate into multiple skin cell types including keratinocytes, endothelial cells and pericytes. Moreover, acceleration of wound closure by increasing rates of MSC accumulation is mediated by secondary lymphoid-tissue chemokine (SLC/CCL21). Additionally, in the presence of bone morphogenetic protein 4 (BMP-4) MSCs can differentiate into keratinocytes expressing keratin 14 [42]. It has been shown that human haematopoietic SCs (hHSC, CD34+) isolated from peripheral blood (PB) differentiate into keratinocytes in the presence of factors secreted from keratinocytes without cell fusion. Experiments have clearly demonstrated that this conversion is mediated by the plasma environment rather than by direct cell-cell interactions [56]. On the contrary, in vitro studies on human MSC co-culture with keratinocytes indicated that differentiation of these cells into epidermal lineages strictly depends on the proximity of their interaction [57]. The phenotype of BMDK progenitor cells is currently being studied. Nair et al. suggested that p63 is a probable candidate present on lineage-committed keratinocyte progenitor cells. These p63 expressing cells circulating in PB do not express antigen CD34, so their origin is unclear and thus they may have reached circulation either from the bone marrow or from the epidermal compartment. The BMDK progenitor cells represent 4% of the adherent cell population from PB. Interestingly, they can be expanded in vitro and within 12 days of culture these cells co-express p63, CK5, and CK14 [58]. Tamai et al. clearly demonstrated that lineage negative and platelet-derived growth factor receptor alpha positive (Lin-/PDGFRa+) cells from BM (containing a mesenchymal fraction) significantly contribute to the regeneration of the epidermis but only after skin grafting in vivo. Furthermore, one biological repair mechanism involves the Lin-/PDGFRa+cells being mobilised in response to elevated high mobility group protein B, amphoterin (HMGB1) levels in serum, the source of which is the skin graft [59]. At the single-cell level, Kuroda and co-workers showed that adult human skin fibroblasts, MSCs and native BM aspirates contain a distinct type of SC (SSEA3+, CD105+) that is capable of generating cells with characteristics of all three germ layers [55]. This population was termed the multilineage differentiating stress enduring cells (MUSE cells), because of their expression of pluripotency markers (SSEA3, Oct3/4, Sox2) and ability to form clusters in suspension culture. Moreover, these cells differentiate into ectodermal, mesodermal and endodermal cells and they endure through stress conditions (i.e., long-term trypsin incubation for 8 or 16 hours). A noteworthy fact is that they do not form teratomas in immunodeficient mouse testes. The propor-

tion of MUSE cells in bone marrow-derived mononucleated cells is very small. However, a large number of cells can be obtained from mesenchymal cell culture and MUSE cell selection followed by formation of cell clusters in suspension culture and expansion of the cells in adherent culture [55]. Skin regeneration could be explained not only by the presence of endothelial and epidermal progenitors that promote neovasculogenesis and re-epithelialisation but also by the presence of other SCs, including pluripotent, very small embryonic-like SCs (VSELs). A few years ago Ratajczak et al. described a rare population of primitive pluripotent cells called VSELs isolated from adult murine bone marrow and then from several other murine tissues (e.g., brain, liver and heart) [60]. VSELs are small, non-haematopoietic cells expressing markers of pluripotent embryonic and primordial germ cells. In murine bone marrow, VSELs are defined as Lineage-negative, Sca-1-positive and CD45-negative cells of small size (<6 mm in diameter) [60]. These rare cells, slightly smaller than red blood cells, display a distinct morphology with a high nuclear-to-cytoplasmic ratio and open type chromatin. VSELs appear to be quiescent under steady state conditions, apparently due to partially erased imprinting and overexpression of cell cycle inhibitory genes. A similar cell type in humans has begun to be characterised, though with a slightly different phenotype and surface markers. VSELs have been identified in human umbilical cord blood as CXCR4+, CD34+, CD133+, Lin- and CD45- cells enriched for Oct-4 and SSEA-4 [61, 62]. It has been demonstrated that haematopoietic stem/progenitor cells (HSPCs), as well as pluripotent VSELs, are mobilised into PB after myocardial infarction [63, 64], G-CSF treatment [65], stroke [66, 67] and in patients with Crohn's disease [68]. Additionally, we have reported the mobilisation of SCs, including a population of pluripotent VSELs, into PB in patients after skin injury caused by extensive and deep burns [69]. Since this hypothesis assumes that VSELs originate in primordial germ cells (PGM) and are deposited early in embryogenesis in developing organs as a SC reservoir, we attempted to establish if VSELs are present in human skin, especially in the basal layer of the epidermis containing HFs. Our unpublished results from flow cytometry analysis indicated that there is a very rare (<0.01%) population of $CD133^+/SSEA4^+$cells in human epidermis and dermis. With the ImageStream system, we confirmed that these cells are very small (4±1.8 μm in diameter) and express the stemness gene Oct-4. However, we have not managed to isolate and analyse this designated population. Taking it a step further, we attempted to find the precise localisation of VSELs in human epidermis. Using confocal microscopy we searched for cells with a VSEL phenotype in HFs, considered as the most common niche of epidermal SCs. Unfortunately, we only managed to localise single small cells (5 μm diameter) expressing CXCR4 in a sub-bulge area. The obtained data indicate that there is a population of cells deposited in human skin demonstrating characteristic features of VSELs. However, an understanding of the developmental origin of VSELs and their function in vivo will provide important insight related to this goal.

9.5.2 Paracrine Effect of Mesenchymal Stem Cells

In addition to the direct participation of bone marrow cells in tissue regeneration, an important role is also played by paracrine regulation of regeneration processes involving factors secreted by MSCs. MSC paracrine signalling reduces inflammation, promotes angiogenesis, induces cell migration and proliferation [70]. MSCs secrete vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), keratinocyte growth factor (KGF), transforming growth factor beta (TGF-b) and stromal-derived factor 1 (SDF-1). Thus, in vitro studies showed that MSC-conditioned medium acts as a chemoattractant for macrophages, endothelial cells, epidermal keratinocytes and dermal fibroblasts [71]. Moreover, stimulation of cells in damaged organs by microvesicles (MVs) derived from MSCs employed for therapy has recently been widely discussed. MVs may transfer between cells proteins, messenger RNA (mRNA) and miRNA that inhibit apoptosis in damaged cells and are potent proangiogenic factors [72, 73]. This phenomenon has been described in Chap. 10 "Microvesicles and Their Emerging Role in Cellular Therapies for Organ and Tissue Regeneration."

Conclusions

Resident stem and progenitor cells are responsible for skin regeneration and repair processes after injury. SCs circulating in the PB may also be recruited to the injury site in response to wound healing mediators. The presence of multiple epidermal SCs ensures constant and rapid self-renewal of the epidermis. However, in the case of major trauma the resources of resident cells are destroyed, and thus other populations of SCs may be required to take over their functions. Undoubtedly, bone marrow is a unique reservoir of various types of SCs, although they are defined in multiple ways. Interestingly, numerous findings indicate that bone marrow-derived SCs may take part in skin regeneration, and thus provide a useful tool for skin tissue engineering.

Acknowledgments This work was supported by European Union structural funds (Innovative Economy Operational Program POIG.01.01.02-00-109/09-00) to MZR.

References

1. Evans ND, Oreffo ROC, Healy E et al (2013) Epithelial mechanobiology, skin wound healing, and the stem cell niche. J Mech Behav Biomed 28:397–409. doi:10.1016/j.jmbbm.2013.04.023
2. Blanpain C, Fuchs E (2009) Epidermal homeostasis: a balancing act of stem cells in the skin. Nat Rev Mol Cell Bio 10:207–217. doi:10.1038/nrm2636
3. Blanpain C, Fuchs E (2006) Epidermal stem cells of the skin. Annu Rev Cell Dev Biol 22:339–373. doi:10.1146/annurev.cellbio.22.010305.104357

4. Weissman IL, Anderson DJ, Gage F (2001) Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. Annu Rev Cell Dev Biol 17:387–403. doi:10.1146/annurev.cellbio.17.1.387
5. Metcalfe AD, Ferguson MWJ (2007) Tissue engineering of replacement skin: the crossroads of biomaterials, wound healing, embryonic development, stem cells and regeneration. J R Soc Interface 4:413–437. doi:10.1098/rsif.2006.0179
6. Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008) Wound repair and regeneration. Nature 453:314–321. doi:10.1038/nature07039
7. Fuchs E (2008) Skin stem cells: rising to the surface. J Cell Biol 180:273–284. doi:10.1083/jcb.200708185
8. Baker SE, Hopkinson SB, Fitchmun M et al (1996) Laminin-5 and hemidesmosomes: role of the alpha 3 chain subunit in hemidesmosome stability and assembly. J Cell Sci 109:2509–2520
9. Owens DM, Watt FM (2003) Contribution of stem cells and differentiated cells to epidermal tumours. Nat Rev Cancer 3:444–451. doi:10.1038/nrc1096
10. Calista D, Goldstein AM, Landi MT (2000) Origin of the corneocyte lipid envelope (cle): observations in harlequin ichthyosis and cultured human keratinocytes. J Invest Dermatol 115:765–769
11. Kalinin A, Marekov LN, Steinert PM (2001) Assembly of the epidermal cornified cell envelope. J Cell Sci 114:3069–3070
12. Fuchs E (2009) Finding one's niche in the skin. Cell Stem Cell 4:499–502. doi:10.1016/j.stem.2009.05.001
13. Nguyen B-C, Lefort K, Mandinova A et al (2006) Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation. Gene Dev 20:1028–1042. doi:10.1101/gad.1406006
14. Yi R, Poy MN, Stoffel M, Fuchs E (2008) A skin microRNA promotes differentiation by repressing "stemness". Nature 452:225–229. doi:10.1038/nature06642
15. Knoblich JA (2008) Mechanisms of asymmetric stem cell division. Cell 132:583–597. doi:10.1016/j.cell.2008.02.007
16. Lechler T, Fuchs E (2005) Asymmetric cell divisions promote stratification and differentiation of mammalian skin. Nature 437:275–280. doi:10.1038/nature03922
17. Potten CS, Morris RJ (1988) Epithelial stem cells in vivo. J Cell Sci Suppl 10:45–62.
18. Mackenzie IC (1970) Relationship between mitosis and the ordered structure of the stratum corneum in mouse epidermis. Nature 226:653–655
19. 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 73:713–724
20. Lavker RM, Sun TT (1982) Heterogeneity in epidermal basal keratinocytes: morphological and functional correlations. Science 215:1239–1241
21. Jones PH, Harper S, Watt FM (1995) Stem cell patterning and fate in human epidermis. Cell 80:83–93
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 U S A 103:11958–11963. doi:10.1073/pnas.0601886103
23. 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 61:1329–1337
24. Alonso L, Fuchs E (2006) The hair cycle. J Cell Sci 119:391–393. doi:10.1242/jcs02793
25. Oshima H, Rochat A, Kedzia C et al (2001) Morphogenesis and renewal of hair follicles from adult multipotent stem cells. Cell 104:233–245
26. Nowak JA, Polak L, Pasolli HA, Fuchs E (2008) Hair follicle stem cells are specified and function in early skin morphogenesis. Cell Stem Cell 3:33–43. doi:10.1016/j.stem.2008.05.009
27. Morris RJ, Liu Y, Marles L et al (2004) Capturing and profiling adult hair follicle stem cells. Nat Biotechnol 22:411–417. doi:10.1038/nbt950

28. Jaks V, Kasper M, Toftgård R (2010) The hair follicle-a stem cell zoo. Exp Cell Res 316:1422–1428. doi:10.1016/j.yexcr.2010.03.014
29. Inoue K, Aoi N, Sato T et al. (2009) Differential expression of stem-cell-associated markers in human hair follicle epithelial cells. Lab Invest 89:844–856. doi:10.1038/labinvest.2009.48
30. Rochat A, Kobayashi K, Barrandon Y (1994) Location of stem cells of human hair follicles by clonal analysis. Cell 76:1063–1073
31. Ohyama M, Terunuma A, Tock CL et al (2006) Characterization and isolation of stem cell—enriched human hair follicle bulge cells. J Clin Invest 116:249–260. doi:10.1172/JCI26043. tures
32. Wright GJ, Puklavec MJ, Willis AC et al (2000) Lymphoid/neuronal cell surface OX2 glycoprotein recognizes a novel receptor on macrophages implicated in the control of their function. Immunity 13:233–242
33. Wright GJ, Cherwinski H, Foster-Cuevas M et al (2003) Characterization of the CD200 receptor family in mice and humans and their interactions with CD200. J Immunol 171:3034–3046
34. Watt FM, Jensen KB (2009) Epidermal stem cell diversity and quiescence. EMBO Mol Med 1:260–267. doi:10.1002/emmm.200900033
35. Alonso LC, Rosenfield RL (2003) Molecular genetic and endocrine mechanisms of hair growth. Hormone Res 60:1–13. doi:10.1159/000070821
36. Horsley V, O'Carroll D, Tooze R et al (2006) Blimp1 defines a progenitor population that governs cellular input to the sebaceous gland. Cell 126:597–609. doi:10.1016/j.cell.2006.06.048
37. Ghazizadeh S, Taichman LB (2001) Multiple classes of stem cells in cutaneous epithelium: a lineage analysis of adult mouse skin. EMBO J 20:1215–1222. doi:10.1093/emboj/20.6.1215
38. Magnúsdóttir E, Kalachikov S, Mizukoshi K et al (2007) Epidermal terminal differentiation depends on B lymphocyte-induced maturation protein-1. Proc Natl Acad Sci U S A 104:14988–14993. doi: 10.1073/pnas.0707323104
39. Badiavas EV, Abedi M, Butmarc J et al (2003) Participation of bone marrow derived cells in cutaneous wound healing. J Cell Physiol 196:245–250. doi:10.1002/jcp. 10260
40. Chen M, Przyborowski M, Berthiaume F (2009) Stem cells for skin tissue engineering and wound healing. Crit Rev Biomed Eng 37:399–421
41. Falanga V, Iwamoto S, Chartier M et al (2007) Autologous bone marrow-derived cultured mesenchymal stem cells delivered in a fibrin spray accelerate healing in murine and human cutaneous wounds. Tissue Eng 13:1299–1312. doi:10.1089/ten.2006.0278
42. Sasaki M, Abe R, Fujita Y et al (2008) Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type 1. J Immunol 180:2581–2587
43. Wu Y, Chen L, Scott PG, Tredget EE (2007) Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. Stem cells 25:2648–2659. doi:10.1634/stemcells.2007-0226
44. Wu Y, Zhao RCH, Tredget EE (2010) Concise review: bone marrow-derived stem/progenitor cells in cutaneous repair and regeneration. Stem Cells 28:905–915. doi:10.1002/stem.420
45. Asahara T, Masuda H, Takahashi T et al (1999) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 85:221–228. doi:10.1161/01.RES.85.3.221
46. Fathke C, Wilson L, Hutter J et al (2004) Contribution of bone marrow-derived cells to skin: collagen deposition and wound repair. Stem Cells 22:812–822.
47. Oswald J, Boxberger S, Jørgensen B et al (2004) Mesenchymal stem cells can be differentiated into endothelial cells in vitro. Stem Cells 22:377–384. doi:10.1634/stemcells.22-3-377
48. Körbling M, Katz RL, Khanna A et al (2002) Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. New Engl J Med 346:738–746. doi:10.1056/NEJMoa3461002
49. Inokuma D, Abe R, Fujita Y et al (2006) CTACK/CCL27 accelerates skin regeneration via accumulation of bone marrow-derived keratinocytes. Stem Cells 24:2810–2816. doi:10.1634/stemcells.2006-0264

50. Chen JS, Wong VW, Gurtner GC (2012) Therapeutic potential of bone marrow-derived mesenchymal stem cells for cutaneous wound healing. Front Immunol 3:192. doi:10.3389/fimmu.2012.00192
51. Jiang Y, Jahagirdar BN, Reinhardt RL et al (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49. doi:10.1038/nature00870
52. Koç ON, Gerson SL, Cooper BW et al (2000) Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol 18:307–316
53. Dezawa M, Ishikawa H, Itokazu Y et al (2005) Bone marrow stromal cells generate muscle cells and repair muscle degeneration. Science 309:314–317. doi:10.1126/science.1110364
54. Pittenger MF, Mackay AM, Beck SC et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147
55. Kuroda Y, Kitada M, Wakao S et al (2010) Unique multipotent cells in adult human mesenchymal cell populations. Proc Natl Acad Sci U S A 107:8639–8643. doi:10.1073/pnas.0911647107
56. Fujita Y, Inokuma D, Abe R et al (2012) Conversion from human haematopoietic stem cells to keratinocytes requires keratinocyte secretory factors. Clin Exp Dermatol 37:658–664. doi:10.1111/j.1365-2230.2011.04312.x
57. Sivamani RK, Schwartz MP, Anseth KS, Isseroff RR (2011) Keratinocyte proximity and contact can play a significant role in determining mesenchymal stem cell fate in human tissue. FASEB J 25:122–131. doi:10.1096/fj.09-148775
58. Nair RP, Krishnan LK (2013) Identification of p63+ keratinocyte progenitor cells in circulation and their matrix-directed differentiation to epithelial cells. Stem Cell Res Ther 4:38. doi:10.1186/scrt186
59. Tamai K, Yamazaki T, Chino T et al (2011) PDGFRalpha-positive cells in bone marrow are mobilized by high mobility group box 1 (HMGB1) to regenerate injured epithelia. Proc Natl Acad Sci U S A 108:6609–6614. doi:10.1073/pnas.1016753108
60. Kucia M, Reca R, Campbell FR et al (2006) A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 20:857–869. doi:10.1038/sj.leu.2404171
61. Kucia M, Halasa M, Wysoczynski M et al (2007) Morphological and molecular characterization of novel population of CXCR4+SSEA-4+Oct-4+very small embryonic-like cells purified from human cord blood: preliminary report. Leukemia 21:297–303. doi:10.1038/sj.leu.2404470
62. Zuba-Surma EK, Guo Y, Taher H et al (2011) Transplantation of expanded bone marrow-derived very small embryonic-like stem cells (VSEL-SCs) improves left ventricular function and remodelling after myocardial infarction. J Cell Mol Med 15:1319–1328. doi:10.1111/j.1582-4934.2010.01126.x
63. Abdel-Latif A, Zuba-Surma EK, Ziada KM et al (2010) Evidence of mobilization of pluripotent stem cells into peripheral blood of patients with myocardial ischemia. Exp Hematol 38:1131–1142.e1. doi:10.1016/j.exphem.2010.08.003
64. Wojakowski W, Tendera M, Kucia M et al (2009) Mobilization of bone marrow-derived Oct-4+SSEA-4+very small embryonic-like stem cells in patients with acute myocardial infarction. J Am Coll Cardiol 53:1–9. doi:10.1016/j.jacc.2008.09.029
65. Havens AM, Shiozawa Y, Jung Y et al (2013) Human very small embryonic-like cells generate skeletal structures, in vivo. Stem Cell Dev 22:622–630. doi:10.1089/scd.2012.0327
66. Paczkowska E, Kucia M, Koziarska D et al (2009) Clinical evidence that very small embryonic-like stem cells are mobilized into peripheral blood in patients after stroke. Stroke 40:1237–1244. doi:10.1161/STROKEAHA.108.535062
67. Wojakowski W, Ratajczak MZ, Tendera M (2010) Mobilization of very small embryonic-like stem cells in acute coronary syndromes and stroke. Herz 35:467–472. doi:10.1007/s00059-010-3389-0

68. Marlicz W, Zuba-Surma E, Kucia M et al (2012) Various types of stem cells, including a population of very small embryonic-like stem cells, are mobilized into peripheral blood in patients with Crohn's disease. Inflamm Bowel Dis 18:1711–1722. doi:10.1002/ibd.22875
69. Drukała J, Paczkowska E, Kucia M et al (2012) Stem cells, including a population of very small embryonic-like stem cells, are mobilized into peripheral blood in patients after skin burn injury. Stem Cell Rev 8:184–194. doi:10.1007/s12015-011-9272-4
70. Gnecchi M, Zhang Z, Ni A, Dzau VJ (2008) Paracrine mechanisms in adult stem cell signaling and therapy. Circulation Res 103:1204–1219. doi:10.1161/CIRCRESAHA.108.176826
71. Maxson S, Lopez EA, Yoo D et al (2012) Concise review: role of mesenchymal stem cells in wound repair. Stem Cell Transl Med 1:142–149. doi:10.5966/sctm.2011-0018
72. Gatti S, Bruno S, Deregibus MC et al (2011) Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. Nephrol Dial Transpl 26:1474–1483. doi:10.1093/ndt/gfr015
73. Ratajczak J, Miekus K, Kucia M et al (2006) Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. Leukemia 20:847–856. doi:10.1038/sj.leu.2404132

Chapter 10
Stem Cell Therapies in Neonatology

Bogusław Machaliński

10.1 Introduction

Preterm birth, defined as delivery prior to 37 completed weeks, represents the most significant problem in perinatal medicine in the developed countries. Although it is the leading cause of infant mortality and morbidity, its etiology is not precisely identified in a considerable number of cases [1, 2]. The prevalence of preterm birth is still unacceptably high and has not decreased over the last years [1, 2]. Especially, premature birth completed before 32 week of gestation can result in significant morbidities and mortality, hence considerable efforts have been focused on understanding and preventing this devastating pregnancy outcome [3]. Despite improved survival rates of very low birth weight (VLBW) preterm infants weighing less than 1500 g, approximately 50 % of them sustain developmental disabilities [4].

Since, the available treatment options are very limited, the prospect of using stem/progenitor cells (SPCs) for injured tissue repair causes understandably excitement among perinatologists and scientists because it may give hope for improvement of prognosis and reduce sequels of prematurity. In the following text, the pathophysiologic background of prematurity and the rationale for the use of SPCs to regenerate affected tissues will be discussed. It will be further highlighted information on novel therapeutic approach proposed by our group to build a basis for a potential discussion of the future clinical trials.

10.2 The Clinical Problem of Premature Birth: Current State

Preterm labor consists a major pediatric public health problem in aspect of future fate of the premature infant. Premature birth strongly predisposes children to typical complications, including intraventricular hemorrhage (IVH), retinopathy of prema-

B. Machaliński (✉)
Department of General Pathology, Pomeranian Medical University, Szczecin, Poland
e-mail: machalin@pum.edu.pl

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,*
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_10,

turity (ROP), respiratory distress syndrome (RDS), bronchopulmonary dysplasia (BPD), necritizing enterocolitis (NEC), infections, and anemia, among others [5]. The etiology of these complications is multifactorial and includes a local and general hypoxic state, organ ischemia, and a broad-spectrum lack of adaptation to the extrauterine environment [5].

IVH remains the most frequent cause of central nervous system injury in preterm infants. It has been attributed to changes in cerebral blood flow to the immature germinal matrix microvasculature and secondary periventricular venous infarction. In response to hypotension, hypoxemia, hypercapnia, or acidosis, cerebral blood flow rises, hemorrhage begins within the germinal matrix and blood may rupture into the ventricular system [6]. As IVH belongs to early onset, premature complication is most commonly encountered within the first 24 h after birth, and can progress over 48 h or more. From clinical point of view, the most significant consequences of IVH belong post-hemorrhagic hydrocephalus and periventricular leukomalacia. Of note, neonates of less than 1500 g birth weight (BW) suffer the more severe grades of IVH, and vast majority of them develop mental retardation and/or cerebral palsy [6].

ROP is the primary cause of visual impairment in preterm infants and is observed when premature birth interrupts normal vascular development. It is characterized by a premature arrest of retinal development that can lead to an ischemia-induced proliferative retinopathy [7]. Several risk factors for ROP have been studied over the last years, including low BW and short gestational period as the major and most significant. However, general immaturity and debility of the infant's physical health status, as well as prolonged oxygen-therapy have been also consistently related to disease onset. The major pathogenic factor appears to be exposure of the immature retina to a succession of changing levels of oxygen, culminating in abnormally levels of retinal growth factors, such as vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF). These, in turn, lead to a dysregulation of retinal vascularization [8]. Thus, ROP is a biphasic disease consisting of an initial phase of vessel growth retardation followed by a second phase of vessel proliferation. As ROP does not belong to an early onset, premature complications, the first phase of disease occurs from birth to approximately 30–32 weeks of gestational age (GA). The second phase, characterized by hypoxia-induced retinal neovascularization, begins around 32–34 weeks GA [9]. The most critical in ROP management is therefore timely screening of premature infants at risk of developing ROP, as early treatment can result in improved visual outcome. However, the current treatment of laser ablation therapy has limitations with regard to acute and long term complications, including corneal edema, intraocular hemorrhage, and cataract formation. On the other side, novel treatment approaches, suppressing the neovascularization, like anti-VEGF therapies have not yet been sufficiently evaluated to be broadly recommended for clinical treatment [9].

Neonatal RDS is a progressive respiratory failure that is caused primarily by a deficiency of pulmonary surfactants, which contains a mixture of phospholipids and proteins, with surfactant proteins A, B, and C. It is secreted by type II epithelial cells into the airways of the lung from 24-weeks GA although in sufficient amounts from

35-weeks GA. Its main role is to reduce surface tension in the alveoli following the onset of breathing thereby facilitating lung expansion and preventing alveolar collapse during expiration. The surfactant forms a film at the alveoli-air interface, which is respread after each expiration to maintain a low surface tension and prevent lung collapse [10]. Despite the increased rate of preterm delivery, recent advances in perinatal medical management have decreased the incidence and severity of RDS. Clinical innovations that have remarkably modified the natural history of RDS include antenatal corticosteroid administration, surfactant replacement therapy, and increasingly sophisticated use of assisted ventilation in the delivery room and intensive care nursery [11].

The term "bronchopulmonary dysplasia" was introduced to neonatology by Northway et al. to describe a chronic form of neonatal lung injury associated with delivery of barotrauma to preterm infants [12]. Since that time, the spectrum of disease has changed and the emphasis has moved away from baro- or even volutrauma as fundamental to its etiology. Nonetheless, the etiology remains multifactorial and includes low GA, low BW, baro/volutrauma, genetic susceptibility, nutritional deficits, and others [13]. The most of cases is seen in infants below 30-weeks' GA and less than 1200 g BW [14]. Advances in medical care, including antenatal steroid therapy, surfactant use, novel ventilator modalities and strategies, aggressive treatment of patent ductus arteriosus (PDA), and other factors, have altered the nature of BPD. Nowadays, BPD is believed to represent less of the effects of severe lung injury and its repair and more of a disruption or arrest of lung development, such as decreases in alveolarization and a dysmorphic vascular structure [15]. Abnormalities of airways structure and function also persist during long-term follow-up, which lead to recurrent respiratory hospitalizations, reactive airways disease, exercise intolerance, and other problems [16, 17].

Necrotizing enterocolitis is a common, devastating, and multifactorial disease of premature infants with a poorly understood pathogenesis that appears to result from a combination of immaturity of intestinal defenses, enteral feeding, and dysbiosis [18]. Multiple factors are considered to induce NEC, including hypoxia, feeding, sepsis, abnormal colonization of the bowel, and the release of inflammatory mediators stimulated by an ischemic-reperfusion injury in an immature gut. An inflammatory cascade responsible for the changes within gut comprises neutrophil activation, increased permeability of the vasculature, release of reactive oxygen species, and ultimately vasoconstriction with ischemic-reperfusion injury. The incidence of NEC is inversely related to an infant's GA [19]. If an infant develops NEC, symptoms may appear insidiously over the few days before a NEC diagnosis or they may appear acutely. As the mucosal barrier breaks down and NEC becomes severe, it can lead to overwhelming sepsis and death in the worst cases. Treatment for NEC includes bowel rest, gastric decompression, antibiotics, surgical intervention, supportive management of hydration and perfusion, correction of hypotension, metabolic acidosis. Advances in clinical care have improved prognosis, but still nearly 12% of infants with BW less than 1500 g develop NEC, from which about 30% die [19].

Preterm infants have a higher risk of infections due to humoral and myeloid cell functions combined with the use of invasive techniques (e.g., central venous catheter and endotracheal intubation) [20]. Since neonatal bacterial sepsis have relatively high mortality rates and causes morbidity in substantial number of survivors, effective and tolerated anti-microbiologic treatment regimens are specially required [20]. Unfortunately, both broad-spectrum antibiotics and prolonged therapy are associated with adverse outcomes including invasive candidiasis, increased antimicrobial resistance, NEC, and death [21].

Neonates experience a decline in circulating red blood cells (RBCs) during the first weeks after birth. This decline results both from physiological factors and, especially in low BW preterm infants, from iatrogenic factors, including phlebotomy blood losses for laboratory testing. In healthy term infants, the nadir hemoglobin value rarely falls below 10 g/dL at an age of 10–12 weeks [22]. This postnatal decrease in hemoglobin level in term infants is usually well tolerated and does not require therapy. In contrast, in premature infants the nadir of this decline is more rapid and occurs mostly at 4–6 weeks of age. Additionally, the blood hemoglobin level falls to noticeable lower values to approximately 8 g/dL in infants with BW of 1.0–1.5 kg and to approximately 7 g/dL in infants with BW below 1 kg [22]. Consequently, the clinically evident decline in hemoglobin concentration in low BW infants, called as “anemia of prematurity,” requires allogeneic RBC transfusions. Commonly, RBC transfusions are given to maintain an optimal level of blood hemoglobin or HCT for the infant’s clinical condition.

Taken together, preterm infants born before 32 weeks of GA are at extremely high risk for neurodevelopmental, respiratory, and other systemic morbidities with lifelong consequences, including brain injury, lung diseases, and others. The risks for adverse outcomes increase with decreasing GA. The economic costs to care for these infants are also substantial. Therefore, the efficient prevention and treatment of these conditions remains a priority in perinatal medicine. However, in the treatment of prematurity-related, acute onset complications, the current therapeutic strategies are very limited.

10.3 Umbilical Cord Blood as a Source of Stem/Progenitor Cells for Transplantation

Human umbilical cord blood (UCB) plays a crucial role in nutrients and oxygen distribution between a mother and fetus. As it has been widely documented, UCB is a rich source of immature SPCs that reveal superior proliferative characteristics. Due to the immaturity of newborn cells UCB offers many theoretical advantages. UCB is enriched in primitive hematopoietic SPCs able to produce in vivo long-term repopulating stem cells (SCs). The properties of UCB cells compensate to certain extend the relatively low number of cells contained in a single UCB and, through rapid expansion, reconstitute myeloablated patients [23]. Therefore, UCB is widely used in the treatment of various hematopoietic diseases. As it has been revealed,

UCB is even richer in hematopoietic stem cells (HSCs), by unit of volume, than the peripheral blood or bone marrow [23]. Of note, our group and others showed that the number of hematopoietic SPCs detectable in UCB is significantly higher in preterm newborns compared with full-term fetuses. Similarly, a proliferative potential of burst forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte/macrophage (CFU-GM) is noticeably enhanced in preterm infants than in full-term neonates, whereas a frequency of clonogenic precursors decreases with advancing GA [5, 24].

UCB also contains a population of mesenchymal stem cells (MSCs), originally described in the bone marrow that provide a supportive microenvironment for maturation and differentiation of HSCs. The frequency of MSCs in UCB is low, however, they reveal certain advantages over bone-marrow that physiologically decrease MSC number and differentiation potential with age. Although, expected progeny of MSCs include adipocytes, reticular, osteogenic, and other connective tissue cells, it is known that UCB MSCs show potential for neural differentiation, as upon sufficient induction, these cells produce cells with neural phenotypes expressing neural markers. Consequently, it was reported that UCB can induce regeneration in the central nervous system [25].

Attention pays a rare population of very small embryonic-like SCs (VSELs) expressing pluripotent SC markers, described recently in UCB by Kucia et al. [26]. It has been previously demonstrated in mice that VSELs are able to form different types of mature cells [27]. These UCB-derived VSELs, similarly to their counterparts identified in adult murine BM are very small (3–5 mm), possess large nuclei that contain embryonic-type unorganized euchromatin. They also express on their surface stage-specific embryonic antigen-4 (SSEA-4) and intra-nuclear two embryonic transcription factors—Oct-4 and Nanog. These cells are also enriched for messenger ribonucleic acid (mRNA) for several genes regulating tissue specification [26]. Interestingly, our group has demonstrated recently that the number of $CD45^{-}lin^{-}CD184^{+}$ cells, resembling VSEL phenotype, circulating in UCB is inversely associated with the BW of premature infants. These observations imply that during the fetal stage of human life, undifferentiated SCs circulate in the blood in a large number and might contribute to organ/tissue formation. Gradually, the number of $CD45^{-}lin^{-}CD184^{+}$ cells decreases and stabilizes along with the infant's maturation [5].

Recent studies suggest that human UCB is a rich source for endothelial progenitor cells (EPCs) that play a critical role in the formation, maintenance, and repair of the vascular system. They have been shown to incorporate into sites of ischemia and differentiate into mature endothelial cells [28]. It is biologically reasonable that progenitor cells are more readily available in fetal life and more active than those in the adult. Accordingly, a direct comparison of UCB and adult endothelial colony-forming cells (ECFCs) revealed that ECFC concentrations were notably higher in cord blood than in the adult peripheral circulation. In culture, fetal colonies emerged a week earlier than adult colonies and they were consistently larger while the cells forming them were smaller in size [29, 30]. As it has been demonstrated recently by our group, premature infants contain higher numbers of EPCs circulating in their

UCB compared with full-term babies. Additionally, the numbers of UCB EPCs are inversely associated with the Apgar score of preterm infants. A similar trend with inverse correlation was observed between UCB EPC number and GA [31]. As EPCs are critical for vascular development, it could be inferred that circulating EPCs influence the maturation process of vascularized organs. It might be assumed that the number of circulating EPCs depends on systemic demand; therefore, a higher concentration of these cells in the UCB of preterm infants reflects the prematurity of the infants' vascularised organs [31]. Prospective analyses of EPC concentrations in peripheral blood until 6 weeks after birth revealed that the number of circulating EPCs in full-term infants is maintained at a constant, relatively low-level, whereas EPC concentration gradually decreases in preterm infants over time [31]. It is possible that the pool of EPCs diminishes with gradual maturation of the infant's tissues/organs.

During prenatal development, various types of SCs migrate, proliferate, and differentiate to form tissues and organs. Tissue and peripheral blood SPCs pools are in dynamic equilibrium with each other, allowing SCs to migrate from extravascular sites or marginal pools into the circulation and vice versa. Whereas 37–42 weeks of gestation provide an optimal period of time for an infant's maturation to extrauterine life, preterm birth deeply disturbs normal development [5]. Independently of the cause of preterm labor, the infant within seconds sustains a substantial loss because 50% or more of his blood volume with circulating SPC pool is removed with umbilical cord and placental blood vessels. To date, premature infant experiences considerably more significant SPC loss compared to healthy term neonate. Due to more advanced, physiological development, the cord blood and placenta of full-term infant contain approximately 23–27% of total blood volume with relatively lower SPC concentrations, as mentioned above.

Clearly, the question arises as to whether or not a number of SCs circulating in UCB is associated with the development of premature birth complications. Consequently, our group has provided evidence that the number of HSCs circulating in UCB is the independent predictor inversely associated with the development of premature birth complications. These include infections, anemia, IVH, and RDS, complications associated with blood and vascular systems origin, the development of which appears to be conditioned either directly (anemia, infections) or indirectly (other complications) by HSCs activity [5]. In contrast, a positive association between the number of EPC with "early" phenotype ($CD133^+CD34^+CD144^+$) and the risk of RDS, ROP, BPD, and infections was found [31]; however, related to higher EPC number in preterm infants with extremely low BW. Furthermore, we observed that UCB concentrations of $CD45^-lin^-CD184^+$ cells are inversely associated with the BW of preterm infants, suggesting these cells might be involved in the maturation of fetal organism [5]. Thus, it might be speculated that differences in the number of CB-circulating SPCs would contribute to impaired organ growth and the pathogenesis of selected prematurity complications.

10.4 Regenerative Management in Neonatology: Experimental Studies

Since many years allogenic or autogenic HSCs from peripheral blood, bone marrow, or UCB are applied as standard procedure in pediatric oncology for hematopoietic reconstitution after myeloablative therapies or treatment of bone marrow failure syndromes. Furthermore, SC transplantations are frequently performed to treat children affected by diseases with known molecular basis and assured diagnosis, such as inborn, severe anemia, or immunodeficiency disorders of infants [32]. Advances in research on SC biology have provided a perspective for regenerative treatment modalities in neonatology to overcome long term sequelae of prematurity, since it is associated with a high risk of adverse outcome leading to lifelong severe disability. Despite significant progress in medical care of preterm infants, short- and long-term outcome is essentially influenced by neurological damages and respiratory diseases. The risk for the development of sensoric, neurologic, cognitive, and behavioral deficits as well as chronic pulmonary disorders for the survivors remains still noticeable [32]. However, the use of SC-based therapy in specific diseases of neonates is still very limited and most experiences refer to preclinical studies.

The preclinical studies consistently demonstrate beneficial effects of cell therapy applied in the treatment of hypoxic-ischemic encephalopathy (HIE) and BPD. From among different SC population, MSCs are most commonly and preferably used in the experimental approaches. Whether MSCs or other SCs structurally replace defective cells and promote tissue regeneration is still a question requiring further studies. However, the release of growth factors or cytokines by SCs and their progenitors may promote angiogenesis and protect surrounding cells from apoptosis. Paracrine factors can also regulate the homing of other circulating SPCs. Regarding neonatal brain damage, the therapeutic potential of MSCs has been studied in several rodent models of ischemic brain injury [33–35]. The data show that MSC transplantation improves functional outcome and can also restore brain structure [33, 36]. Recently, it has been also shown that transplantation of bone marrow-derived MSCs markedly improves functional outcome after neonatal hypoxic-ischemic (HI) in rodents [33, 36, 37], as the neonatal HI brain injury model has proved to be useful as an animal model of perinatal HIE [38]. Furthermore, intracranial MSC administration after neonatal HI enhanced neurogenesis and oligodendrogenesis and reduced lesion volume [33, 36]. Of note, in preclinical studies, experimental SC-based therapies occurred to be more successful in neonatal than in adult animal models, due to higher plasticity of neonatal compared to adult neuronal cells, as it could be speculated [32].

Hyperoxic exposure of the developing lung is one of the main contributing factors to the development of BPD [39]. Although, currently no effective treatments for BPD have been developed in the clinic, bone marrow-derived MSCs have been shown to ameliorate hyperoxia-induced lung injury (HILI), but the underlying mechanism remains elusive. It has been reported however, that hyperoxia upregulates receptor for advanced glycation end products (RAGE) with transthyretin triggers nuclear transcription factor κB (RAGE/NF-κB) signal pathway in lung tissue

of newborn rats, and transplantation of MSCs attenuates HILI, which may be mediated by the anti-inflammatory effects [40]. Other studies performed in rodents suggest that the acute effects of MSC are mainly paracrine mediated, and thus administration of a cocktail of factors secreted by the MSCs could improve HILI [39]. Altogether, preclinical studies with animals after induction of experimental BPD consistently show that treatment with MSCs may lead to preservation of alveolar structure and lung vascularization, decreased inflammation, and improved pulmonary outcome and survival [32].

Cell-based therapeutic strategies might also be important for neonatal diseases affecting other organs than brain and lungs. The potential therapeutic use of bone marrow-derived MSCs in a rat model of NEC indicated that MSCs successfully homed to injured sites in the bowel and reduced pathological damage [41]. While, immunosuppressive activity of MSCs might well promote bacterial overgrowth and increase transmural bacterial translocation, further studies are necessary to examine long-term effects and risks of this approach.

Overall, the advances in the field of experimental studies suggest that SC-based therapy might represent a novel treatment modality for the repair and regeneration of injured tissues in preterm infants. However, further extensive studies are definitely required to understand the mechanism of SPC action in particular disorders and to present them as a new treatment option for clinical approaches.

10.5 Adjuvant Cell Therapy to Prevent Prematurity Complication Development: A Novel Therapeutic Approach

As it has been broadly described, premature neonates are rapidly and ahead of time deprived from a large amount of CB-circulating SPCs. As a consequence, preterm infants experience deep anemia necessitating RBC transfusion, mostly within few days since the birth. Accordingly, allogenic matched RBCs are given to prevent severe signs and symptoms of anemia. Noteworthy, UCB RBCs are equipped with fetal hemoglobin (HGBF), which is optimal for oxygen and carbon dioxide transport and tissue distribution in newborns. Unlike UCB, adult peripheral blood RBCs possess HGBA (mostly A1) with slightly different physical and chemical properties. However, one of the essential issues is that together with adult RBCs certain volume of liquid is infused into the preterm infant's peripheral circulation. This may lead to additional dilution of already reduced SPC number, still remaining in the infant's blood. Altogether, it might be speculated that differences in the number of circulating SPCs could contribute to impaired organ growth and to the pathogenesis of selected, early-onset prematurity-related complications.

In light of these issues, we propose to replace the SPCs lost with placenta and umbilical cord into peripheral blood of the preterm newborn, by providing the autogenic whole UCB transfusion performed in the first days after birth as soon as the hematological indications for RBC transfusion occur (Fig. 10.1). This manage-

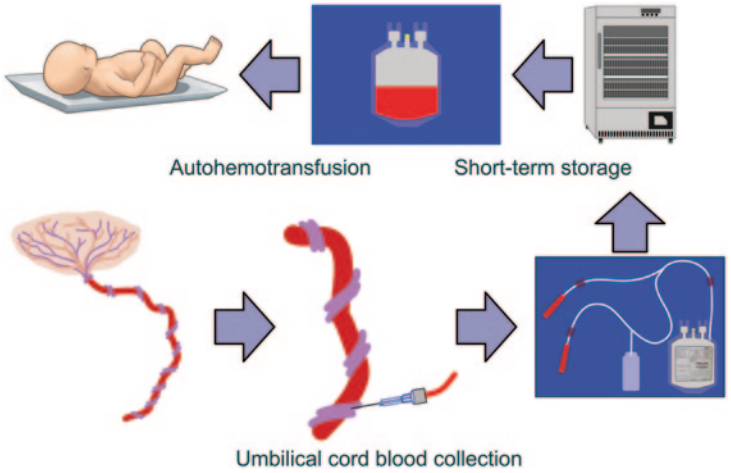

Fig. 10.1 Schematic overview of the novel therapeutic approach

ment may supply not only RBCs with optimal HGBF, but more importantly, restore the initial number of circulating SPCs and in this aspect re-establish physiological conditions for infant development. No systematic clinical investigations concerning the efficacy, recovery, and safety of autologenic UCB transfusion for prevention of prematurity complications have been published to date. We primarily focused on the risk of development of early-onset disorders, such as IVH, RDS, NEC, sepsis, and overall survival in the 1st month after birth. The secondary objective of this study was to evaluate the feasibility of UCB collection, and its short-term storage for subsequent autogenic transfusion to potential preterm UCB donors. Based on the above mentioned concept, 14 anemic preterm newborns (all below 1250 g BW) were included to the preliminary study of which 5 received transfusion of autogenic whole UCB and were compared to preterm neonates, matched according to GA and BW, who were administrated with allogeneic RBCs due to anemia of prematurity.

A principal factor affecting the feasibility of the UCB collection procedure and subsequent its short term storage for transfusion is the volume of the UCB sample. In our study, the average volume of harvested whole UCB was 20 ± 12 mL. While UCB was preserved in the collection bags up to 5 days in the standardized cooler, no signs of clot formation or hemolysis were observed macroscopically. Of note, in two cases the collected UCB units were disqualified from the subsequent transfusion procedure because of the very low HCT (14%) or low HGB concentration (4.3 g/dL), respectively. Both preterm infants were therefore excluded primarily from the study due to the study protocol violation. Besides the feasibility of UCB collection, the main criterion in clinical use is the safety of blood components for the patient. To evaluate the safety of UCB units for transfusion, the ratio of nonsterile preparations was investigated. We did not find bacterial contamination in any of the collected UCB samples in our study, probably due to the fact, that UCB was taken during cesarean section under sterile conditions.

Our preliminary data revealed that none of the premature infants included to the study developed infectious complications such as sepsis or local organ infection. Moreover, only two UCB recipients and two controls (allogenic RBC recipients) met the requirements to diagnose the NEC during the first weeks of their life. However, all UCB recipients and 100% of control children were suffering from RDS. Of note, only 40% of premature autogenic CB recipients compared to 67% control newborns developed neurological complications in the form of IVH (manuscript in preparation). This might indicate that autogenic UCB possesses the potential to reduce the rate of prematurity complications, mostly related to neurodevelopmental processes. While RDS and NEC strongly depend on the GA and affect vast majority of extremely low BW (less than 1000 g BW) preterm infants, IVH might reflect the maturity of blood vessels and vascular development in general. Infusion of autogenic UCB cells, with all SPC populations including EPCs, as a potential treatment strategy might well support physiological blood vessel maturation and effectively prevent IVH onset.

Thus, the implementation of the concept of autogenic UCB collection and subsequent early transfusion seems to be valuable idea with the high impact on prematurity outcome. Likewise, the first clinical experience of an innovative approach is encouraging and has shown feasibility and safety of the described method. Autogenic whole UCB transfusion reveals the potential to be a substitute source of SPCs for protection of physiological preterm infant development, especially concerning central nervous system.

Conclusions and Future Perspectives

The clinical need for therapeutic approach aimed to alleviate symptoms and complications of premature birth is growing. Our preliminary study that included a small number of preterm neonates does not authorize us to draw a substantially clear conclusion. Yet, many hurdles remain to be overcome before safe translation of UCB-based therapies in newborns can be achieved. Nevertheless, promising initial experience led us to believe that further clinical trial, with longer follow-up and well-selected endpoints would help us to fully determine safety and effectiveness of using autogenic UCB in the first days of life for decreasing the risk of systemic complications developing in prematurely born infants.

Although cell-based therapy is relatively novel approach to the treatment of prematurity complications, our encouraging initial experience together with new data derived from novel animal studies widen the horizons for this novel strategy. Having accepted the limitations of our current knowledge, it seems appropriate to state that SC therapies in neonatology hold exceedingly great promise.

Acknowledgments The work was supported by European Union structural funds—Innovative Economy Operational Program POIG.01.01.02-00-109/09-00.

References

1. Wadhwa PD, Entringer S, Buss C, Lu MC (2011) The contribution of maternal stress to preterm birth: issues and considerations. Clin Perinatol 38:351–384
2. Muglia LJ, Katz M (2010) The enigma of spontaneous preterm birth. N Engl J Med 362:529–535
3. Spong CY, Mercer BM, D'Alton M, Kilpatrick S, Blackwell S, Saade G (2011) Timing of indicated late-preterm and early-term birth. Obstet Gynecol 118:323–333
4. Scafidi J, Fagel DM, Ment LR, Vaccarino FM (2009) Modeling premature brain injury and recovery. Int J Dev Neurosci 27:863–871
5. Kotowski MJ, Safranow K, Kawa MP, Lewandowska J, Dziedziejko V, Paczkowska E, Czajka R, Celewicz Z, Rudnicki J, Machalinski B (2012) Circulating hematopoietic stem cell count is a valuable predictor of prematurity complications in preterm newborns. BMC Pediatr 12:148
6. McCrea HJ, Ment LR (2008) The diagnosis, management and postnatal prevention of intraventricular hemorrhage in the preterm neonate. Clin Perinatol 35:777–792
7. Machalińska A, Modrzejewska M, Kotowski M, Dziedziejko V, Kucia M, Kawa M, Safranow K, Baśkiewicz-Masiuk M, Modrzejewska A, Karczewicz D, Rudnicki J, Machaliński B (2010) Circulating stem cell populations in preterm infants: implications for the development of retinopathy of prematurity. Arch Ophtalmol 128:1311–1319
8. Lee J, Dammann O (2012) Perinatal infection, inflammation, and retinopathy of prematurity. Semin Fetal Neonatal Med 17:26–29
9. Chen J, Stahl A, Hellstrom A, Smith LE (2011) Current update on retinopathy of prematurity: screening and treatment. Curr Opin Pediatr 23:173–178
10. McFetridge L, McMorrow A, Morrison PJ, Shields MD (2009) Surfactant metabolism dysfunction and childhood interstitial lung disease (chILD). Ulster Med J 78:7–9
11. Agrons GA, Courtney SE, Stocker JT, Markowitz RI (2005) From the archives of the AFIP: lung disease in premature neonates: radiologic-pathologic correlation. Radiographics 25:1047–1073
12. Northway WH Jr, Rosan RC, Porter DY (1967) Pulmonary disease following respirator therapy of hyaline-membrane disease: bronchopulmonary dysplasia. N Engl J Med 276:357–368
13. Reyburn B, Martin RJ, Prakash YS, MacFarlane PM (2012) Mechanisms of injury to the preterm lung and airway: implications for long-term pulmonary outcome. Neonatology 101:345–352
14. Bhandari A, Bhandari V (2009) Pitfalls, problems, and progress in bronchopulmonary dysplasia. Pediatrics 123:1562–1573
15. Jobe AH, Bancalari E (2001) Bronchopulmonary dysplasia. Am J Respir Crit Care Med 163:1723–1729
16. Abman SH, Mourani PM, Sontag M (2008) Bronchopulmonary dysplasia: a genetic disease. Pediatrics 122:658–659
17. Baraldi E, Filippone M (2007) Chronic lung disease after premature birth. N Engl J Med 357:1946–1955
18. Underwood MA (2012) Paneth cells and necrotizing enterocolitis. Gut Microbes 3:562–565
19. Gephart SM, McGrath JM, Effken JA, Halpern MD (2012) Necrotizing enterocolitis risk: state of the science. Adv Neonatal Care 12:77–87
20. Pacifici GM, Allegaert K (2012) Clinical pharmacokinetics of vancomycin in the neonate: a review. Clinics (Sao Paulo) 67:831–837
21. Tripathi N, Cotten CM, Smith PB (2012) Antibiotic use and misuse in the neonatal intensive care unit. Clin Perinatol 39:61–68
22. Strauss RG (2010) Anaemia of prematurity: pathophysiology and treatment. Blood Rev 24:221–225
23. Gluckman E, Rocha V (2009) Cord blood transplantation: state of the art. Haematologica 94:451–454

24. Opie TM, Shields LE, Andrews RG (1998) Cell-surface antigen expression in early and term gestation fetal hematopoietic progenitor cells. Stem Cells 16:343–348
25. Ali H, Bahbahani H (2010) Umbilical cord blood stem cells—potential therapeutic tool for neural injuries and disorders. Acta Neurobiol Exp (Wars) 70:316–324
26. Kucia M, Halasa M, Wysoczynski M, Baskiewicz-Masiuk M, Moldenhawer S, Zuba-Surma E, Czajka R, Wojakowski W, Machalinski B, Ratajczak MZ (2007) Morphological and molecular characterization of novel population of CXCR4(+) SSEA-4(+) Oct-4(+) very small embryonic-like cells purified from human cord blood—preliminary report. Leukemia 21:297–303
27. Kucia M, Reca R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J, Ratajczak MZ (2006) A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+stem cells identified in adult bone marrow. Leukemia 20:857–869
28. Hunting CB, Noort WA, Zwaginga JJ (2005) Circulating endothelial (progenitor) cells reflect the state of the endothelium: vascular injury, repair and neovascularization. Vox Sang 88:1–9
29. Sipos PI, Crocker IP, Hubel CA, Baker PN (2010) Endothelial progenitor cells: their potential in the placental vasculature and related complications. Placenta 31:1–10
30. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K et al (2004) Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood 9:2752–2760
31. Safranow K, Kotowski M, Lewandowska J, Machalińska A, Dziedziejko V, Czajka R, Celewicz Z, Rudnicki J, Machaliński B (2012) Circulating endothelial progenitor cells in premature infants: is there an association with premature birth complications? J Perinat Med 40:455–462
32. Haase R, Körholz D, Herting E, Sorg RV, Müller LP, Göbel U (2012) Rationale for regenerative treatment in neonatology. Klin Padiatr 224:230–232
33. van Velthoven CT, Kavelaars A, van Bel F, Heijnen CJ (2010a) Nasal administration of stem cells: a promising novel route to treat neonatal ischemic brain damage. Pediatr Res 68:419–422
34. Chen J, Li Y, Wang L, Lu M, Zhang X, Chopp M (2001) Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. J Neurol Sci 189:49–57
35. Kurozumi K, Nakamura K, Tamiya T, Kawano Y, Ishii K, Kobune M, Hirai S, Uchida H, Sasaki K, Ito Y, Kato K, Honmou O, Houkin K, Date I, Hamada H (2005) Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model. Mol Ther 11:96–104
36. van Velthoven CT, Kavelaars A, van Bel F, Heijnen CJ (2010b) Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration. Brain Behav Immun 24:387–393
37. Lee JA, Kim BI, Jo CH, Choi CW, Kim EK, Kim HS, Yoon KS, Choi JH (2010) Mesenchymal stem-cell transplantation for hypoxic-ischemic brain injury in neonatal rat model. Pediatr Res 67:42–46
38. Wang F, Maed N, Yasuhara T, Kameda M, Tsuru E, Yamashit T, Shen Y, Tsud M, Date I, Sagara Y (2012) The therapeutic potential of human umbilical cord blood transplantation for neonatal hypoxic-ischemic brain injury and ischemic stroke. Acta Med Okayama 66:429–434
39. Sutsko RP, Young KC, Ribeiro A, Torres E, Rodriguez M, Hehre D, Devia C, McNiece I, Suguihara C (2013) Long-term reparative effects of mesenchymal stem celltherapy following neonatal hyperoxia-induced lung injury. Pediatr Res 73:46–53
40. Tian Z, Li Y, Ji P, Zhao S, Cheng H (2013) Mesenchymal stem cells protects hyperoxia-induced lung injury in newborn rats via inhibiting receptor for advanced glycation end-products/nuclear factor κB signaling. Exp Biol Med (Maywood) 238:242–247
41. Tayman C, Uckan D, Kilic E, Ulus AT, Tonbul A, Murat Hirfanoglu I, Helvacioglu F, Haltas H, Koseoglu B, Tatli MM (2011) Mesenchymal stem celltherapy in necrotizing enterocolitis: a rat study. Pediatr Res 70:489–494

Chapter 11
Pluripotent Very Small Embryonic-like Stem Cells in Adult Mammalian Gonads

Deepa Bhartiya, Seema Parte, Hiren Patel, Sandhya Anand, Kalpana Sriraman and Pranesh Gunjal

11.1 Development of Mammalian Gonads

Both testis and ovary develop from the gonadal ridge and partly from the mesonephros. The primordial germ cells (PGCs) appear in the yolk sac from the epiblast cells and migrate along the dorsal mesentery to reach the gonadal ridge. PGCs are pluripotent in nature and express nuclear octamer-binding transcription factor 4 (OCT-4) along with other pluripotent markers. During migration, the PGCs divide rapidly but do not differentiate. They are termed gonocytes and differentiate into spermatogonia (which do not enter meiosis) in males and oogonia in the females [1, 2]. Oogonia enter meiosis and are arrested in prophase I, get surrounded by pregranulosa cells, and assemble as a fixed population of primordial follicles at birth [3]. Recent emerging data suggests that PGCs persist into adulthood in the gonads [4, 5]. Moreover, it has also been proposed that PGCs or their precursors during their migration not only settle in the gonads to give rise to germ cells but also settle in various other organs in the body including heart, liver, kidney, pancreas, brain, etc. and persist throughout life as very small embryonic-like stem cells (VSELs)

D. Bhartiya (✉) · S. Parte · H. Patel · S. Anand · K. Sriraman · P. Gunjal
Stem Cell Biology Department, National Institute for Research in Reproductive Health, Indian Council of Medical Research, Jehangir Merwanji Street, Parel, Mumbai 400012, India
e-mail: deepa.bhartiya@yahoo.in; bhartiyad@nirrh.res.in

S. Parte
e-mail: seema.parte@gmail.com

H. Patel
e-mail: hiren_p85@rediffmail.com

S. Anand
e-mail: sandhya0810@gmail.com

K. Sriraman
e-mail: kalpanasriraman@yahoo.com

P. Gunjal
e-mail: praneshgunjal@gmail.com

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,*
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_11,

[6]. These cells maintain lifelong tissue homeostasis and are easily mobilized from the bone marrow under disease conditions [7–11].

It has been suggested by few groups that a novel population of pluripotent stem cells with a potential to self-renew may exist in adult mammalian ovary [2, 5]. Similarly, Virant-Klun's group reported the presence of pluripotent stage-specific embryonic antigen-4 (SSEA-4) positive cells of germ-line lineage in adult human ovaries [12]. Presence of pluripotent VSELs were reported in adult mammalian testis [13] and ovary [14]. Ovarian VSELs are responsible for postnatal neo-oogenesis and primordial follicle assembly [15] and are modulated by follicle stimulating hormone (FSH) [16] via alternatively spliced receptor growth factor type isoform FSH R3 [17]. Similar to their bone marrow counterparts resistant to total body irradiation [18], we observed that gonadal VSELs also are relatively quiescent, remain unaffected by oncotherapy, and survive in germ-cell-depleted mouse and human azoospermic testis [19] as well as in mouse ovary [20]. These recent advances in our understanding of pluripotent VSELs in mammalian gonads are set to alter basic paradigms in reproductive biology and are the main focus of this chapter which will be discussed in greater detail under relevant sections.

11.2 Current Understanding of Testicular Stem Cell Biology

Spermatogonial stem cells (SSCs) were first reported by Brinster and Zimmerman [21]. They are responsible for continued spermatogenesis and are known to have role in restoration of spermatogenesis after transplantation in the seminiferous tubules. Indeed, testicular spermatogenesis is a classic example of tissue-specific stem cell activity involving self-renewal, proliferation, and differentiation. In other organs with high turnover of stem cells like hair follicle, intestinal epithelium, and bone marrow, two distinct stem cell populations exist comprising a quiescent and an actively dividing one [22, 23]. However, the fact whether two populations of stem cells similarly exist in the testis remains controversial [24]. It is believed that division of SSCs is symmetrical in nature [25, 26], whereas a true stem cell is expected to undergo asymmetric cell divisions. Thus, stem cell biology in testicular tissue remains poorly studied at present. VSELs in mouse testis were first reported using flow cytometry-based quantification by Ratajczak's group [27]. Several groups have provided evidence supporting the presence of a sub-population of primitive stem cells among SSCs, but have not specifically employed the term VSELs and thus these stem cells have indeed remained somehow elusive. These reports are listed below:

- Lim et al. [28, 29] reported that in SSCs culture, SSEA-4, and GFRα do not co-localize rather co-exist as two distinct cell populations.
- Izadyar et al. [30] reported the presence of a subpopulation of SSCs with pluripotent characteristics which can repopulate the human testis.

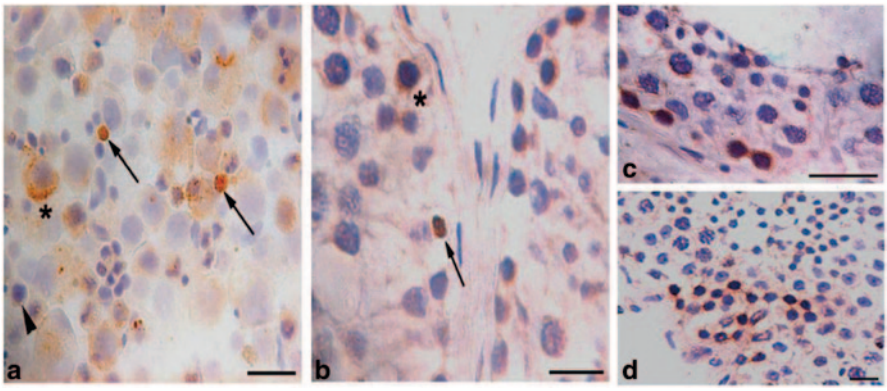

Fig. 11.1 Two populations of stem cells in adult human testis including very small embryonic-like stem cells (VSELs) and spermatogonial stem cells (SSCs) [13]. Immuno-localization of octamer-binding transcription factor 4 OCT-4A and OCT-4B in (**a**) testicular cell smear and (**b**–**d**) testicular sections. Note the presence of very small, spherical VSELs with nuclear OCT-4 (*arrow*) and slightly bigger cells with cytoplasmic OCT-4 (*asterix*) and dark hematoxylin stained nuclei which are the A_{dark} SSCs (**c**) in pairs and (**d**) chains. Nuclear OCT-4 in a pluripotent VSEL shows cytoplasmic localization in the SSCs which initiate differentiation and is eventually lost as cells become more committed (arrowhead). Scale bar = 20 μm

- Bhartiya et al. [13] reported the presence of nuclear OCT-4A positive VSELs which are relatively quiescent and actively dividing SSCs with cytoplasmic OCT-4B in adult human testis.
- Grisanti et al. [31] reported previously unrecognized cell heterogeneity in murine A_s spermatogonia *in vivo.* Have shown that a subset of A_s spermatogonia do not express GFRA1 and were clonogenic by germ cell transplantation assay and that A_s subsets arise by asymmetric cell divisions.
- Kubota et al. [32] reported that SSCs and side population (SP) in testis are two distinct populations and that SSCs are not enriched in the testis SP. The SP reported by them indeed comprises stem cell antigen $(SCA)^+$ pluripotent stem cells (SCA is a pluripotent stem cell marker in mice).
- Huckins [33, 34] reported the existence of both quiescent and actively cycling SSCs based on identification of heterogeneity within the A_s spermatogonial population that was found to contain both long-term and short-term label-retaining cells.

VSELs in adult human testes (Fig. 11.1) are spherical, small-sized cells with high nucleo-cytoplasmic ratio and express various pluripotent markers including nuclear OCT-4A, Nanog, and Sox-2. Large numbers of SSCs with cytoplasmic OCT-4B are also detected which are actively dividing and also appear as chains as a result of rapid cell division with incomplete cytokinesis [13].

Mice testicular VSELs are studied by flow cytometry as 2–10 μm in size, lineage negative, CD45 negative, and SCA-1 positive (Fig. 11.2). They express nuclear OCT-4A, SSEA-1, and SCA-1. They are negative for GFRα, a characteristic marker for SSCs (more than 10 μm in size) personal observations.

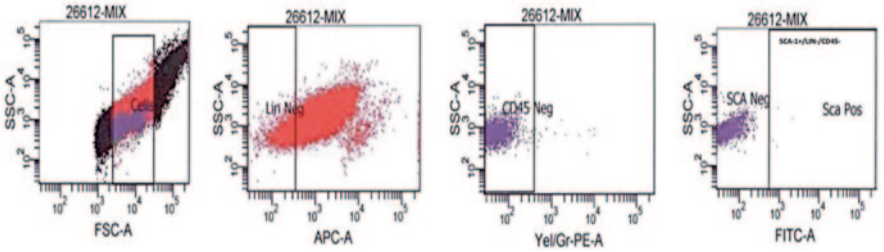

Fig. 11.2 Presence of small sized VSELs which are LIN-/CD45-/SCA-1+ in mouse testis. All events between size 2–10 μm were gated using size calibration beads. This population was further gated as Lineage-negative followed by gating for CD45. Lineage-negative and CD45-negative cell gate was then gated for SCA-1 positive cells

Several groups have reported that testicular tissue culture results in the appearance of embryonic stem cell-like colonies *in vitro* [28, 35–37], possibly due to dedifferentiation of SSCs to pluripotent state. But the efficiency of this reprogramming remains extremely poor and recently several questions have been raised about the pluripotent status of these colonies [38, 39]. It is possible that rather than reprogramming and dedifferentiation of SSCs, VSELs being embryonic in nature start growing as ES-like colonies [40]. We need to contemplate that if all SSCs are exposed to similar culture environment, why only few SSCs dedifferentiate and get reprogrammed? On the other hand, VSELs may be present in very few numbers and hence the poor efficiency for deriving ES-like colonies can be easily explained. Moreover we have observed similar colonies when we culture ovarian tissue [14] and also human endometrial tissue (personal observations). Thus, the growth of ES-like colonies is not something unique to testis, but rather similar VSELs exist in ovary and endometrium also and may be giving rise to ES-like colonies in all the three organs.

11.3 Current Understanding of Ovarian Stem Cell Biology

Female germ line stem cells have been identified and isolated in mice and human ovaries and their functionality has also been demonstrated by several groups [5, 41–44]. These stem cells spontaneously differentiate into oocyte-like structures *in vitro* [14, 45], develop into embryos after long-term culture [46] and have also produced live offsprings [47]. These female oogonial stem cells are located in the ovary surface epithelium (OSE) of adult mammalian ovary and have been equated to SSCs in the testis [41]. Recently Woods and Tilly [42] published protocols to isolate mouse and human ovarian stem cells (OSCs), their culture and detailed characterization. However, we have documented the presence of two distinct populations of stem cells in OSE (Figs. 11.3, 11.4 and 11.5) including VSELs with nuclear OCT-4 and SSEA-4 and slightly bigger ovarian germ stem cells (OGSCs) with cytoplasmic OCT-4 and minimal SSEA-4 expression similar to that in the testis (Fig. 11.1). OSCs and OGSCs possibly describe the same stem cell.

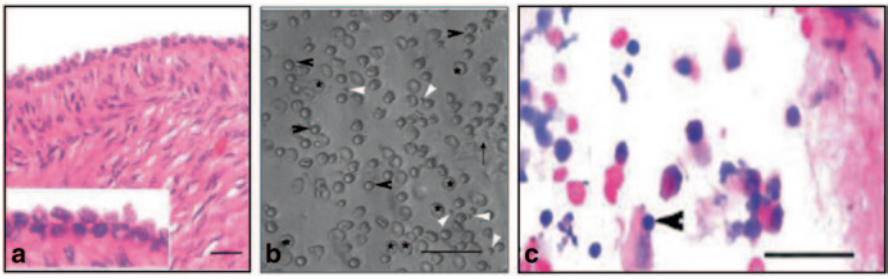

Fig. 11.3 Two populations of stem cells in adult human ovary including very small embryonic-like stem cells (VSELs) and ovarian germ stem cells (OGSCs) [14]. (**a**) H & E-stained section of peri-menopausal human ovarian cortex comprising single layer of cuboidal epithelial cells and is devoid of follicles. (**b**) Scraped ovary surface epithelium (OSE) cells reveal presence of spherical stem cells of two distinct size including the VSELs (*white arrowhead*) and slightly bigger OGSCs (*black arrowhead*) interspersed with epithelial cells (*arrow*) and red blood cells (*asterisk*). (**c**) H & E stained smears of OSE shows the presence of epithelial cells with abundant cytoplasm, red blood cells, and spherical putative stem cells with dark stained nuclei (*arrowhead*) and minimal cytoplasm. Scale bar = 20 μm

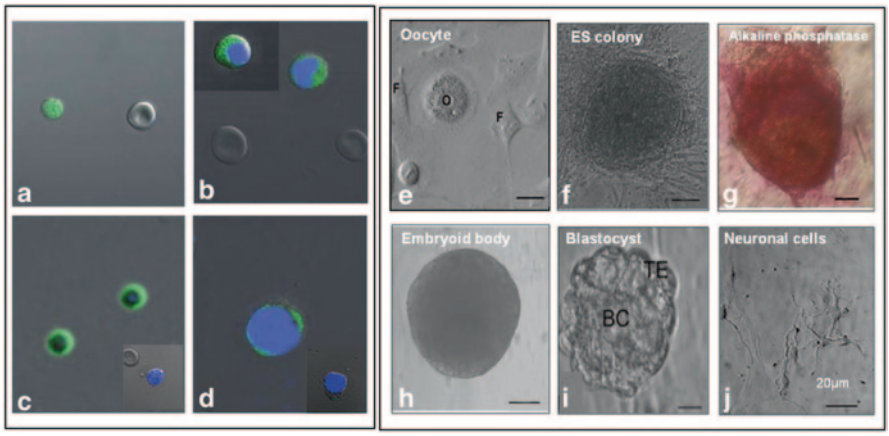

Fig. 11.4 Characterization of ovarian stem cells and spontaneous differentiation *in vitro* [14]. Pluripotent markers nuclear octamer-binding transcription factor 4 (OCT-4; **a**), and cell surface stage-specific embryonic antigen-4 (SSEA-4; **c**) are localized on ovarian VSELs, whereas the slightly bigger OGSCs show cytoplasmic OCT-4 (**b**) and minimal SSEA-4 (**d**). Respective negative controls by omission of primary antibody appear in inset in (**c**) and (**d**). Stem cells spontaneously differentiate within 3 weeks of ovary surface epithelial cells culture into (**e**) oocyte-like (**f**) embryonic stem cell (ES)-like colony (**g**) alkaline phosphatase positive ES-like colony, (**h**) embryoid body-like, (**i**) blastocyst-like structure, and (**j**) neuronal phenotype cells as described earlier [14]. Magnification in **(a)** and **(b)** is X520 with 5X optical zoom and **(c)** and **(d)** is X882 with 5X optical zoom. Merged image of DAPI, FITC, and DIC. Scale bar in e–j = 20 μm. *DAPI* 4′,6-diamidino-2-phenylindole, *FITC* Fluorescein isothiocyanate, *DIC* Differential Interference Contrast

Ovarian stem cells upon three weeks culture differentiate into oocyte-like, parthenote-like, embryoid body-like structures and cells with neuronal-like phenotype, and ES cell-like colonies (Fig. 11.4) [14].

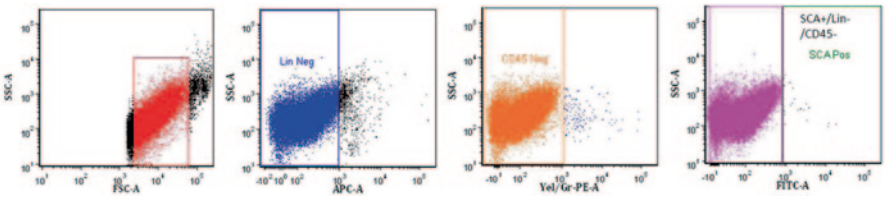

Fig. 11.5 Presence of small sized VSELs which are LIN-/CD45-/SCA-1+ in mouse ovary. Flow cytometry analysis of mouse ovarian VSELs using SCA-1, Lineage and CD45 markers. All events between size 2–10 μm were gated using size calibration beads. This population was further gated as Lineage negative followed by gating for CD45. Lineage negative and CD45 negative cell gate was then gated for SCA-1 positive cells.

Similar to the testicular VSELs, we have studied VSELs in mouse ovary [20] by flow cytometry approach as cells between 2–10 μm in size and LIN-/CD45-/SCA-1+ (Fig. 11.5).

The disparity between the results published by Tilly's group and ours is intriguing and is evidently because of the very small size of VSELs. Similar disparity of results exists in the bone marrow system also where Prof. Ratajczak's group has elegantly shown the presence of VSELs [48] whereas other groups have denied their presence [49]. Thus to summarize, both adult mammalian testis and ovary house relatively quiescent VSELs and actively dividing progenitors termed SSCs in testis and OGSCs in ovary (Fig. 11.6). Presence of VSELs in the gonads needs to be acknowledged in addition to the recent understanding of gonadal stem cell biology provided by Woods and Tilly [41, 42].

11.4 Ovarian Stem Cells are Modulated by FSH through FSH Receptor R3 Isoform

Stem cells are lodged in the OSE. But the question arises is, how these stem cells function *in situ* in an adult mammalian ovary and how does postnatal oogenesis and primordial follicle assembly occur? We know so much about spermatogenesis but apparently oogenesis in adult ovary has not yet been completely described!

Since ovaries are directly under the control of gonadotropins and are routinely stimulated by follicle stimulating hormone (FSH) during assisted reproduction, we studied the effect of pregnant mare serum gonadotropin (PMSG) on adult mouse ovary with a focus on stem cells [15]. Mice were treated with PMSG (5 IU, subcutaneously) and the ovaries were studied on day 2 and 7 (Fig. 11.7). On day 2, focal areas of OSE showed proliferation and clusters of oocytes (either single or with cytoplasmic continuity) were present in these protuberances. By day 7, the protuberances were not observed any longer, however several cohorts of primordial follicles (PF) were evident all over the surface of treated ovary compared to an ovary in estrus with only 1–2 cohorts of follicles. Pluripotent stem cell (Oct-4A, Nanog), meiosis (Stra-8, Scp3, Dmc-1), and developing oocyte (Oct-4B, Mvh, HoxA10,

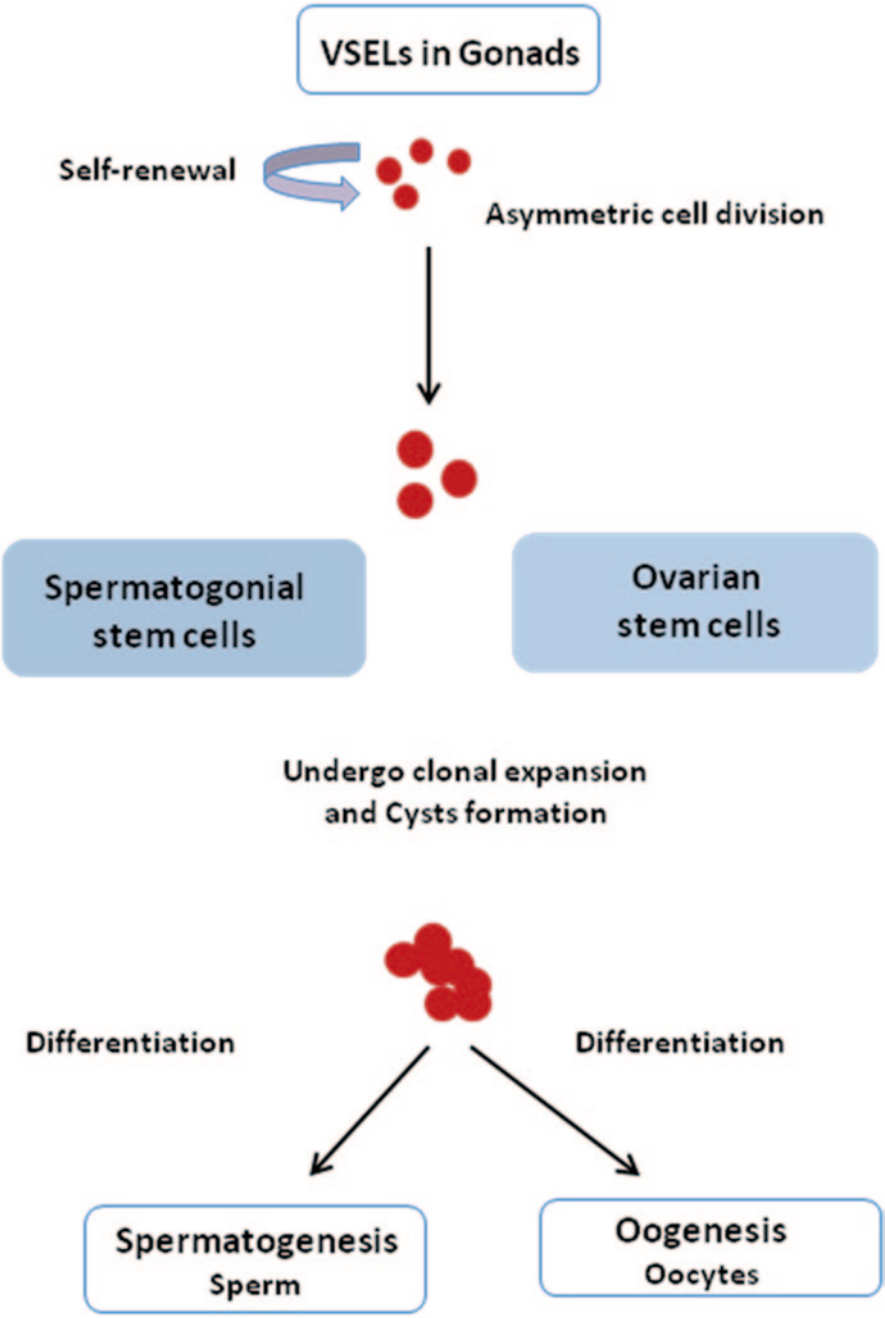

Fig. 11.6 Schematic representation of the stem cells in the mammalian gonads. VSELs are present in both adult ovary and testis. In the ovary the stem cells are lodged in the OSE whereas they are located in the basal epithelial layer in the testicular seminiferous tubules. VSELs are relatively quiescent, undergo asymmetric cell division, and give rise to the progenitors which divide rapidly, undergo clonal expansion and symmetric cell divisions to form cysts, and eventually differentiate into oocytes and sperm, respectively, in the ovary and testis. *VSELs* Very small embryonic-like stem cells, *OSE* Ovary surface epithelium

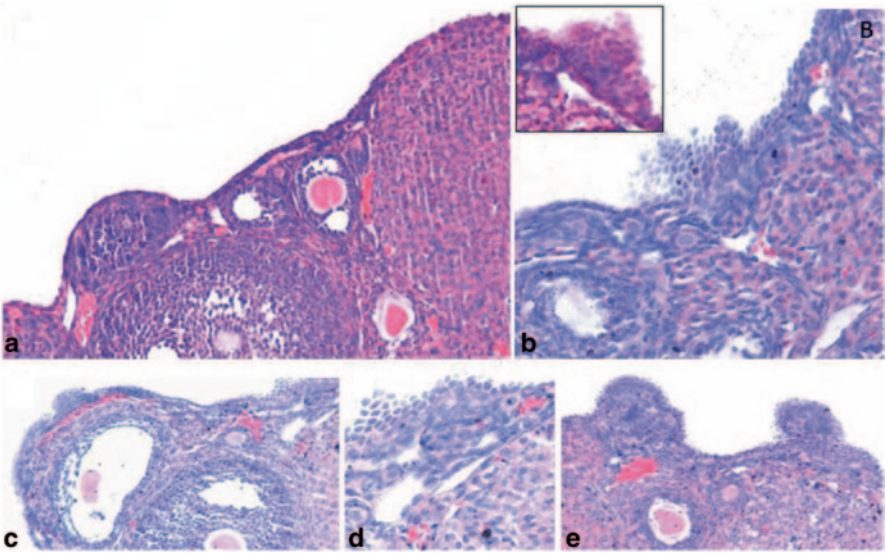

Fig. 11.7 Effect of PMSG (FSH analog) on mouse ovary surface epithelium (OSE) [15]. Besides the well-known effect of FSH on the granulosa cells of developing follicles, it also exerts a direct effect on OSE. Mouse ovary sections from **(a)** untreated ovary and **(b–e)** 2 days after PMSG treatment. Note focal proliferation of OSE at various sites. 10× magnification. *PMSG* Pregnant mare serum gonadotropin, *FSH* Follicle stimulating hormone

Nobox)-specific markers were employed to characterize at protein and messenger ribonucleic acid (mRNA) expression levels. Although FSH did exert a very subtle effect on the OSE in normal estrus cycle, its effect was augmented and became very prominent and obvious after PMSG treatment [15].

Thus, it becomes apparent that the ovarian nuclear OCT-4A positive VSELs are stimulated by FSH, undergo asymmetric cell division to give rise to OGSCs which in turn undergo clonal expansion as germ cell nests/cysts and meiosis to form cohort of primordial follicles (Fig. 11.8). The developing oocytes get surrounded by granulosa cells resulting in primordial follicle assembly. We have earlier proposed that possibly the epithelial cells in OSE undergo epithelial-mesenchymal transition to form granulosa cells [14], in agreement with earlier reports [50, 51].

Then, why such a massive follicle assembly occurs during fetal life? Are the high FSH levels in female fetuses during mid to late gestation responsible for this [52–54]? Also, we need to look more closely as to how do ovaries respond to FSH treatment in an IVF lab! Do the infertility experts actually stimulate the ovarian stem cells to obtain eggs? Our clinical collaborators with several decades of experience tell us that during routine IVF procedure when a woman is stimulated with FSH, the initial follicles that mature are asynchronous at times and the oocytes are of poor quality. Hence these follicles are often ignored and the patients are given additional FSH treatment, which leads to a synchronously growing cohort of follicles that yield high quality of eggs. We have no proof but it can be speculated that the initial follicles arise from pre-existing follicles and the later synchronous cohort

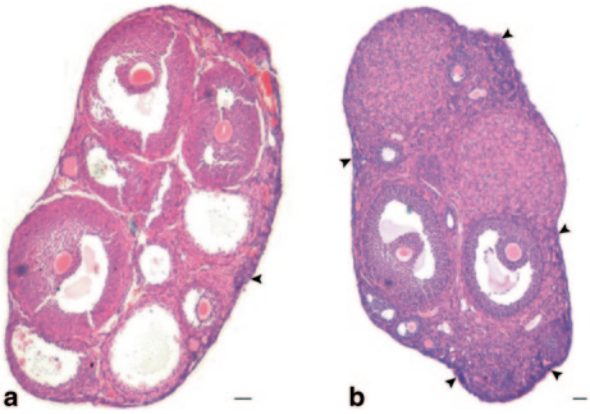

Fig. 11.8 PMSG treatment results in increased number of primordial follicle cohorts on day 7 [15]. Comparative histo-architecture of mouse ovary during normal estrus stage **(a)** and 7 days after PMSG treatment **(b)**. As evident, focal areas where OSE gives multilayer appearance and has associated cohort of PF are markedly increased after PMSG treatment (arrow head) as compared to control. Scale bar = 100 μm. PMSG Pregnant mare serum gonadotropin, OSE Ovary surface epithelium

of follicles possibly arise as a result of stem cell activity in response to FSH. Also sometimes a woman may not respond to FSH treatment and show no follicular development. She is sent home for few days and when she returns after 4–5 days rest—beautifully growing follicles are observed! Where did they originate from? More perplexing question that comes to mind is that how many days does it take for VSELs to assemble into primordial follicles and develop further into a mature follicle? All these questions need to be answered in order to better understand the ovary, which has been exploited for several decades resulting in the birth of millions of babies by IVF.

Action of FSH on the ovarian stem cells was further confirmed by culturing sheep OSE cells with and without FSH (Fig. 11.9). The stem cells underwent clonal expansion and cyst formation. Further we showed that FSH acts on the stem cells through a novel receptor isoform FSH-R3 rather than the canonical isoform FSH-R1 [17].

Ovarian cortical tissue slices are cryopreserved as a source of primordial follicles to achieve biological parenthood in cancer survivors [55]. Careful observation of the cortical tissue pieces growing on cell culture inserts revealed that large numbers of OSE cells interspersed with stem cells were released on the surface of these cell inserts and the stem cells retained their ability to spontaneously differentiate into oocyte-like structures during *in vitro* culture (Figs. 11.10 and 11.11).

In addition to growth of primordial follicles during cortical tissue culture, we observed a prominent proliferation of OSE (which harbors stem cells) after treatment with FSH and basic fibroblast growth factor (bFGF). Thus, we concluded that cortical tissue slices not only serve as source of primordial follicles but may also serve as source for ovarian stem cells which have the potential to spontaneously differentiate into oocyte-like structures. Ovarian cortical tissue cultures have been

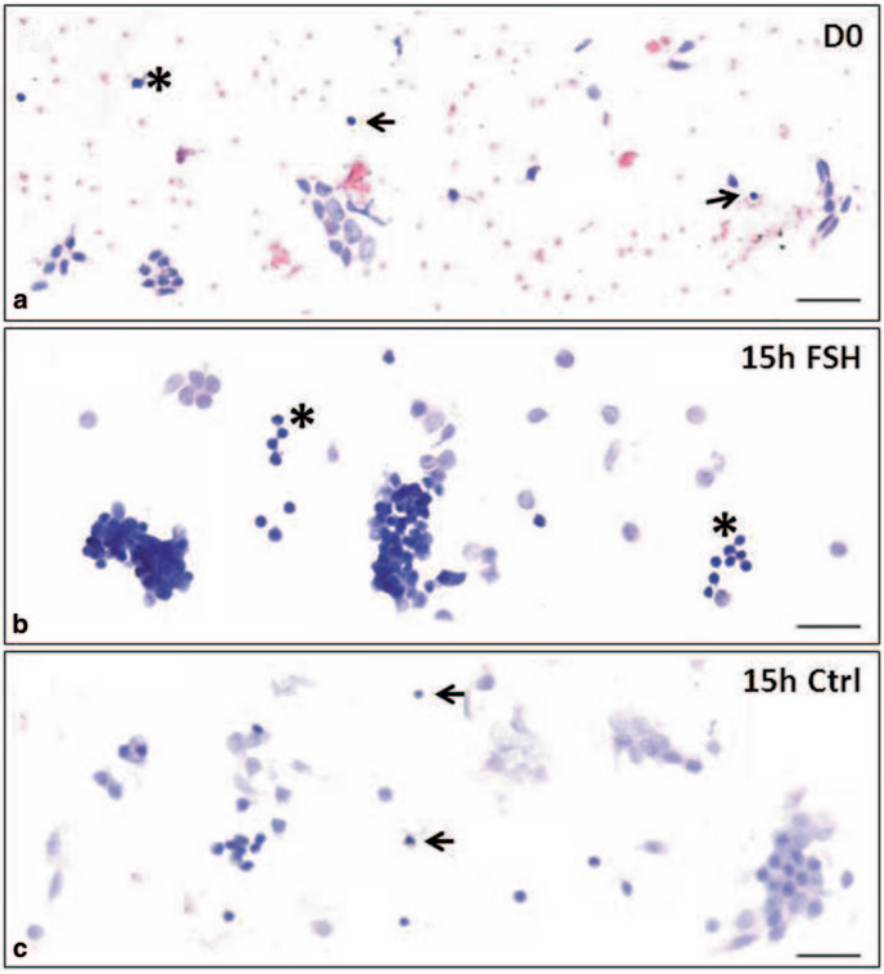

Fig. 11.9 Effect of FSH treatment (5 IU) on sheep ovarian stem cells *in vitro* [17]. (**a)** Freshly prepared sheep OSE smear after H & E staining. Epithelial cells (large spindle shaped cells with abundant cytoplasm) and distinct populations of putative stem cells including the VSELs (*arrow*) and OGSCs (*asterisk*) are evident along with red blood cells. (**b**) Note the increase in number of stem cells and germ cell 'cysts' after 15 h of FSH treatment. (**c)** OSE smear after 15 h culture without FSH appears similar to control (**a**). *OSE* Ovary surface epithelium, *VSELs* Very small embryonic-like stem cells, *OGSCs* Ovarian germ stem cells

studied for variable time including upto 3 weeks whereas the primordial follicle transition into primary and secondary follicles occurs within first 2–3 days [56]. Cortical tissue sections from cultured tissue revealed extensive disintegration by 3 days in untreated samples, whereas FSH and basic FGF addition to culture medium resulted in a healthy OSE with increased stem cell activity [16]. In this context, it is indeed intriguing to note that Garor et al. [57] (please refer to Table 2 of their paper) reported that number of primordial follicles increased from 137 to 247 and

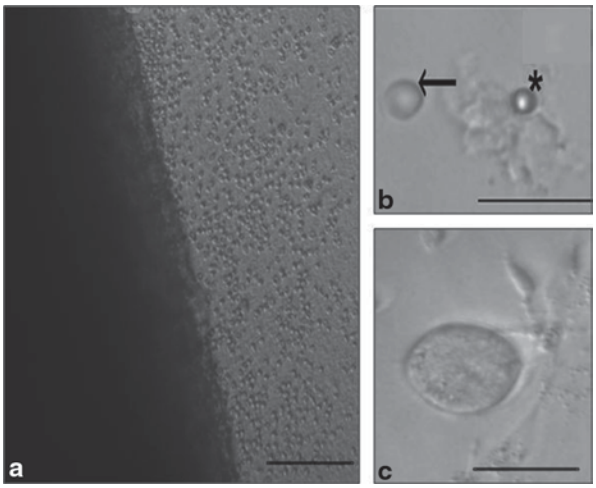

Fig. 11.10 Human ovarian cortical tissue is also a source of stem cells [16]. (**a)** Ovarian cortical tissue showing cells released on cell culture insert surface during organotypic culture. (**b)** Putative stem cells (*asterix*) within released cells on the cell culture inserts along with RBCs (*arrow*) and epithelial cells are visible at a higher magnification. (**c)** Three weeks culture results in spontaneous differentiation of the stem cells into oocyte-like structure similar to our earlier findings [14]. Scale bar = 20 μm in (**a**) and 10 μm in (**b**) and (**c**). *RBCs* red blood cells

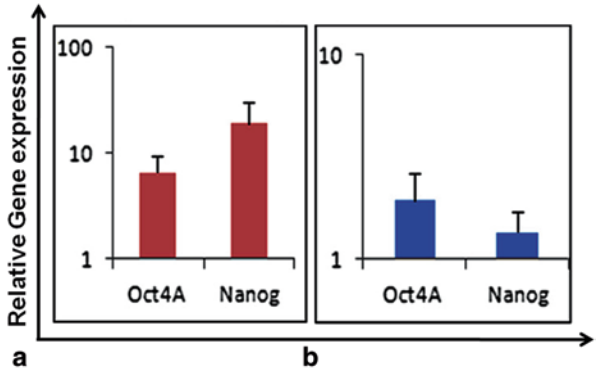

Fig. 11.11 Effect of basic fibroblast growth factor (bFGF) and FSH on pluripotent transcripts by q-RTPCR in human ovarian cortical tissues [16]. An increase of > 8–9 and > 2–3 folds in expression of OCT-4A and Nanog is observed after treatment with (**a)** bFGF and (**b**) FSH treatment respectively with respect to untreated control group. Please note the mean fold change values are expressed over untreated control value taken as one in logarithmic scale (log to the base 10; normalized to 18S house-keeping gene). The results show that both bFGF and FSH stimulate the stem cells present in the OSE (for further details please refer to reference 16). *q-RTPCR* Quantitative real-time reverse-transcription polymerase chain reaction, *OCT-4* Octamer-binding transcription factor 4, *OSE* Ovary surface epithelium

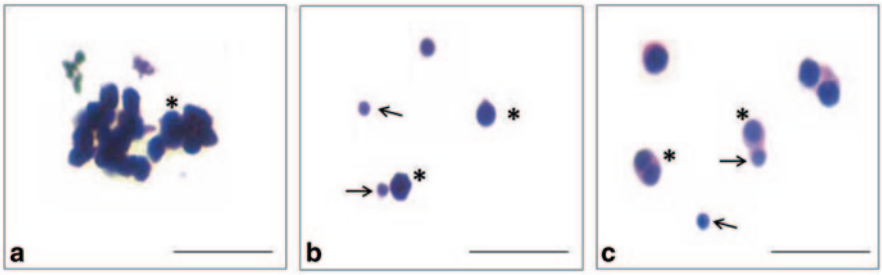

Fig. 11.12 PMSG treatment induces germ cell nest formation in adult mouse after ovary [60]. (**a**) Germ cell nest formed from the ovarian germ stem cells (OGSCs) in PMSG treated mice ovary after H & E staining. Note that the OGSCs stain dark and have minimal cytoplasm. (**b**) Two types of stem cells in mouse ovary surface epithelium (OSE) comprising relatively small VSELs (*arrow*) and slightly bigger OGSCs. (**c**) Stem cells in busulphan treated mouse testicular smear showing similar two kinds of stem cells including smaller (VSELs) and slightly bigger spermatogonial stem cells (SSCs). Dividing cells with incomplete cytokinesis can be clearly seen in the testicular smear. The dividing cells in the centre are of unequal size and whether they represent asymmetric cell division versus the other two dividing pair of cells which are of equal size remains to be determined. Scale bar = 20 μm. *PMSG* Pregnant mare serum gonadotropin, *VSELs* Very small embryonic-like stem cells

301 after treating with 50 ng/ml of bFGF over 3 and 4 weeks in culture. Have they unknowingly reported a stem cell connection? How did the number of follicles increase over time? It is possible that the stem cells undergo neo-oogenesis and primordial follicle assembly *in vitro* during cortical tissue culture, which has remained unnoticed till now!

One of the crucial evidence for stem cells resulting in postnatal oogenesis is the formation of germ cell cysts (or nests), which represent clonal expansion and rapid proliferation of germ cells with incomplete cytokinesis are well-studied structures in the fetal ovary [3]. Lei and Spradling [58] could not detect cysts in adult mouse ovary whereas we have reported cysts in human, sheep and mouse ovaries. The cysts become more evident after PMSG treatment in mice ovaries (Fig. 11.12) [59]. Recently our group has reported that the process of germ cell nest formation, Balbiani body and cyclosis which are well studied and reported in fetal ovaries are recapitulated during spontaneous differentiation of stem cells into oocyte-like structures during human OSE culture [60].

11.5 Novel Action of FSH on Ovary

Data mentioned in above sections provides altogether a new perspective to FSH action on ovary, not yet reported in literature. Existing paradigm suggests that initial follicle growth is independent of FSH action and that FSH acts on the granulosa cells of preantral follicles via the canonical FSH receptor through cyclic adenosine monophosphate (cAMP) pathway resulting in their growth. However, our results

suggest that in addition to this, FSH exerts a direct action on the ovarian stem cells lodged in the OSE via novel growth factor type I receptor transcript FSH R3. Seminal work done by Sairam's group on the detection and functional biology of FSHR transcripts needs to be acknowledged [61]. They have reported that FSH R3 is a growth factor Type I receptor that acts via calcium signaling and mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) pathway. Thus, our results provide a likely mechanism underlying the gonadotropin theory of ovarian cancer and also provide an explanation why majority of ovarian cancers arise from the OSE (possibly the stem cells in OSE give rise to ovarian cancers). Many groups have studied mutations in exon 10 of FSH receptor in women with premature ovarian failure (POF) on the premise that being transmembrane and intracellular in nature may be more crucial for FSH action [62, 63] with negative results. Logically R3 isoform which lacks exon 9 and 10 and spans a novel exon 11 [61] needs to be studied since it is implicated in neo-oogenesis and follicular assembly from stem cells and is likely to be responsible for any defect leading to primary ovarian failure or ovarian cancers rather than canonical R1 receptor isoform which localizes to the granulosa cells and has a role during follicle maturation. FSH receptor isoform R3 is well studied in sheep and we have reported its involvement in sheep ovarian stem cell biology [17]; this transcript in mouse and humans is not yet reported except a few reports in mice [64–67]. The complete azoospermia achieved by Rao's group [68] in non-human primates and the lack of primordial follicles in hamster ovaries by Roy and Albee [69] when they suppressed FSH action by various approaches could possibly be attributed to blocked stem cell function. However, more research in these directions needs to be undertaken.

11.6 Oncofertility and Gonadal Stem Cells

The basic understanding of VSELs in the adult mammalian gonads has provided an altogether new perspective to fertility restoration in infertile individuals with compromised gonadal function including cancer survivors. Infertility is a side effect of cancer treatment since cancer therapy is not specifically targeted to the tumor, but the various actively dividing cells in the body, are affected, leading to hair fall, germ cell depletion, etc. As a result men are rendered azoospermic, whereas the women face premature ovarian failure. Extent of damage depends on the drugs used and the widely accepted concept is to cryopreserve embryos, gametes, or gonadal tissue prior to oncotherapy, for use later on in life when the cancer survivors wish to achieve biological parenthood [69].

Being relatively quiescent in nature, VSELs were found to persist in adult mouse gonads after treatment with busulphan and cyclophosphamide. The gonads were completely depleted of germ cells, but nuclear OCT-4A positive VSELs were present along with the Sertoli cells in testis and in the ovary (Fig. 11.13).

These results are completely in agreement with an earlier report where total body irradiation in mice resulted in complete depletion of actively dividing he-

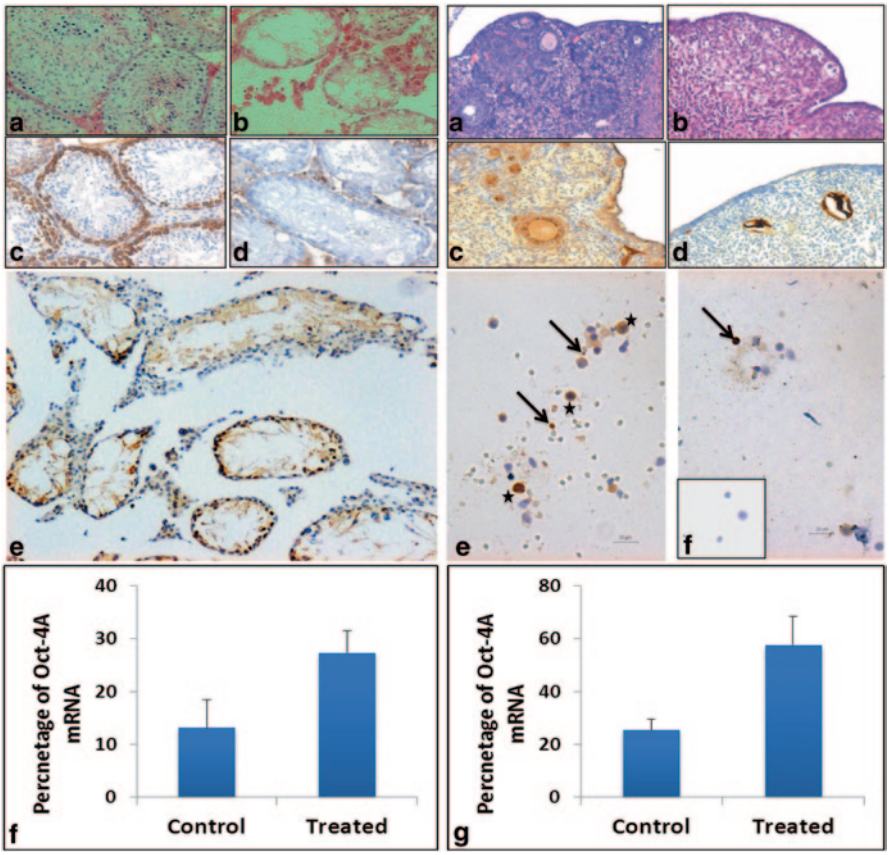

Fig. 11.13 VSELs persist despite chemotherapy in adult mouse testis (**a**–**f**) and ovary (**a**–**g**). H& E stained sections of normal (**a**) and busulphan (25 mg/Kg) treated (**b**) testis. Germ cells are depleted as evident by absent DAZL (germ cell marker) immunolocalization in treated (**d**) versus control (**c**) sections. Few scattered cells in the tubules stain positive for nuclear OCT-4 confirming the presence of VSELs. H & E stained sections of normal (**a**) and treated (**b**) ovary showing absence of follicles after treatment. Immunolocalization for DAZL shows follicles in normal ovary (**c**) and complete absence post treatment (**d**). Immunolocalization for OCT-4 was done on ovarian smears from normal (**e**) and treated ovary (**f**) showing presence of OCT-4 positive cells. Inset in (**f**) is negative control for immunolocalization experiment. Quantitative RT-PCR analysis of OCT-4 (all isoforms) and OCT-4A (isoform specific for pluripotent state) in control versus treated testis (**f**) and ovary (**g**). Percentage of OCT-4A transcript is increased after chemotherapy. *VSELs* Very small embryonic-like stem cells, *OCT-4* Octamer-binding transcription factor 4, *RT-PCR* Real-time reverse-transcription polymerase chain reaction. DAZL Deleted in Azoospermic-like

matopoietic stem cells in the bone marrow, whereas the VSELs persisted and even proliferated as shown by increased BrdU uptake [70]. The reason as to why these persisting VSELs in the testis, ovary, or the bone marrow are unable to differentiate into desired cell types is because of the compromised microenvironment/somatic niche due to the treatment. Transplanting somatic cells to reconstitute the niche resulted in the restoration of spermatogenesis from the persisting VSELs [19] and

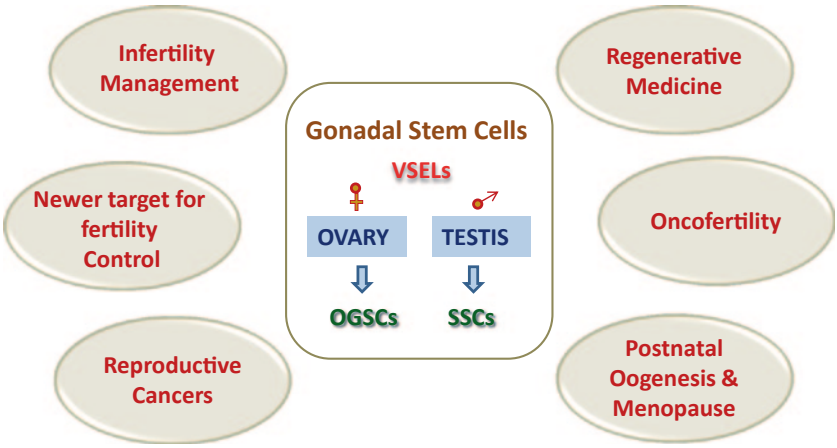

Fig. 11.14 Clinical and translational potential of gonadal stem cells

similarly persisting VSELs in chemoablated ovaries differentiate into oocytes *in vitro* [20]. This success is mind-boggling since it will completely change the field of oncofertility. Basic understanding of VSELs biology in mammalian gonads has translational value (Fig. 11.14), and further research must be pursued with an open mind in order to unfold secrets of Mother Nature, on gonadal biology and combat the various aspects of infertility, menopause, reproductive cancers, etc.

Conflict of interest None

Acknowledgements Would like to acknowledge various groups whose work may be relevant to the chapter but we have made a conscious effort to cite most recent articles. The work was done as part of financial support provided by Indian Council of Medical Research and Department of Biotechnology, Government of India, New Delhi.
NIRRH accession number is OTH/24/10-2013.

References

1. Culty M (2013) Gonocytes, from the fifties to the present: is there a reason to change the name? Biol Reprod 89(2):46
2. De Felici M, Barrios F (2013) Seeking the origin of female germline stem cells in the mammalian ovary. Reproduction 146:R125–R130
3. Pepling ME (2012) Follicular assembly: mechanisms of action. Reproduction 143:139–149
4. De Felici M (2010) Germ stem cells in the mammalian adult ovary: considerations by a fan of the primordial germ cells. Mol Hum Reprod 16:632–636
5. Bhartiya D, Unni S, Parte S, Anand S (2013) Very small embryonic-like stem cells: implications in reproductive biology. Biomed Res Int 2013:682326
6. Ratajczak MZ, Liu R, Ratajczak J, Kucia M, Shin DM (2011) The role of pluripotent embryonic-like stem cells residing in adult tissues in regeneration and longevity. Differentiation 81(3):153–161

7. Wojakowski W, Landmesser U, Bachowski R, Jadczyk T, Tendera M (2012) Mobilization of stem and progenitor cells in cardiovascular diseases. Leukemia 26:23–33
8. Drukała J, Paczkowska E, Kucia M, Młyńska E, Krajewski A, Machaliński B et al (2012) Stem cells, including a population of very small embryonic-like stem cells, are mobilized into peripheral blood in patients after skin burn injury. Stem Cell Rev 8:184–194
9. Ratajczak MZ, Liu R, Marlicz W, Blogowski W, Starzynska T, Wojakowski W (2011) Identification of very small embryonic/epiblast-like stem cells (VSELs) circulating in peripheral blood during organ/tissue injuries. Methods Cell Biol 103:31–54
10. Paczkowska E, Kucia M, Koziarska D, Halasa M, Safranow K, Masiuk M et al (2009) Clinical evidence that very small embryonic-like stem cells are mobilized into peripheral blood in patients after stroke. Stroke 40:1237–1244
11. Kucia MJ, Wysoczynski M, Wu W, Zuba-SurmaEK, Ratajczak J, Ratajczak MZ (2008) Evidence that very small embryonic-like stem cells are mobilized into peripheral blood. Stem Cells 26:2083–2092
12. Virant-Klun I, Skutella T, Hren M, Gruden K, Cvjeticanin B, Vogler A et al (2013) Isolation of small SSEA-4-positive putative stem cells from the ovarian surface epithelium of adult human ovaries by two different methods. Biomed Res Int 2013:690415
13. Bhartiya D, Kasiviswanathan S, Unni SK, Pethe P, Dhabalia JV, Patwardhan S et al (2010) Newer insights into pre-meiotic development of germ cells in adult human testis using Oct-4 as a stem cell marker. J Histochem Cytochem 58:1093–1106
14. Parte S, Bhartiya D, Telang J, Daithankar V, Salvi V, Zaveri K et al (2011) Detection, characterization and spontaneous differentiation *in vitro* of very small embryonic-like putative stem cells in adult mammalian ovary. Stem Cells Dev 20(8):1451–1464
15. Bhartiya D, Sriraman K, Gunjal P, Modak H (2012) Gonadotropin treatment augments postnatal oogenesis and primordial follicle assembly in adult mouse ovaries? J Ovarian Res 5(1):32
16. Parte S, Bhartiya D, Manjramkar DD, Chauhan A, Joshi A (2013) Stimulation of ovarian stem cells by follicle stimulating hormone and basic fibroblast growth factor during cortical tissue culture. J Ovarian Res 6(1):20
17. Patel H, Bhartiya D, Parte S, Gunjal P, Yedurkar S, Bhatt M (2013) Follicle stimulating hormone modulates ovarian stem cells through alternately spliced receptor variant FSH-R3. J Ovarian Res 6(1):52
18. Ratajczak J, Zuba-Surma E, Paczkowska E, Kucia M, Nowacki P, Ratajczak MZ (2011) Stem cells for neural regeneration-a potential application of very small embryonic-like stem cells. J Physiol Pharmacol 62:3–12
19. Anand S, Bhartiya D, Sriraman K, Patel H, Manjramkar DD, Bakshi G et al (2014) Quiescent very small embryonic-like stem cells resist oncotherapy and can restore spermatogenesis in germ cell depleted mammalian testis. Manuscript under review
20. Sriraman K, Bhartiya D, Anand S, Bhutda S (2014) Mouse ovarian very small embryonic-like stem cells resist chemotherapy and initiate oocyte-specific differentiation *in vitro* in response to follicle stimulating hormone. Manuscript under review
21. Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 91:11298–11302
22. Li L, Clevers H (2010) Coexistence of quiescent and active adult stem cells in mammals. Science 327:542–545
23. De Rosa L, De Luca M (2012) Cell biology: dormant and restless skin stem cells. Nature 489:215–217
24. Oatley JM, Brinster RL (2012) The germline stem cell niche unit in mammalian testes. Physiol Rev 92:577–595
25. Caires K, Broady J, McLean D (2010) Maintaining the male germline: regulation of spermatogonial stem cells. J Endocrinol 205:133–145
26. deRooij DG, Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask. J Androl 21:776–798

27. Zuba-Surma EK, Kucia M, Wu W, Klich I, Lillard JW Jr, Ratajczak J et al (2008) Very small embryonic-like stem cells are present in adult murine organs: ImageStream-based morphological analysis and distribution studies. Cytometry A 73 A:1116–1127
28. Lim JJ, Kim HJ, Kim KS, Hong JY, Lee DR (2013) *In vitro* culture-induced pluripotency of human spermatogonial stem cells. Biomed Res Int 2013:143028
29. Lim JJ, Sung SY, Kim HJ, Song SH, Hong JY, Yoon TK et al (2010) Long-term proliferation and characterization of human spermatogonial stem cells obtained from obstructive and non-obstructive azoospermia under exogenous feeder-free culture conditions. Cell Prolif 43:405–417
30. Izadyar F, Wong J, Maki C, Pacchiarotti J, Ramos T, Howerton K et al (2011) Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. Hum Reprod 26(6):1296–1306
31. Grisanti L, Falciatori IG, Muciaccia B et al (2009) Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. Stem Cells 27:3043–3052
32. Kubota H, Mary R, Brinster RL (2003) Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. PNAS 100:6487–6492
33. Huckins C (1971) The spermatogonial stem cell population in adult rats. 3. Evidence for a long-cycling population. Cell Tissue Kinet 4:335–349
34. Mays-Hoopes LL, Bolen J, Riggs AD, Singer-Sam J (1995) Preparation of spermatogonia, spermatocytes, and round spermatids for analysis of gene expression using fluorescence-activated cell sorting. Biol Reprod 53:1003–1011
35. Mizrak SC, Chikhovskaya JV, Sadri-Ardekani H, van Daalen S, Korver CM, Hovingh SE et al (2010) Embryonic stem cell-like cells derived from adult human testis. Hum Reprod 25:158–167
36. deRooij DG, Mizrak SC (2008) Deriving multipotent stem cells from mouse spermatogonial stem cells: a new tool for developmental and clinical research. Development 135:2207–2213
37. Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A et al (2005) Germline niche transplantation restores fertility in infertile mice. Hum Reprod 20:2376–2382
38. Chikhovskaya JV, Jonker MJ, Meissner A, Breit TM, Repping S, van Pelt AM (2012) Human testis-derived embryonic stem cell-like cells are not pluripotent, but possess potential of mesenchymal progenitors. Hum Reprod 27:210–221
39. Warthemann R, Eildermann K, Debowski K, Behr R (2012) False-positive antibody signals for the pluripotency factor OCT4A (POU5F1) in testis-derived cells may lead to erroneous data and misinterpretations. Mol Hum Reprod 18:605–612
40. Bhartiya D, Kasiviswananthan S, Shaikh A (2012) Cellular origin of testis-derived pluripotent stem cells: a case for very small embryonic-like stem cells. Stem Cells Dev 21:670–674
41. Woods DC, Tilly JL (2013) An evolutionary perspective on adult female germline stem cell function from flies to humans. Semin Reprod Med 31:24–32
42. Woods DC, Tilly JL (2013) Isolation, characterization and propagation of mitotically active germ cells from adult mouse and human ovaries. Nat Protoc 8(5):966–988
43. Virant-Klun I, Stimpfel M, Skutella T (2012) Stem cells in adult human ovaries: from female fertility to ovarian cancer. Curr Pharm Des 18:283–292
44. Bukovsky A, Caudle MR (2012) Immunoregulation of follicular renewal, selection, POF, and menopause *in vivo*, vs. neo-oogenesis *in vitro*, POF and ovarian infertility treatment, and a clinical trial. Reprod Biol Endocrinol 10:97
45. Virant-Klun IR, Rulicke TR et al (2009) Parthenogenetic embryo-like structures in the human ovarian surface epithelium cell culture in postmenopausal women with no naturally present follicles and oocytes. Stem Cells Dev 18:137–149
46. White YA, Woods DC, Takai Y, Ishihara O, Seki H, Tilly JL (2012) Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. Nat Med 18:413–421
47. Zou K, Yuan ZY, Zhou L et al (2009) Production of offspring from a germline stem cell line derived from neonatal ovaries. Nat Cell Biol 11:631–636

48. Ratajczak MZ, Zuba-Surma E, Wojakowski W, Suszynska M, Mierzejewska K, Liu R et al (2013) Very small embryonic like stem cells (VSELs) represent a real challenge in stem cell biology. Recent pros and cons in the midst of a lively debate. Leukemia. doi:10.1038/leu.2013.255 [Epub ahead of print]
49. Abbott A (2013) Doubt cast over tiny stem cells. Nature 499:390. doi:10.1038/499390a
50. Chang HL, MacLaughlin DT, Donahoe PK (2009) Somatic stem cells of the ovary and their relationship to human ovarian cancers (April 30, 2009), Stem Book, ed. The stem cell research community, Stem Book. doi/10.3824/stembook.1.43.1, http://www.stembook.org
51. Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC (2001) Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr Rev 22:255–288
52. Lei L, Jin S, Mayo KE, Woodruff TK (2010) The interactions between the stimulatory effect of follicle-stimulating hormone and the inhibitory effect of estrogen on mouse primordial folliculogenesis. Biol Reprod 5:13–22
53. Debieve F, Beerlandt S, Hubinont C, Thomas K (2000) Gonadotropins, prolactin, inhibin A, inhibin B, and activin A in human fetal serum from mid-pregnancy and term pregnancy. J Clin Endocrinol Metab 85:270–274
54. Vomachka AJ, Greenwald GS (1979) The development of gonadotropin and steroid hormone patterns in male and female from birth to puberty. Endocrinology 5:960–966
55. Telfer EE, Zelinski MB (2013) Ovarianfollicleculture: advances and challenges for human and nonhuman primates. Fertil Steril 99:1523–1533
56. McLaughlin M, Telfer EE (2010) Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system. Reproduction 139:971–978
57. Garor R, Abir R, Erman A, Felz C, Nitke S, Fisch B (2009) Effects of basic fibroblast growth factor on *in vitro* development of human ovarian primordial follicles. Fertil Steril 91:1967–1975
58. Lei L, Spradling AC (2013) Female mice lack adult germ-line stem cells but sustain oogenesis using stable primordial follicles. Proc Natl Acad Sci U S A 110:8585–8590
59. Bhartiya D, Sriraman K, Parte S, Patel H (2013) Ovarian stem cells: absence of evidence is not evidence of absence. J Ovarian Res 6(1):65
60. Parte S, Bhartiya D, Patel H, Daithankar V, Chauhan A, Zaveri K, Hinduja I (2013) Dynamics associated with spontaneous differentiation of ovarian stem cells *in vitro*. J Ovarian Res 7(1):25
61. Sairam MR, Babu PS (2007) The tale of follitropin receptor diversity: a recipe for fine tuning gonadal responses? Mol Cell Endocrinol 260–262:163–171
62. Desai SS, Roy BS, Mahale SD (2013) Mutations and polymorphisms in FSH receptor: functional implications in human reproduction. Reproduction 146(6):R235–R248
63. Woad KJ, Prendergast D, Winship IM, Shelling AN (2013) FSH receptor gene variants are rarely associated with premature ovarian failure. Reprod Biomed Online 26:396–399
64. Babu PS, Jiang L, Sairam AM, Touyz RM, Sairam MR (1999) Structural features and expression of an alternatively spliced growth factor type I receptor for follitropin signaling in the developing ovary. Mol Cell Biol Res Commun 2:21–27
65. Babu PS, Danilovich N, Sairam MR (2001) Hormone-induced receptor gene splicing: enhanced expression of the growth factor type I follicle-stimulating hormone receptor motif in the developing mouse ovary as a new paradigm in growth regulation. Endocrinology 142:381–389
66. Sairam MR, Jiang LG, Yarney TA, Khan H (1997) Alternative splicing converts the G-protein coupled follitropin receptor gene into a growth factor type I receptor: implications for pleiotropic actions of the hormone. Mol Reprod Dev 48:471–479
67. Li Y, Ganta S, Cheng C, Craig R, Ganta RR, Freeman LC (2007) FSH stimulates ovarian cancer cell growth by action on growth factor variant receptor. Mol Cell Endocrinol 267:26–37
68. Rao AJ, Ramachandra SG, Ramesh V, Couture L, Abdennebi L, Salesse R et al (2004) Induction of infertility in adult male bonnet monkeys by immunization with phage-expressed peptides of the extracellular domain of FSH receptor. Reprod Biomed Online 8:385–391

69. Roy SK, Albee L (2000) Requirement for follicle-stimulating hormone action in the formation of primordial follicles during perinatal ovarian development in the hamster. Endocrinology 141:4449–4456
70. Lange S, Tait D, Matthews M (2013) Oncofertility: an emerging discipline in obstetrics and gynecology. Obstet Gynecol Surv 68:582–593
71. Ratajczak J, Wysoczynski M, Zuba-Surma E, Wan W, Kucia M, Yoder MC et al (2011) Adult murine bone marrow-derived very small embryonic-like stem cells differentiate into the hematopoietic lineage after co-culture over OP9 stromal cells. Exp Hematol 39:225–237

Chapter 12
Molecular Signature of Very Small Embryonic-like Stem Cells

Hyunsook Kang, Jisun Lim, Jinbeom Heo, Jaeho Jeong, YongHwan Kim and Dong-Myung Shin

12.1 Introduction

A population of stem cells (SCs) regulate embryonic development and latter on rejuvenation of adult tissues. These cells self-renew/maintain their own pool and at the same time give rise to differentiated progenitors that replace cells used up during the life [1]. Thus SCs are guardians of tissue/organ integrity and regulate life span of an adult organism.

The most primitive SC in development is fertilized oocyte (zygote) and first blastomeres in morula [2]. These cells called as totipotent cells possess ability to give rise to both embryo and placenta. During further development, inner cell mass (ICM) of blastocyst is specified as first line of pluripotent SCs (PSCs), which could give rise to all three germ layers of developing embryo; however, they lose the ability to differentiate into placenta [3]. PSCs from ICM of blastocyst could be expanded ex vivo as immortalized embryonic SCs (ESCs) lines [4]. From regenerative potential of view, the PSCs are most important SC population. According to definition, PSCs have to fulfill some in vitro as well as in vivo criteria, such as (i)

D.-M. Shin (✉) · H. Kang · J. Lim · J. Heo · J. Jeong · Y. Kim
Department of Biomedical Sciences, Department of Physiology,
University of Ulsan College of Medicine, Pungnap-2 dong, Songpa-gu, Seoul 138-736, Korea
e-mail: d0shin03@amc.seoul.kr

H. Kang
e-mail: ssook4311@korea.ac.kr

J. Lim
e-mail: wow312000@snu.ac.kr

J. Heo
e-mail: hjb0328@naver.com

J. Jeong
e-mail: jjh2853@naver.com

Y. Kim
e-mail: myhome1232@naver.com

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,*
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_12,

give rise to cells from all three germ layers, (ii) complete blastocyst development, and (iii) form teratomas after inoculation into experimental animals [3].

After implantation of the blastocyst, first-line PSCs from ICM give rise to pluripotent epiblast SCs (EpiSCs) that will form in the future entire embryo proper [5, 6]. These cells exhibit features of pluripotency both in vitro and in teratoma assays; however, they show the reduced developmental potency in blastocyst complementation. From gastrulation in which cell lineage determination programs initiate, EpiSCs differentiate into several types of monopotent tissue committed SCs (TCSCs), which restrict their differentiation potential to cells for one tissue only (e.g., epidermis, intestinal epithelium, liver, skeletal muscles, hematopoiesis) [1]. The pluripotent-specific gene expression and epigenetic programs are terminated in differentiated TCSCs. Instead, they turn on the resulting lineage-specific molecular programs. However, primordial germ cells (PGCs) emerged from proximal epiblast around embryonic days 7.25 (E7.25) still maintain the pluripotency by reactivating the pluripotency-specific molecular programs [7]. Therefore, PGCs are the only SC population that expresses the pluripotency-specific genes after gastrulation. Indeed, PGCs could be captured as embryonic germ cells (EGCs) which similarly as ESC can be expanded indefinitely when they are cultured over murine embryonic fibroblasts and exposed ex vivo to three growth factors, kit ligand, leukemia inhibitory factor, and basic fibroblast growth factor [8]. During further embryogenesis, PGCs also lose the pluripotency and initiate the commitment to gametes (oocyte and sperm) after migration into genital ridge around E12.5 [7]. Therefore, PSCs could be specific population detected only during the narrow period of early embryogenesis.

12.2 Molecular Feature of PSCs Detected in Embryogenesis

Unlike differentiated somatic cells, PSCs are commonly characterized as expressing the pluripotency core transcription factors (TFs) such as Oct4, Nanog, and Sox2 [3, 9]. These TFs form the pluripotent core circuitry by reinforcing the expression of genes, which are involved to keep PSCs undifferentiated status but repressing the differentiation inducing transcription. The significance of these core factors is experimentally proven by the success of inducible PSCs (iPSCs) protocol, in which fully differentiated somatic cells can be reprogrammed into ESC-like SCs by transduction of so-called Yamanaka factors (Oct4, Sox2, Klf4, and cMyc) [10, 11]. To support the expression of pluripotency-specific genes, PSCs exhibit the open chromatin structures for their promoters at the level of deoxyribonucleic acid (DNA) methylation and histone modifications [9]. Therefore, their promoters are DNA demethylated and also highly enriched with histone codes such as acetylated histones and trimethylated on lysine4 of histone3 (H3K4me3), which are associated with active transcription. In addition, it is well known that female PSCs (e.g., murine and human ESCs isolated from ICM of blastocyst as well as PGCs) reactivate X

chromosomes that becomes inactivated after fertilization (XIC), and, for example, female PSCs display equivalently activated two X chromosomes (Xa) [9, 12]. Thus, like open chromatin in Oct4 promoter, reactivation of XIC is one of the "golden standards" to evaluate female SC pluripotency [13].

Mounting evidence accumulates that the PSCs exhibit the distinct epigenetic signature for their remarkable properties of self-renewal and pluripotency [3, 9]. PSCs exhibit significantly higher nuclear/cytoplasm (N/C) ratio and possess unorganized chromatin (euchromatin) in large nuclei. During differentiation, PSCs display increased heterochromatin in the nucleus [14]. As correlated with the morphologic feature, PSCs from ICM show global DNA demethylation, which leads to the reactivation of (i) inactive X chromosome (Xi), (ii) the germ-line lineage genes (e.g., Stella, Mvh, Dazl, Sycp3), and (iii) repetitive sequences families (e.g., LINE1, SINE, IAP) [15].

As respect to the modification of histone proteins, undifferentiated PSCs exhibit the specific epigenetic marks, so-called bivalent domain (BD) [16–19]. This phenomenon is based by a presence of transcriptionally active histone codes such as H3K4me3 physically coexisting with repressive histone codes like trimethylated lysine27 of histone3 (H3K27me3) in promoters of some homeodomain containing development master TFs, for example, Dlx, Irx, Lhx, Pou, Pax, and Sox family proteins. Due to overwhelming effect of repressive H3K27me3 activity, the transcription in BDs in genes associated with H3K27me3 is transiently blocked [16–19]. However, in the response to development stimuli, BD of these promoters is switched into monovalent to determine their genes expression (activation versus repression). Therefore, the BD is essential not only to keep PSCs undifferentiated but also to enable them to respond dynamically to developmental stimuli.

Most important concept for SC biology is that they show hierarchy as respect to their developmental potency [1]. PSCs emerged during embryogenesis also show this hierarchy where populations of most primitive SCs exists and give rise to more differentiated SC populations with ability to self-renew but with more and more limited ability for multi-lineage differentiation. Of importance, the SC hierarchy is orchestrated by distinct epigenetic programs which affect the profile of gene expression [9, 20]. First, EpiSCs differentiated from ICM after implantation perform the DNA methylation of X chromosome, the promoters for ICM enriched (Rex1) and germ-line (Stella) genes, and the repetitive sequences, which are hallmarks of the epigenetic state of this population. As result of epigenetic reprogramming during implantation of blastocyst, EpiSCs exhibit different transcription profiles, distinct with ESCs. For example, the expression of Nanog, Sox2, and Stella is reduced in EpiSCs through DNA methylation of their promoters [21]. Accordingly, EpiSC show a restricted capacity about blastocyst complementation [5, 6].

After gastrulation, most EpiSCs lose the expression of pluripotency-specific TFs except for some precursor cells for PGCs located in proximal epiblast. The founder of PGCs revert to a state that resembles the ICM population by undergoing DNA demethylation of the promoter for pluripotency and germ-line master regulators (e.g., Fragilis, Blimp1, and Stella) in response to signals from extra-embryonic tissues [7]. Thus, around 40 alkaline phosphatase (AP)-positive PGCs emerge in

extra-embryonic mesoderm at the base of allantois, an appendage arising from the posterior primitive streak around E7.25 [22]. At E8.5, PGCs enter back into embryo proper through the primitive streak and start migration through hindgut endoderm, mesentry in aorta-gonads-mesonephros (AGM) region, and around E11.5, they reach the genital ridge in which PGCs differentiate monopotent gametes (sperm and egg). It should be noted that PGCs performed genome-wide DNA demethylation and remodeling of repetitive sequences, resulting in the reactivation of Xi and erasure of genomic imprinting during migration into genital ridge [23].These epigenetic reprogramming could play an important role to regulate the accurate timing for the expression of genes, which are involved in germ cells development such as Blimp1, Stella, Mvh, Dazl, and Sycp3 [23, 24] and also discriminate PGCs from the neighboring somatic cells [7].

Taken together, PGCs emerged on early embryogenesis show the distinguished characteristics about the epigenetic status and gene expression profiles and the molecular signature of these cells could be fingerprint for identifying their developmental ontology. Thus, the precise molecular signature of the putative PSC candidates should be investigated not only to understand their nature but also to increase the regenerative power of these cells

12.3 Isolation of VSELs SCs from Adult Tissues

As mentioned before, it has been considered that most PSCs emerge during early embryogenesis and disappear in adulthood as they differentiate monopotent somatic or germ-line cells. However, several attempts have been made in the past few years to purify a population of PSCs from adult tissues. Potential PSCs in adult tissues were described as (i) mesenchymal SCs (MSCs), (ii) multipotent adult progenitor cells (MAPCs), (iii) marrow-isolated adult multi-lineage inducible (MIAMI) cells, (iv) multipotent adult (MASCs), or (v) OmniCytes [25–28]. The presence of primitive PSCs in adult tissues may much better explain the controversy for the reports that monopotent TCSCs are plastic and can change differentiation commitment (trans-dedifferentiate) into SCs for other tissues [1].

We also identified very small embryonic-like SCs (VSELs) by multi-parameter fluorescence-activated cell sorter (FACS) as a population of Sca-1^{+}Lin^{-}CD45^{-} in several other adult murine organs (e.g., brain, liver, skeletal muscles, heart, and kidney [29, 30]. Murine bone marrow (BM)-derived VSELs are morphologically featured as they (i) are very rare (~0.01 % of nucleated BM cells); (ii) small in size (~3–5 μm); (iii) express several PSCs markers such as Oct4, Nanog, Rex1, and SSEA-1; (iv) exhibit significantly higher nuclear/cytoplasm (N/C) ratio and a lower cytoplasmic area as compared to hematopoietic SCs (HSCs); and (iv) possess unorganized chromatin (euchromatin) in large nuclei [29]. Human tissues including human umbilical cord blood (UCB), mobilized peripheral blood (mPB), and adult BM also contain small (~4–7 μm) sized population of CD133^{+}Lin^{-}CD45^{-} cells [31]. Human VSELs similarly as murine counterparts display embryonic-like

morphology (e.g., large nuclei containing primitive unorganized euchromatin and relatively small rim of cytoplasm with numerous round mitochondria). They also express Oct4 and Nanog in nuclei and on the surface display SSEA-4 antigen [31].

When freshly isolated VSELs are cultured under C2C12 myoblast feeder layer, they form the VSEL-derived spheres (VSEL-DSs) that correspond to embryoid bodies (EBs) from ESCs [29, 32]. After replating of cells from VSEL-DSs into specific differentiation inducing media, they expand into cells from all three germ-cell layers (e.g., neural cells, cardiomyocytes, and insulin-producing cells), demonstrating that VSELs show the pluripotency at the criteria of in vitro differentiation [29, 32]. We also prove that VSELs can be differentiated into MSCs [33], cardiomyocyte [34], and long-term engrafting HSCs [35, 36] in vivo tissue regeneration animal models, supporting the pluripotency of VSELs in vivo condition. Furthermore, VSELs are mobilized into peripheral blood during several models of organ injury and circulate there in an attempt to enrich and regenerate damaged tissues (e.g., heart infarct, stroke) [37–39].

These in vitro and in vivo data suggest that the pluripotent VSELs probably change differentiation commitment (trans-dedifferentiate) into SCs for other tissues to plays a more significant role in the regeneration of some small tissue and organ injuries. However, several enigmas remain to be solved for successful application of the adult PSCs into clinic. First, it should be answered whether VSELs found in adult tissues cells are functional in steady state conditions or are merely a remnants from developmental embryogenesis that reside in a dormant state in the tissues. Second, dormant status of these cells could be regulated by cell-intrinsic epigenetic reprogramming similarly to other PSCs during embryogenesis or their microenvironment, unfavorable to proliferation, for example, (i) located in non-physiological niches, (ii) exposed to inhibitors, (iii) deprived of some appropriate stimulatory signals. Precise molecular signature of VSELs in adult tissues could give us some clues to these important enigmas.

12.4 Molecular Signature of VSELs Residing in BM

To address this issue, we have tried to investigate the relationship between VSELs and embryonic type of PSCs (e.g., ESCs, EpiSCs, PGCs, and EGCs) by employing several molecular strategies to evaluate molecular signature of VSELs (Fig. 12.1). Highly purified Sca-1^{+}Lin^{-}CD45^{-} VSELs from murine BM were evaluated for expression of (i) pluripotent genes, (ii) epiblast, and (iii) germ-line markers including developmentally crucial imprinted genes.

12.4.1 The Expression of PSCs Genes in VSELs

All the PSCs should express the Oct4, essential pluripotency TF and VSELs also express the Oct4 at messenger ribonucleic acid (mRNA) and protein level [40].

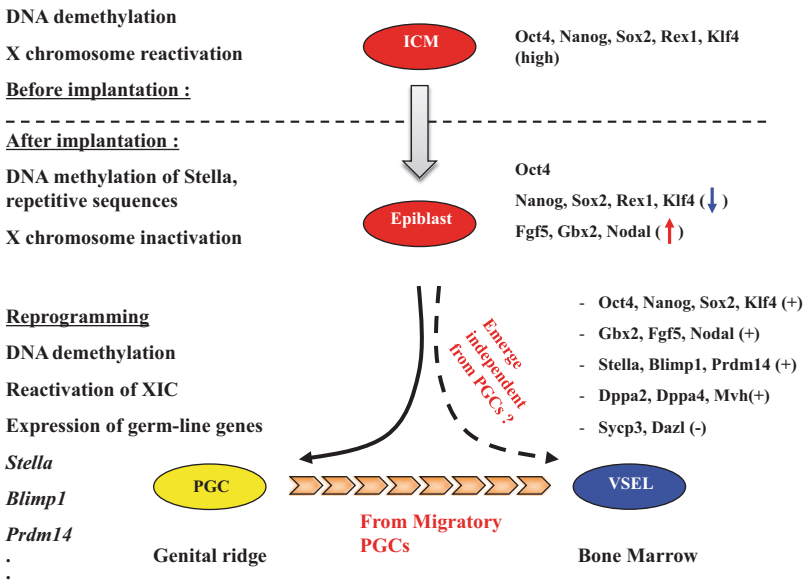

Fig. 12.1 Proposed developmental ontology of VSEL population. PSCs from ICM of blastocyst differentiate into epiblast after implantation. PSCs from epiblast give rise to not only cells of entire somatic tissue but also PGCs, germ-line lineage stem cells. Migratory PGCs-like PSCs become specified in proximal epiblast and migrate and colonize into genital ridge around embryonic day 12.5. During the embryogenesis, PSCs from ICM, epiblast, and PGCs show the distinct gene expression and epigenetic programs. Some of migratory PGCs-like PSCs might go astray from the main migratory route and finally seed to adult bone marrow by following the developmental route of hematopoietic stem cells. We cannot exclude that some VSELs emerge in other parts of the epiblast as precursors for TCSCs. Due to this proposed developmental ontology, VSELs could exhibit the unique gene-expression pattern like (i) expression of common PSC-specific TFs (Oct4, Nanog, Sox2, Klf4), epiblast (Gbx2, Fgf5, Nodal), germ-line specification (Stella, Blimp1, Prdm14), migratory PGCs genes (Dppa2, Dppa4, Mvh), but little expression of post-migratory PGCs ones (Sycp3, Dazl). *VSEL* very small embryonic/epiblast-like stem cell, *PSCs* pluripotent stem cells, *ICM* inner cell mass, *PGCs* primordial germ cells, *TCSCs* tissue committed stem cells, *TFs* transcription factors

However, debate whether cells isolated from adult tissues may express Oct4 has been recently raised [41, 42]. It has been postulated that positive polymerase chain reaction (PCR) data showing Oct4 expression in putative candidates of PSCs may be due to amplification of Oct4 pseudogenes or various alternative spliced Oct4 transcripts, which are highly expressed in non-pluripotent differentiated cells [43, 44]. The epigenetic status of Oct4 promoter (e.g., DNA methylation and histone codes) is considered the most definite in vitro prove for the true Oct4 expression in putative candidates of PSCs. Thus, to prove true expression of the Oct4 gene in VSELs, we investigated their epigenetic status for Oct4 promoter. When DNA methylation status of the Oct4 promoter were examined by bisulfate sequencing using the highly purified Sca-1^{+}Lin^{-}CD45^{-} murine VSELs, we noticed that the Oct4 promoter in VSELs, similar to cells isolated from ESCs-derived EBs, is hypomethylated (28 % and 13.2 %, respectively) [40].

Next, to evaluate the status of histone codes for Oct4 promoter, we performed the chromatin-immunoprecipitation (ChIP) assay for checking its association with acetylated-histone3 (H3Ac) and dimethylated-lysine-9 of histone-3 (H3K9me2), the molecular features for open- and closed-type chromatin, respectively [45]. However, the experimental strategies for ChIP assay require a significant number of cells (at least 5×10^6 per assay) in order to ensure reproducible and quantitative results. It is why this assay has not been so far applied in adult SCs field. To omit this obstacle, we have employed a sensitive ChIP assay for a small number of cells (carrier-ChIP assay) [46]. By employing carrier-ChIP assay using human hematopoietic cell-line THP-1 as carrier, we found that Oct4 promoter chromatin is associated with H3Ac and its association with H3K9me2 is relatively very low [40]. In case of epigenetic status of the Nanog promoter, it was methylated (~50%), however, quantitative ChIP data confirmed that the H3Ac/H3K9me2 ratio supports the active status of the Nanog promoter in these cells [40]. Based on these results, VSELs truly express Oct4 and Nanog. Of note, we also reported that VSELs express also several other markers of PSCs such as SSEA-1 antigen as well as Sox2 and Klf4 TFs.

12.4.2 Expression of Epiblast Markers

Unlike ESCs, but similarly to EpiSCs, highly purified BM-derived Oct4$^+$VSELs show little activity on blastocyst complementation although they express Oct4 [29, 32]. In addition, pluripotent EpiSCs differentiate into TCSCs, which have an important role in organogenesis during embryogenesis and later on in adult life in tissue rejuvenation and regeneration. Therefore, we have hypothesized that VSELs could be epiblast-derived precursors of TCSCs [47]. Since EpiSCs perform epigenetic reprogramming during implantation, they exhibit different transcription profiles. It is known that Gbx2, Fgf5, and Nodal are upregulated in EpiSCs, but expressed at lower levels in ESCs isolated from the ICM of blastocysts [21]. In contrast, the level of *Rex1/Zfp42* transcripts is highly expressed in ICM cells. Thus, to investigate the similarity between VSELs and EpiSCs, we examined in adult BM-derived VSELs the expression of genes which are characteristic for EpiSCs or ICM cells. VSELs highly express Gbx2, Fgf5, and Nodal, but express less Rex1/Zfp42 transcript as compared to established ESCs cell line, ESC-D3, suggesting that VSELs are more differentiated than ICM-derived ESCs and share several markers with more differentiated EpiSCs [48].

12.4.3 Expression of Germ-Line Markers

After gastrulation, only PGCs derived from posterior proximal epiblast maintain the molecular signature that resembles the ICM population by undergoing epigenetic reprogramming [7]. Thus, we also investigate whether VSELs could exhibit the similar feature to PGCs. VSELs highly expressed the transcripts of genes in-

volved in the germ-line specification of the epiblast (e.g., Stella, Prdm14, Fragilis, Blimp1, Nanos3, and Dnd1) and we also confirmed the expression of the Stella, Blimp1, and Mvh at the protein level by immunostaining [48]. Furthermore, the Stella promoter in VSELs is partially demethylated at the DNA methylation level and also displays transcriptionally active histone modifications (H3Ac and H3K4me3) and was less enriched for transcriptionally repressive histone markers (H3K9me2 and H3K27me3) [48]. Taken together, VSELs express specific genes and display a chromatin structure that is characteristics for germ-line specification. Furthermore, VSELs also highly express Dppa2, Dppa4, and Mvh, which characterize late migratory PGCs; however, they do not express Sycp3, Dazl, and LINE1 genes that are highly expressed in post-migratory PGCs [48]. Thus, our results in toto support a concept that VSELs deposited into murine BM show some similarities in gene expression and epigenetic signatures to epiblast-derived migratory PGCs (~E10.5–E11.5).

12.4.4 Bivalent Domain

PSCs should self-renew themselves and at the same time differentiate into all the types of cells in the response to the external cues. This means that the open chromatin structure at the promoter of PSC-specific TFs should be maintained and the developmental master ones should be poised to be activated. Indeed, the promoter of most homeodomain-containing developmental TFs in PSCs are marked by specific epigenetic status, called BDs, representing status of chromatin structure where transcriptional opposite histone codes physically co-exist in the same promoter [16–19]. In undifferentiated ESCs, BD epigenetic codes temporally repress targeted developmental TFs, preventing the premature differentiation of primitive SCs. During differentiation, the transient repressive epigenetic marks in TFs would become monovalent to activate or repress expression of certain TFs.

In order to maintain BDs, the undifferentiated ESCs highly express polycomb group (PcG) and Trithorax group (TrxG) proteins, which mediating transcription-repressive trimethylation of lysine 27 of histone H3 (H3K27me3) and transcription-promoting trimethylation of lysine 4 of histone H3 (H3K4me3) modifications, respectively [49–51]. The essential role of these proteins in the stability of BDs was confirmed by gene-targeting and RNA interference (RNAi) studies [16–19].

PcG proteins mediate the transcription repression by involving two distinct repressive complexes: polycomb repressive complex 1 (PRC1) and 2 (PRC2) [49, 50]. Instead, PRC1 induces the chromatin condensation by mono-ubiquitination of lysine119 of H2A (H2AK119-Ub), PRC2 acts by inducing the H3K27me3 at specific sites in chromatin recognized by the complex. Enhancer of zeste drosophila homolog 2 (Ezh2) is a core member of PRC2 together embryonic ectoderm development (Eed) and suppressor of Zeste 12 (Suz12) and it actually show the histone methyltransferases (HMTase) enzyme activity. The PRC-mediated repression can be counter-balanced by trithorax group (TrxG) protein, which activates the tran-

scription by mediating trimethylation of lysine 4 of histone H3 (H3K4me3) at TrxG targeted genes [51]. The epigenetic histone code and marks generated by PcG and TrxG proteins are stably inherited during cell proliferation and are considered to be a major mechanism of "cellular memory" [51]. Thus, the balance between PcG and TrxG protein activity establishes transcriptional memory that decides cell fate.

When we investigated the VSEL-specific transcriptome analysis (will be discussed below), we found that murine BM-derived VSELs exhibit characteristically high expression of Ezh2 at the mRNA and protein level [52]. VSELs as similar to other PSCs possess BDs in promoters of homeodomain-containing developmental TFs (Sox21, Nkx2.2, Dlx1, Lbx14, Hlx9, and Pax5) [52]. By differentiation and RNA interference experiments, we prove that Ezh2 in VSELs is indispensable to maintain the BD in the promoter of lineage committing TFs, thus preventing premature activation of them.

VSELs, when plated over C2C12 cells, proliferate and form characteristic spheres, VSEL-DSs, which progressively enriched for more differentiated SCs. Thus, Oct4 promoters in VSEL-DSs show the progressive DNA methylation, enrichment for H3K27me3 paralleled by a decrease in promoter-associated H3K4me3-modified histones, and they also progressively decreased the expression of most stemness genes [52]. Interestingly, we also observed that the Ezh2 gene was downregulated during VSEL-DS formation, concomitantly with the disappearance of BDs at promoters of developmentally important TFs [52]. As result, cells isolated from VSEL-DSs de-repressed the expression of some BD-regulated genes (e.g., Hlxb9, HoxA3, and Evx1) in VSELs. Therefore, downregulation of Ezh2 during VSEL-DS formation could destabilize BD structures, leading to de-repression of some BD target genes and promoting cell differentiation. Therefore, both positive (expression of Oct4-Nanog-Sox2 pluripotency circuitry) and negative (repression of differentiation inducing TFs by BD) regulatory mechanisms are indispensable to maintain pluripotency of SCs.

12.4.5 Partial Activation of Inactive X Chromosome in VSELs

As mentioned before, female ESCs and PGCs reactivate X chromosomes that becomes inactivated after fertilization (XIC). Our initial studies in murine female VSELs show that these cells partially reactivate inactivated X chromosome. XIC is known as the epigenetic process for transcriptional silencing of one of two X chromosomes in female cells in order to compensate for gene dosage [12]. Thus, female cells similarly to genomic imprinting show the differential epigenetic marks on each X chromosome [9]. The XIC process is mediated by the expression of large noncoding RNA—Xist that is transcribed on the Xi. The coating of DNA and spreading of Xist on Xi induces the silenced chromatin structure (e.g., H3K4 hypomethylation, hypoacetylation of H3K9 and H4, enrichment for H4K20me, H2AK119-Ub, H3K27me3, presence of variant histone macroH2A1.2), leading to the transcriptionally inactive state of Xi. Therefore, DNA of Xist promoter is unmethylated at Xi but

fully methylated at Xa. As mentioned above, we noticed that female mice VSELs partially hypermethylate the Xist promoter (~80%), unlike to HSCs and bone marrow mononuclear cells (BMMNCs; which as expected show 50% of DNA methylation). This result strongly suggests that murine VSELs similarly to ESCs and PGCs could undergo the process of XIC reactivation.

In summary, murine BM-derived VSELs at the molecular level (i) express several markers of pluripotency (e.g., Oct4 and Nanog), (ii) possess BDs in promoters of developmentally crucial TFs, and (iii) reactivate the X chromosome (in female PSCs), which becomes silenced at early stages of embryogenesis or reactivated during germ-line development.

12.5 Epigenetic Changes of Imprinted Genes that Regulate VSELs Pluripotency

Unlike PSCs from embryonic tissues, murine Oct-4$^+$VSELs do not proliferate in vitro if cultured alone and do not grow teratomas in vivo. On the other, proliferation capacity can be restored during VSEL-DS formation, suggesting that the proliferation of VSELs should be tightly regulated and could be modulated by some mechanisms [29, 32]. It is interesting that similarly to VSELs, PGCs in cultures freshly isolated from embryos proliferate for a few days only, and then disappear either because they differentiate or die if not when PGC are cultured over feeder layer in the presence of proper growth factors [53]. Furthermore, VSELs exhibits several molecular features similar to PGCs at the level of gene expression and epigenetic status [40, 48]. Therefore, it is possible that two SC populations could employ similar molecular mechanism to regulate their pluripotency and to prevent the unleashed cell proliferation.

The hallmark for epigenetic reprogramming during PGCs development is erasure of genomic imprinting [7, 9, 20, 23] (Fig. 12.2). Genomic imprinting is epigenetic program that ensures the parent-of-origin-specific monoallelic transcription of so-called imprinted genes [54]. Around 80 imprinted genes (expressed from maternal or paternal chromosomes only) have been reported in mouse genome and they play a crucial role in embryogenesis, fetal growth, totipotential status of the zygote, and pluripotency of developmentally early SCs. The majority of imprinted genes exist as gene clusters enriched for CpG islands and their monoallelic expression is regulated by DNA methylation on differential methylated regions (DMRs), which are CpG-rich cis-elements in their loci [55]. DNA methyltransferase (Dnmts) is responsible for the parental origin-specific differential methylation status of DMRs [55]. The genomic imprints should be maintained in all the somatic lineage cells throughout whole developmental period. In contrast, PGCs erase the genomic imprints during migration into genital ridge as result of the epigenetic reprogramming programs, resulting in global DNA demethylation and changes in histone modifications [23]. During differentiation into gametes after migration, they mark new genomic imprints in the sex-dependent manner. The erasure of genomic imprints

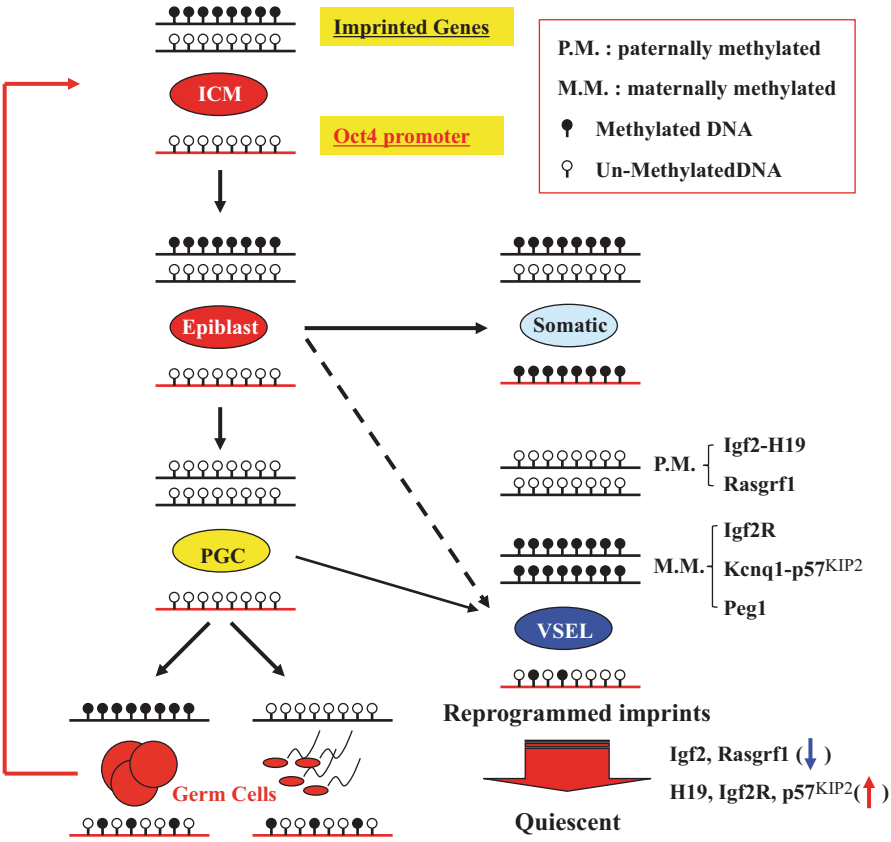

Fig. 12.2 Overview of genomic imprinting reprogramming. The genomic imprints, parental origin-specific differential DNA methylation are well maintained in somatic lineage cells throughout whole developmental stages from ICM to all somatic cells in adult tissues. In contrast, the epigenetic marks are erased in PGCs during migration into genital ridge where they reset the sex-dependent genomic imprints during gametogenesis. VSELs similarly as PGCs reprogram methylation of imprinted genes by erasing the paternally methylated (P.M.) imprints (e.g., Igf2-H19, Rasgrf1 loci), but by hypermethylating the maternally methylated (M.M.) ones (e.g., Igf2 receptor (Igf2R), Kcnq1-p57KIP2, Peg1 loci). As a result, VSELs highly express growth-repressive genes (H19, $p57^{KIP2}$, Igf2R) and downregulate growth-promoting ones (Igf2, Rasgrf1), which explains the quiescent status of VSELs. *Red line* and *black line* indicate the Oct4 promoter and imprinted loci, respectively. *VSEL* very small embryonic/epiblast-like stem cell, *PGCs* primordial germ cells, *DNA* deoxyribonucleic acid

could be potential mechanism to protect the Oct4 expressing germ-line SCs from uncontrolled expansion and teratoma formation. For example, while the nuclei of early migrating PGC at E8.5–9.5 can be successfully used as donors for nuclear transfer, nuclei from post-migratory PGC after E11.5 are incompetent to support full-term development [56].

Since VSELs share similar molecular signature to PGCs, we have postulated that VSELs similarly as PGCs may modify methylation of imprinted genes that prevents

them from unleashed proliferation and may explain their quiescent status in adult tissues. We noticed that VSELs freshly isolated from murine BM erase the paternally methylated imprints (e.g., Igf2-H19, Rasgrf1 loci); however, they hypermethylate the maternally methylated ones (e.g., Igf2 receptor (Igf2R), Kcnq1-p57KIP2, Peg1 loci) [40]. According to the parental conflict theory, paternally expressed imprinted genes (Igf2, Rasgrf1) enhance the embryo growth and maternally expressed genes (H19, $p57^{KIP2}$, Igf2R) inhibit cell proliferation [40]. Thus, the unique genomic imprinting pattern observed on VSELs demonstrates growth-repressive imprints in these cells. As supported, VSELs highly express growth-repressive genes (H19, $p57^{KIP2}$, Igf2R) and downregulate growth-promoting ones (Igf2, Rasgrf1), which explains the quiescent status of VSELs [40]. Importantly, all the growth repressive patterns of genomic imprinting could be progressively recovered during VSEL-DS formation, which lead to the proliferation and differentiation of VSELs [40].These results suggest that epigenetic reprogramming of genomic imprinting should be a mechanism that keeps VSELs quiescent and prevents them from teratoma formation. Therefore, the investigation of potential modulation of mechanisms controlling genomic imprinting in VSELs would give us some clues about the biological significance of VSELs such as ageing process and also developing more powerful strategies to unleash the regenerative potential of these cells for efficient employment in the clinical setting.

Interestingly, most imprinted genes reprogrammed in VSELs are involved in insulin/insulin-like growth factor (Ins/Igf) signaling [57]. As a result of unique genomic imprinting reprogramming, VSELs tends to upregulate the Ins/Igf signaling favorable genes like (e.g., Igf2 and Rasgrf1, a small GTP exchange factor for Ras protein for Ins/Igf signaling); however, they downregulate the non-signaling receptor Igf2R which functions as decoy receptor for Igf2, thus prevents its availability for Igf1R. Overall, our data indicate that genomic imprints could regulate a quiescent status of VSELs by decrease in Ins/Igf signaling. It is well known that changes in Ins/Igf signaling molecules play a crucial role on aging. Accordingly, (i) insulin-like growth factor 1 (Igf-1) signaling negatively regulates the lifespan from worms, flies, to mammals [58] and that (ii) Igf-1 and insulin level in blood is regulated positively by calorie uptake [59]. Thus, we can assume that prolonged Ins/Igf signaling may lead to premature depletion of VSELs in adult tissues and we hypothesize that VSELs "are burned during our life in a fire of Ins/Igf factors." The detail for a role of genomic imprints reprogramming on VSEL ageing would be discussed in other chapters (*14. Novel view on stem cells and aging—Prof. Magdalena Kucia*).

12.6 Transcriptome Analysis from Highly Purified VSELs

VSELs are very rare primitive SCs, existing around 0.02% of mononuclear cells in murine adult bone marrow [29]. Although VSELs can be isolated as a relative homogenous population by FACS sorting with Sca-1^{+}Lin^{-}CD45^{-} phenotype, they

still could exhibit some heterogeneity. The heterogeneity and limited cell number of primitive VSELs has been hindering the genome-wide the gene expression profiling of VSELs, thus leading to lack of systemic understanding of their molecular nature. Recently, we overcome the issues by employing single-cell-based gene expression and microarray protocol, which we have already successfully established for the study of rare PGCs isolated from embryonic tissue [60, 61]. We established several complementary DNA (cDNA) libraries, which were created from RNA isolated from 20 FACS-sorted VSELs and analyzed the genome-wide transcriptome of them by Affymetirx microarray. This study on murine VSELs expanded for gene-ontology and canonical pathway analysis of microarray results allowed us to gets more information on developmental origin of VSELs (epiblast/germ line) and identified several pathways that regulate their quiescence and differentiation that we are currently studying [52]. For example, VSELs upregulate cell-cycle checkpoint genes and several tissue-specific gene sets (neural cells, adipocyte, and hepatocytes) [52]. In contrast, they downregulate genes involved in protein turnover, ultraviolet (UV) response, mRNA processing, and mitogenic/growth factors pathways including MAPK, PI3K, Igf1, Wnt, and TRKA pathway. Particularly, VSEL generally showed at a low level the gene expression of several components for Igf1 signaling cascade (e.g., Fos, Jun, Jak1, Kras, Sos2, Srf, Socs3, and Shc1). This study indicates that VSELs show the similar, yet non-identical characteristic transcriptome compared with ESCs and HSCs.

One gene of particular interest as characteristic high expression in VSELs is *Ezh2*, a PcG protein as mentioned before. As like Ezh2 plays an essential role on maintaining BD epigenetic marker in undifferentiated ESCs, Ezh2 in VSELs is indispensable for maintaining BD marks in several developmental regulator gene promoters, which keeps them poised for activation [52]. Furthermore, VSELs highly expressed some other cellular memory machinery transcripts from PcG family (Suz12, Cbx3, Phc1, and Phc2) and also TrxG one, such as Ash1L Ash2L and Mllt4 [52]. We are investigating to search for other bio-markers, characteristic for VSELs by analyzing the database from VSELs transcriptome. We expect that this study would not only advance our understanding of biological process for their pluripotency, differentiation, and quiescence but should also help to develop better protocols for ex vivo expansion of VSELs.

12.7 Developmental Origin of VSELs

As mentioned before, molecular signature of the corresponding SCs could be fingerprint for identifying the developmental ontology for PSCs. The epigenetic status of pluripotency TFs and reprogrammed genomic imprinting led us to hypothesize that VSELs deposited in adult BM originate from migratory PGCs, which could go astray from orthodoxy migration route [15]. During embryogenesis, VSELs are found as Sca-1^{+}Lin^{-}CD45^{-} population in fetal-liver from second trimester [62]. As like their BM-derived counterparts, FL-VSELs express a similar gene expression

profile and epigenetic status of pluripotency TFs, epiblast, and germ-line genes [48]. Accordingly, the promoters for Oct4, Nanog, and Sox2 show the significant DNA demethylation and enriched histone modifications for open, transcriptionally active structure of Oct4 promoter. FL-VSELs show the erasure of DMR for both paternally methylated (*Igf2-H19, Rasgrf1*) and maternally methylated (*Kcnq1, Igf2R*) loci [48]. The status of genomic imprints in FL-VSELs was similar to PGCs, but does not correspond exactly to that observed in BM-VSELs, which show the parent-of-origin-specific methylation pattern (the demethylated paternally methylated imprints, but hypermethylation of maternally methylated ones) [48]. This would indicate that VSELs deposited in the adult BM microenvironment later in development increase DNA methylation of maternal imprints, which increases their quiescence.

Fetal liver is main embryonic hematopoiesis tissue. Interestingly, accumulating evidence also indicates that PGCs could somehow be related to HSCs, another population of highly migratory SCs [63–66]. The following evidence supports this hypothesis: (i) a tight spatio-temporal overlap exists between the migration route of PGCs and the developmental origin of HSCs, first in extra-embryonic tissues in yolk sac blood islands and then in the aorta-gonad-mesonephros (AGM) region; (ii) PGCs isolated from murine embryos have been described as being able to grow HSC colonies; and (iii) robust hematopoietic differentiation has been observed in many classical germ tumors. Furthermore, as we have already reported both adult BM- and E12.5 fetal liver-derived $Oct4^+$VSELs may differentiate over OP9 cells into $CD45^+$HSCs with long-term engrafting capacity [35, 36] and VSELs follow developmental route of HSCs [48, 62]. Thus, PGCs, HSCs, and VSELs form all together a unique highly migratory population of interrelated SCs as respect to their developmental ontology. Due to this unique developmental origin, VSELs show characteristic epigenetic reprogramming and gene expression in stemness, germline, and imprinted genes that maintain their pluripotency, but also prevent their unleashed proliferation and teratoma formation.

Conclusions

Several attempts have been made in the past few years to purify a population of PSCs from adult tissues. The very primitive VSELs in adult tissues could function as precursor for monopotent TCSCs. It is very likely that these cells play a physiological role in rejuvenation of a pool of TCSCs under steady state conditions. Molecular signature of VSELs demonstrates that they VSELs exhibit the common features for pluripotency, (i) open chromatin structure in the promoters of PSC-specific TFs (Oct4 and Nanog), (ii) BD domain in the promoters of developmental master regulator TFs, and (iii) partial reactivation of Xi. As respect to gene expression and epigenetic programs of germ-line markers, VSELs share several molecular signatures with epiblast and migrating PGCs, suggesting that VSELs developmentally originate from epiblast-derived migrating PGCs. Based on the similarity to PGCs, the capacity for proliferation and differentiation of VSELs is orchestrated

by DNA methylation status of some of the developmentally crucial imprinted genes (e.g., H19, Igf2, Igf2R, $p57^{KIP2}$, and Rasgrf1). The progress loss of the proliferation-repressive epigenetic marks in VSELs results in the increased sensitivity to Ins/Igf signaling and concomitantly depletion of primitive VSELs population during ageing process. VSELs could function as a reserve pool of primitive stem by regeneration into several tissue residing TCSCs (e.g., MSCs, HSCs, cardiac SCs) in response to tissue/organ injury. Thus, VSELs isolated from adult tissues are safe and ethically noncontroversial source of SCs as an alternative to embryonic types of PSCs for regenerative medicine. To successfully employ VSELs in the realm of regenerative medicine, it is very important to establish experimental protocols for unleashing the regenerative potential of VSELs like reprogramming of their growth repressive genomic imprints status into regular somatic pattern and the study about precise molecular signature of VSELs could advance the development of ex vivo expansion protocol for these SCs.

Conflicts Of Interest Statement The authors declare that they have no competing financial interests.

Acknowledgments This work was supported by a grant from the Asian Institute for Life Sciences, Seoul, Korea (2014–528) and a grant from the Korean Health Technology R & D Project, Ministry of Health & Welfare, Republic of Korea (A120301) to DMS.

References

1. Ratajczak MZ, Machalinski B, Wojakowski W, Ratajczak J, Kucia M (2007) A hypothesis for an embryonic origin of pluripotent Oct-4+ stem cells in adult bone marrow and other tissues. Leukemia 21(5):860–867
2. Tam PPL, Loebel DAF (2007) Gene function in mouse embryogenesis: get set for gastrulation. Nat Rev Genet 8(5):368–381. doi:http://www.nature.com/nrg/journal/v8/n5/suppinfo/nrg2084_S1.html
3. Niwa H (2007) How is pluripotency determined and maintained? Development 134(4):635–646. doi:10.1242/dev.02787
4. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292(5819):154–156
5. Brons IGM, Smithers LE, Trotter MWB, Rugg-Gunn P, Sun B, Chuva de Sousa Lopes SM, Howlett SK, Clarkson A, Ahrlund-Richter L, Pedersen RA, Vallier L (2007) Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448(7150):191–195. doi:http://www.nature.com/nature/journal/v448/n7150/suppinfo/nature05950_S1.html
6. Tesar PJ, Chenoweth JG, Brook FA, Davies TJ, Evans EP, Mack DL, Gardner RL, McKay RDG (2007) New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448(7150):196–199
7. Hayashi K, de Sousa LSMC, Surani MA (2007) Germ Cell Specification in Mice. Science 316(5823):394–396. doi:10.1126/science.1137545
8. Matsui Y, Zsebo K, Hogan BLM (1992) Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70(5):841–847
9. Surani MA, Hayashi K, Hajkova P (2007) Genetic and epigenetic regulators of pluripotency. Cell 128(4):747–762
10. 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

11. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448(7151):318–324. doi: http://www.nature.com/nature/journal/v448/n7151/suppinfo/nature05944_S1.html
12. Payer B, Lee JT (2008) X chromosome dosage compensation: how mammals keep the balance. Annu Rev Genet 42(1):733–772. doi:10.1146/annurev.genet.42.110807.091711
13. Tchieu J, Kuoy E, Chin MH, Trinh H, Patterson M, Sherman SP, Aimiuwu O, Lindgren A, Hakimian S, Zack JA, Clark AT, Pyle AD, Lowry WE, Plath K (2010) Female human iPSCs retain an inactive X chromosome. Cell Stem Cell 7(3):329–342. doi:http://dx.doi.org/10.1016/j.stem.2010.06.024
14. Meshorer E, Misteli T (2006) Chromatin in pluripotent embryonic stem cells and differentiation. Nat Rev Mol Cell Biol 7(7):540–546
15. Ratajczak MZ, Liu R, Ratajczak J, Kucia M, Shin D-M (2011) The role of pluripotent embryonic-like stem cells residing in adult tissues in regeneration and longevity. Differentiation 81(3):153–161. doi:http://dx.doi.org/10.1016/j.diff.2011.01.006
16. Azuara V, Perry P, Sauer S, Spivakov M, Jorgensen HF, John RM, Gouti M, Casanova M, Warnes G, Merkenschlager M, Fisher AG (2006) Chromatin signatures of pluripotent cell lines. Nat Cell Biol 8(5):532–538. doi:http://www.nature.com/ncb/journal/v8/n5/suppinfo/ncb1403_S1.html
17. Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, Bell GW, Otte AP, Vidal M, Gifford DK, Young RA, Jaenisch R (2006) Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441(7091):349–353. doi: http://www.nature.com/nature/journal/v441/n7091/suppinfo/nature04733_S1.html
18. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125(2):315–326
19. Stock JK, Giadrossi S, Casanova M, Brookes E, Vidal M, Koseki H, Brockdorff N, Fisher AG, Pombo A (2007) Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. Nat Cell Biol 9(12):1428–1435. doi:http://www.nature.com/ncb/journal/v9/n12/suppinfo/ncb1663_S1.html
20. Hayashi K, Surani MA (2009) Resetting the epigenome beyond pluripotency in the germline. Cell Stem Cell 4(6):493–498. doi:http://dx.doi.org/10.1016/j.stem.2009.05.007
21. Hayashi K, Lopes SMCdS, Tang F, Surani MA (2008) Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. Cell Stem Cell 3(4):391–401
22. Wylie C (1999) Germ cells. Cell 96(2):165–174
23. Seki Y, Yamaji M, Yabuta Y, Sano M, Shigeta M, Matsui Y, Saga Y, Tachibana M, Shinkai Y, Saitou M (2007) Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice. Development 134(14):2627–2638. doi:10.1242/dev.005611
24. Maatouk DM, Kellam LD, Mann MRW, Lei H, Li E, Bartolomei MS, Resnick JL (2006) DNA methylation is a primary mechanism for silencing postmigratory primordial germ cell genes in both germ cell and somatic cell lineages. Development 133(17):3411–3418. doi:10.1242/dev.02500
25. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418(6893):41–49
26. D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC (2004) Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. J Cell Sci 117(14):2971–2981. doi:10.1242/jcs.01103

27. Pochampally RR, Smith JR, Ylostalo J, Prockop DJ (2004) Serum deprivation of human marrow stromal cells (hMSCs) selects for a subpopulation of early progenitor cells with enhanced expression of OCT-4 and other embryonic genes. Blood 103(5):1647–1652. doi:10.1182/blood-2003-06-1967
28. Beltrami AP, Cesselli D, Bergamin N, Marcon P, Rigo S, Puppato E, D'Aurizio F, Verardo R, Piazza S, Pignatelli A, Poz A, Baccarani U, Damiani D, Fanin R, Mariuzzi L, Finato N, Masolini P, Burelli S, Belluzzi O, Schneider C, Beltrami CA (2007) Multipotent cells can be generated in vitro from several adult human organs (heart, liver, and bone marrow). Blood 110(9):3438–3446. doi:10.1182/blood-2006-11-055566
29. Kucia M, Reca R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J, Ratajczak MZ (2006) A population of very small embryonic-like (VSEL) CXCR4+SSEA-1+Oct-4+stem cells identified in adult bone marrow. Leukemia 20(5):857–869
30. Zuba-Surma EK, Kucia M, Wu W, Klich I, Jr. JWL, Ratajczak J, Ratajczak MZ (2008) Very small embryonic-like stem cells are present in adult murine organs: ImageStream-based morphological analysis and distribution studies. Cytometry Part A 73A (12):1116–1127
31. Kucia M, Halasa M, Wysoczynski M, Baskiewicz-Masiuk M, Moldenhawer S, Zuba-Surma E, Czajka R, Wojakowski W, Machalinski B, Ratajczak MZ (2006) Morphological and molecular characterization of novel population of CXCR4 + SSEA-4 + Oct-4 + very small embryonic-like cells purified from human cord blood-preliminary report. Leukemia 21(2):297–303
32. Kucia M, Wysoczynski M, Ratajczak J, Ratajczak M (2008) Identification of very small embryonic like (VSEL) stem cells in bone marrow. Cell Tissue Res 331(1):125–134. doi:10.1007/s00441-007-0485-4
33. Taichman RS, Wang Z, Shiozawa Y, Jung Y, Song J, Balduino A, Wang J, Patel LR, Havens AM, Kucia M, Ratajczak MZ, Krebsbach PH (2010) Prospective identification and skeletal localization of cells capable of multilineage differentiation in vivo. Stem Cells Dev 19 (10):1557–1570. doi:10.1089/scd.2009.0445
34. Dawn B, Tiwari S, Kucia MJ, Zuba-Surma EK, Guo Y, SanganalMath SK, Abdel-Latif A, Hunt G, Vincent RJ, Taher H, Reed NJ, Ratajczak MZ, Bolli R (2008) Transplantation of bone marrow-derived very small embryonic-like stem cells attenuates left ventricular dysfunction and remodeling after myocardial infarction. Stem Cells 26(6):1646–1655. doi:10.1634/stemcells.2007-0715
35. Ratajczak J, Wysoczynski M, Zuba-Surma E, Wan W, Kucia M, Yoder MC, Ratajczak MZ (2011) Adult murine bone marrow-derived very small embryonic-like stem cells differentiate into the hematopoietic lineage after coculture over OP9 stromal cells. Exp Hematol 39(2):225–237
36. Ratajczak J, Zuba-Surma E, Klich I, Liu R, Wysoczynski M, Greco N, Kucia M, Laughlin MJ, Ratajczak MZ (2011) Hematopoietic differentiation of umbilical cord blood-derived very small embryonic/epiblast-like stem cells. Leukemia 25(8):1278–1285
37. Kucia MJ, Wysoczynski M, Wu W, Zuba-Surma EK, Ratajczak J, Ratajczak MZ (2008) Evidence that very small embryonic-like stem cells are mobilized into peripheral blood. Stem Cells 26(8):2083–2092. doi:10.1634/stemcells.2007-0922
38. Wojakowski W, Tendera M, Kucia M, Zuba-Surma E, Paczkowska E, Ciosek J, Halasa M, Kr M, Kazmierski M, Buszman P, Ochala A, Ratajczak J, Machalinski B, Ratajczak MZ (2009) Mobilization of bone marrow-derived oct-4 + ssea-4 + very small embryonic-like stem cells in patients with acute myocardial infarction. J Am Coll Cardiol 53(1):1–9
39. Paczkowska E, Kucia M, Koziarska D, Halasa M, Safranow K, Masiuk M, Karbicka A, Nowik M, Nowacki P, Ratajczak MZ, Machalinski B (2009) Clinical evidence that very small embryonic-like stem cells are mobilized into peripheral blood in patients after stroke. Stroke 40(4):1237–1244. doi:10.1161/strokeaha.108.535062
40. Shin DM, Zuba-Surma EK, Wu W, Ratajczak J, Wysoczynski M, Ratajczak MZ, Kucia M (2009) Novel epigenetic mechanisms that control pluripotency and quiescence of adult bone marrow-derived Oct4+ very small embryonic-like stem cells. Leukemia 23(11):2042–2051
41. Lengner CJ, Camargo FD, Hochedlinger K, Welstead GG, Zaidi S, Gokhale S, Scholer HR, Tomilin A, Jaenisch R (2007) Oct4 expression is not required for mouse somatic stem cell self-renewal. Cell Stem Cell 1(4):403–415

42. Liedtke S, Enczmann J, Waclawczyk S, Wernet P, K?ler G (2007) Oct4 and its pseudogenes confuse stem cell research. Cell Stem Cell 1(4):364–366
43. Atlasi Y, Mowla SJ, Ziaee SAM, Gokhale PJ, Andrews PW (2008) OCT4 spliced variants are differentially expressed in human pluripotent and nonpluripotent cells. Stem Cells 26(12):3068-3074. doi:10.1634/stemcells.2008-0530
44. Guo C-l, Liu L, Jia Y-d, Zhao X-y, Zhou Q, Wang L (2012) A novel variant of Oct3/4 gene in mouse embryonic stem cells. Stem Cell Res 9(2):69–76. doi:http://www.nature.com/ncb/journal/v8/n5/suppinfo/ncb1403_S1.html
45. Margueron R, Trojer P, Reinberg D (2005) The key to development: interpreting the histone code? Curr Opin Genet Dev 15(2):163–176
46. O'Neill LP, VerMilyea MD, Turner BM (2006) Epigenetic characterization of the early embryo with a chromatin immunoprecipitation protocol applicable to small cell populations. Nat Genet 38(7):835–841
47. Shin D-M, Liu R, Klich I, Ratajczak J, Kucia M, Ratajczak M (2010) Molecular characterization of isolated from murine adult tissues very small embryonic/epiblast like stem cells (VSELs). Mol Cells 29(6):533–538. doi:10.1007/s10059-010-0081-4
48. Shin DM, Liu R, Klich I, Wu W, Ratajczak J, Kucia M, Ratajczak MZ (2010) Molecular signature of adult bone marrow-purified very small embryonic-like stem cells supports their developmental epiblast/germ line origin. Leukemia 24(8):1450–1461. doi:http://www.nature.com/leu/journal/v24/n8/suppinfo/leu2010121s1.html
49. Simon JA, Kingston RE (2009) Mechanisms of Polycomb gene silencing: knowns and unknowns. Nat Rev Mol Cell Biol 10(10):697–708
50. Schwartz YB, Pirrotta V (2007) Polycomb silencing mechanisms and the management of genomic programmes. Nat Rev Genet 8(1):9–22
51. Ringrose L, Paro R (2004) Epigenetic regulation of cellular memory by the polycomb and trithorax group proteins. Annu Rev Genet 38(1):413–443. doi:10.1146/annurev.genet.38.072902.091907
52. Shin DM, Liu R, Wu W, Waigel SJ, Zacharias W, Ratajczak MZ, Kucia M (2012) Global gene expression analysis of very small embryonic-like stem cells reveals that the Ezh2-dependent bivalent domain mechanism contributes to their pluripotent state. Stem Cells Dev 21 (10):1639–1652. doi:10.1089/scd.2011.0389
53. De Felici M, McLaren A (1983) In vitro culture of mouse primordial germ cells. Exp Cell Res 144(2):417–427
54. Reik W, Walter J (2001) Genomic imprinting: parental influence on the genome. Nat Rev Genet 2(1):21–32
55. Delaval K, Feil R (2004) Epigenetic regulation of mammalian genomic imprinting. Curr Opin Genet Dev 14(2):188–195
56. Yamazaki Y, Mann MR, Lee SS, Marh J, McCarrey JR, Yanagimachi R, Bartolomei MS (2003) Reprogramming of primordial germ cells begins before migration into the genital ridge, making these cells inadequate donors for reproductive cloning. Proc Natl Acad Sci U S A 100 (21):12207–12212. doi:10.1073/pnas.2035119100, 2035119100[pii]
57. Ratajczak MZ, Shin DM, Ratajczak J, Kucia M, Bartke A (2010) A novel insight into aging: are there pluripotent very small embryonic-like stem cells (VSELs) in adult tissues overtime depleted in an Igf-1-dependent manner? Aging 2 (11):875–883. 100231 [pii]
58. Russell SJ, Kahn CR (2007) Endocrine regulation of ageing. Nat Rev Mol Cell Biol 8(9):681–691
59. Piper MDW, Bartke A (2008) Diet and aging. Cell Metab 8(2):99–104
60. Kurimoto K, Yabuta Y, Ohinata Y, Saitou M (2007) Global single-cell cDNA amplification to provide a template for representative high-density oligonucleotide microarray analysis. Nat Protoc 2(3):739–752
61. Yabuta Y, Kurimoto K, Ohinata Y, Seki Y, Saitou M (2006) Gene expression dynamics during germline specification in mice identified by quantitative single-cell gene expression profiling. Biol Reprod 75(5):705–716. doi:10.1095/biolreprod.106.053686
62. Zuba-Surma EK, Kucia M, Rui L, Shin D-M, Wojakowski W, Ratajczak J, Ratajczak MZ (2009) Fetal liver very small embryonic/epiblast like stem cells follow developmental migra-

tory pathway of hematopoietic stem cells. Ann N Y Acad Sci 1176 (Hematopoietic Stem Cells VII):205–218
63. Rich IN (1995) Primordial germ cells are capable of producing cells of the hematopoietic system in vitro. Blood 86(2):463–472
64. Ohtaka T, Matsui Y, Obinata M (1999) Hematopoietic development of primordial germ cell-derived mouse embryonic germ cells in culture. Biochem Biophys Res Commun 260(2):475–482
65. Kritzenberger M, Wrobel K-H (2004) Histochemical in situ identification of bovine embryonic blood cells reveals differences to the adult haematopoietic system and suggests a close relationship between haematopoietic stem cells and primordial germ cells. Histochem Cell Biol 121(4):273–289
66. Saito A, Watanabe K, Kusakabe T, Abe M, Suzuki T (1998) Mediastinal mature teratoma with coexistence of angiosarcoma, granulocytic sarcoma and a hematopoietic region in the tumor: a rare case of association between hematological malignancy and mediastinal germ cell tumor. Pathol Int 48(9):749–753

Chapter 13
Role of Extracellular Vesicles in Tissue/Organ Regeneration

Maria Chiara Deregibus, Alessandra Iavello, Ciro Tetta and Giovanni Camussi

13.1 Introduction

A lot of evidence supports the notion that stem cells exert a regenerative effect on damaged tissues/organs by paracrine mechanisms [1, 2]. The beneficial effect observed is not, however, supported by concrete evidence that stem cells used in regenerative medicine generate a significant amount of organ-specific cells in vivo despite their in vitro plasticity and ability to transdifferentiate into different cell types. In some cases, cell plasticity events have been ascribed to cell fusion [3], but incidences of cell fusion are extremely rare and can only partially account for tissue regeneration observed after stem cell therapy. An alternative mechanism has been therefore proposed of a paracrine action of stem cells that may favor self-tissue/organ repair by means of soluble factor production such as growth factors, chemokines, cytokines, and bioactive lipids. Many studies have supported the paracrine hypothesis of stem cell action; above all, the observation that conditioned media of stem cells retain the same beneficial properties of stem cells [4–6]. The paracrine hypothesis may therefore provide an explanation for the beneficial effects achieved by stem cell therapies in the regeneration of damaged organs or tissues as an alternative to transdifferentiation. Stem cells produce many growth factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 and -2 (IGF-1 and -2), and stromal-derived factor 1

G. Camussi (✉) · A. Iavello
Department of Medical Sciences, University of Turin,
Via Nizza 52, Turin, Italy
e-mail: giovanni.camussi@unito.it

M. C. Deregibus
Department of Medical Sciences and Translational Center for Regenerative Medicine,
University of Turin, Via Nizza 52, Turin, Italy

C. Tetta
Translational Center for Regenerative Medicine,
University of Turin, Via Nizza 52, Turin, Italy

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,*
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_13,

(SDF-1). Moreover, they produce cytokines such as tumor necrosis factor-α, certain interleukins, and interferon, as well as chemokines including monocyte chemotactic protein-1 and regulated on activation normal T-cell expressed and secreted (RANTES) [7–10].

Beside soluble factors, stem cells, as well as other cells, release small vesicles into the extracellular space which facilitate information exchange between all cells [11–16]. Microvesicles present in biological fluids and released by cultured cells are a heterogeneous population which includes cell surface-shed vesicles and exosomes; for this reason, it has been suggested to call them collectively extracellular vesicles (EVs).

The release of EVs, which are able to interact with neighboring cells, is now recognized as an important means of intercellular communication in stem/progenitor cell biology [17–19]. Soluble mediators as well as EVs may reduce damage by limiting cell apoptosis in the injured tissues/organs and may stimulate the proliferation of cells survived to injury.

13.2 Extracellular Vesicles

EVs are small vesicles released by eukaryotic cells, are heterogeneous in size and origin, and are known in the literature with different names such as microvesicles, microparticles, ectosomes, and exosomes. Most cells produce EVs which can be detected in many body fluids (e.g., blood, urine, saliva, bronchoalveolar lavage fluid, breast milk, cerebrospinal fluid, and malignant pleural effusions) or released in the conditioned medium of cell cultures [12, 19–27]. The release of EVs from most cells, including stem cells, occurs in basal condition and increases during cell proliferation and cell activation after exposure to shear stress, hypoxia, activated complement components, oxidative and irradiation injury [13].

EVs can be classified into two major groups: shedding vesicles and exosomes. Shedding vesicles, secreted by healthy cells, comprise small vesicles generated by direct budding of the plasma membrane of the cell of origin, and are distinct from, and much smaller in size than preapoptotic vesicles and apoptotic bodies which are released by dying cells and contain deoxyribonucleic acid (DNA) [13]. Release of shedding vesicles relies on cytoskeleton activation and on increased levels of intracellular calcium capable of changing the equilibrium of calpain, flippase, floppase, scramblase, and gelsolin enzymes with consequent modification of the membrane asymmetry, and creation of nanodomains enriched in molecules such as phosphatydilserine, flotillin, and cholesterol that make these sites more prone to vesiculation [13, 15, 28] (Table 13.1). In microglia cells, EV shedding is due to acid sphingomyelinase activity which induces sphingomyelin hydrolysis and consequent destabilization of the membrane [29]. Surface markers of shedding vesicles mainly depend on the membrane composition of the cells from which they are released [12]. The bloodstream is enriched in shedding vesicles, most of which are released from platelets and known in the literature as microparticles; a small amount of shedding vesicles in the blood of healthy subjects derives from erythrocytes, leukocytes, and

Table 13.1 Characterization of extracellular vesicles

	Shedding vesicles	Exosomes
Size	100–1000 nm	30–120 nm
Origin	Generated by plasma membrane budding	Generated by exocytosis of multi-vesicular bodies
Mechanisms involved in EV biogenesis	Cytoskeleton reorganization dependent on increased calcium concentration in cytosol, loss of cell membrane phospholipid asymmetry dependent on altered balance of floppase, fippase, scramblase, and calpain enzymes	Process calcium concentration independent, dependent on cytoskeleton activation; Involvement of ESCRT, ceramide, tetraspanins, high-order oligomerization, Syndecan
EV markers	High exposure of PS Lipid raft-associated molecules Markers specific to the cell of origin	Low exposure of PS Expression of CD63, CD9, CD81, CD82, Hsp70, Tsg101, Alix
Carried molecules	Lipids, proteins, mRNA, miRNA, long noncoding RNA, rarely DNA	Lipids, proteins, mRNA, miRNA, long noncoding RNA, rarely DNA

Hsp70 heat shock protein 70, *PS* phosphatidylserine, *Tsg101* tumor susceptibility gene 101, *ESCRT* endosomal sorting complex required for transport, *EV* extracellular vesicle

endothelial cells [15, 30, 31]. This proportion may be altered in pathological conditions.

The second group of vesicles is represented by exosomes which are smaller than shedding vesicles (30–120 nm) and originate from the late endosomal compartment of the cells [13, 15]. The vesicles contained inside the multivesicular bodies may be directed to lysosomes with subsequent degradation of their contents, or alternatively, their membranes may fuse with the cell plasma membrane and be discharged into the extracellular space. A lot of evidence supports the notion that the biogenesis of multivesicular bodies and exosomes is under the control of the endosomal sorting complex required for transport (ESCRT) which could be responsible for membrane invagination and internalization of the multivesicular bodies with formation of the intraluminal vesicles [32]. Molecular mechanisms to determine the fate of intraluminal vesicles either for degradation or extracellular release are not yet fully understood. Nevertheless, it seems that some guanosine triphosphate (GTP)-ases, such as Rab27a and Rab27b, play a key role in different stages of exosome secretion, promoting the docking of internal vesicles of the multivesicular bodies to plasma membranes [33]. Another line of evidence supports the notion of a ceramide-dependent mechanism in vesicle biogenesis, independent of the ESCRT machinery [34]. Ceramide is one of the main lipid components of the cell membrane lipid bilayer and is produced by sphingomyelin hydrolysis catalyzed by sphingomyelynases. Ceramide is involved in the generation of intraluminal vesicles of multivesicular bodies destined to be released as exosomes. Another ESCRT- and ceramide-independent mechanism of EV sorting has been described in melanocytes [35] where the localization of protein cargo to multivesicular bodies depends on the

co-localization of LMP1 with CD63 tetraspanin in the intraluminal vesicles [36]. A similar mechanism has been reported in lymphoblastoid cell lines (LCL). Latent membrane protein 1 (LMP1) is a protein encoded by the Epstein Barr virus which results in constitutive activation of nuclear factor kappa beta (NFkB). It has been proposed that LMP1 avoids degradation by release, via exosomes, to the extracellular space, leading to a consequent reduction in activated NFkB [36], which is dependent on the interaction of LMP1 with CD63 tetraspanin [36].

In Jurkat T cells, a sorting pathway of exosomal proteins, involving higher-order oligomerization, has been described [37], suggesting to be involved in targeting plasma membrane proteins to exosomes with a mechanism similar to that of the sorting of human immunodeficiency virus (HIV) and other retroviruses [38].

A role of heparan sulfate in exosome biogenesis has also been described. Syndecan is one of the most expressed cell-surface-bound heparan sulfates, and syntenin is a soluble protein able to bind to the cytosolic domain of syndecan. It has been established that the interaction between syndecan and syntenin, and the subsequent interaction of syntenin with an ESCRT-III binding protein, named Alix, determines the biogenesis of intraluminal vesicles and exosomes [39].

Proteomic analysis have shown that exosomes have a unique protein composition, shared among exosomes of different cell origin, as well as a cell-type-specific protein content. The tetraspanin family members CD63, CD9, CD81, and other molecules such as CD82, Tsg101, and Alix are considered as specific markers of exosomes (Table 13.1). Moreover, the heat shock 70 kDa protein 8, and Rab GTPase are frequently found within exosomes [15, 40].

Cell-to-cell communication is an important mechanism for preserving the cell/tissue homeostasis and for appropriate responses in order to restore this equilibrium when its stability is altered. In this regard, EVs represent a crucial mechanism for the horizontal exchange of information between cells [11–16]. Independently from their formation and characteristics, both shedding vesicles and exosomes express surface receptors which facilitate interactions with surrounding cells, leading to the stimulation of target cells, as well as to the transfer into recipient cells of biologically active molecules such as proteins, lipids, and nucleic acids, in particular extracellularly secreted ribonucleic acid (exRNA) [17, 18, 41, 42] (Fig. 13.1). Recent studies have suggested that RNA molecules secreted in the extracellular space may transport information that alters the phenotype of recipient cells [43–45]. The exRNA can act locally and at distant sites, conveying paracrine/endocrine signals. ExRNA is present in all human biological fluids in a degradative enzyme-protected form, being either encapsulated within EVs or nonencapsulated, but associated with protein carriers such as Ago2 and high-density lipoprotein (HDL) [46–50]. It has been suggested that the majority of RNA is carried within EVs and delivered to target cells. Indeed, it has been shown that EVs contain diverse populations of exRNA including messenger RNA (mRNA), long noncoding RNA, and microRNA (miRNA). Transfer of exRNA from the cell of origin to the recipient cell may have important physiological and pathological implications. In particular, long non-coding RNA has been implicated in the regulation of the epigenome, and miRNAs are known to regulate up to 80% of all protein-encoding genes [51].

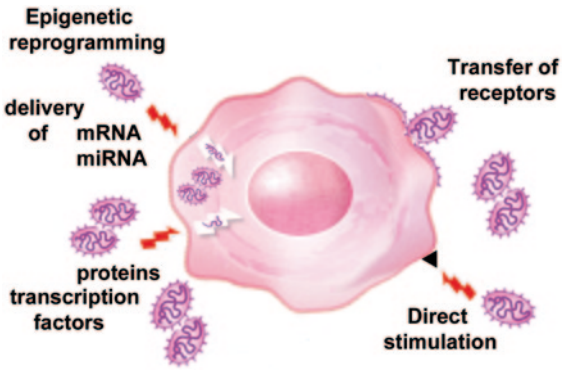

Fig. 13.1 Schematic representation of mechanisms involved in extracellular vesicle (EV)-mediated intercellular communication. EVs may induce epigenetic reprogramming of recipient cell by transfer of genetic information via messenger ribonucleic acid (*mRNA*), microRNA (*miRNA*), or proteins/transcription factors. In addition, EVs may transfer receptors between cells or act as a "signaling complex" through surface expressed ligands that directly stimulate the target cells

13.3 Cell-to-Cell Cross Talk via EVs

In 2006, Ratajczak et al. showed a horizontal transfer of proteins and mRNA between cells, demonstrating that murine embryonic stem cell-derived microvesicles could transfer proteins and mRNA for early transcription factors, such as Oct-4, Rex-1, Nanog, and HoxB4, to hematopoietic progenitors, and highlighted the importance of these vesicles, specifying that they were undervalued as mediators of cell-to-cell communication [11, 17]. A subsequent study of our group evidenced that microvesicles released from endothelial progenitors cells (EPCs) were able to activate an angiogenic program in quiescent endothelial cells by transferring functional mRNA [18]. Valadi et al. demonstrated that exosomes from mouse and human mast cell lines may not only transport mRNA but also specific subsets of miRNAs [41]. Yuan et al. showed that microvesicles derived from embryonic stem cells carried abundant miRNA and were therefore capable of transferring a subset of miRNA to mouse embryonic fibroblasts in vitro [52]. Since miRNAs play a role in regulating protein translation, the authors suggested that stem cells can modify gene expression of target cells by means of miRNA transferred by EVs [52].

Our group demonstrated that adult stem cells, such as multipotent stromal cells (MSC) from bone marrow and human liver stem cells (HLSC), a cell line of mesenchymal origin with partial hepatic commitment, generate EVs containing specific subsets of mRNA [53, 54] and miRNA [42]. Human MSC-derived EVs were shown to express CD44 and CD29 adhesion molecules, which allowed the internalization of EVs into renal tubular cells and delivery of their RNA content [53]. EV-contained mRNA and miRNA are specifically linked to the mesenchymal phenotype of the cell of origin and to certain important cell processes related to the control

of transcription, proliferation and immune regulation. We also found that vesicle-carried RNAs are functional once transferred to tubular epithelial cells [42, 53]. Aliotta et al. demonstrated direct delivery of mRNA by EVs, following entry into bone marrow cells and induction of transcription [55]. It has been suggested that this horizontal transfer of exRNAs operates as an intercellular mechanism of communication in which the RNA from a donor cell modulates gene expression of a recipient cell [46].

Little is known about the mechanisms by which exRNAs are enriched in EVs. We found, however, that MSC- and HLSC-derived EVs contained ribonucleoproteins involved in the RNA transport, stability, and storage within EVs [42]. EVs released from these stem cells contained proteins such as TIA, TIAR, HuR, and Staufen which were also present in the cytosol foci that form during cellular stress, and known as stress granules, but absent in processing bodies, structures involved in mRNA degradation. In addition, MSC- and HLSC-derived EVs contained Argonaute 2 (Ago2), an essential catalytic component of the RNA-induced silencing complex (RISC), responsible for the transport and functioning of miRNA [42]. Analysis of the miRNA content carried by EVs, compared to that contained in the stem cells of origin, demonstrated that most miRNAs are enriched in EVs, suggesting that cells could shuttle genetic information to other target cells, in a nonrandom manner, following an ordered and precise mechanism of miRNA collection within EVs, prior to the release of these vesicles from cells [42].

miRNAs are transcribed from miRNA genes in several 100 nucleotide long pri-miRNAs with a 5′ cap and 3′ poly-A tail. In the nucleus, Drosha, an RNase III family member, converts pri-miRNA into 70-nucleotide-long pre-miRNA with a characteristic hairpin loop [56]. This nuclear Drosha step is bypassed by some miRNAs which are instead generated by a process of splicing and debranching. The pre-miRNAs are exported from the nucleus into the cytoplasm by exportin 5, and are cleaved by the RNase III Dicer, with the generation of approximately 22-nucleotide-long mature miRNA duplexes. The guide strand of the miRNA duplex is then integrated into the miRNA-induced silencing complex (miRISC), whereas the passenger strand is degraded. The miRISC core consists of the Ago2 protein that interacts and cooperates with miRNAs in repression of target mRNAs [57]. Translationally repressed mRNAs accumulate within cytoplasmic foci, known as processing bodies and stress granules while waiting to be recycled or degraded. These foci essentially share some components of the miRISC complex [58]. EVs also contain Ago2 and other components of the miRISC complex as well as several RNA-binding proteins, recognized as regulators of RNA traffic between the nucleus and the cytoplasm, suggesting their involvement in the accumulation of selected RNAs during EV biogenesis.

Several studies have shown that exRNA transferred by EVs from the cell of origin to the target cell are functional. Using reporter mRNAs, it has been shown that transferred mRNA is translated into proteins, both in vitro and in vivo [18, 53, 55]. Similarly, the transfer of selected miRNAs via EVs has been shown to be followed by a block of translation of target mRNA [41, 42, 52]. In fact, miRNAs are able to induce inhibition or degradation of target mRNAs, thus influencing the phenotype of the recipient cell [56].

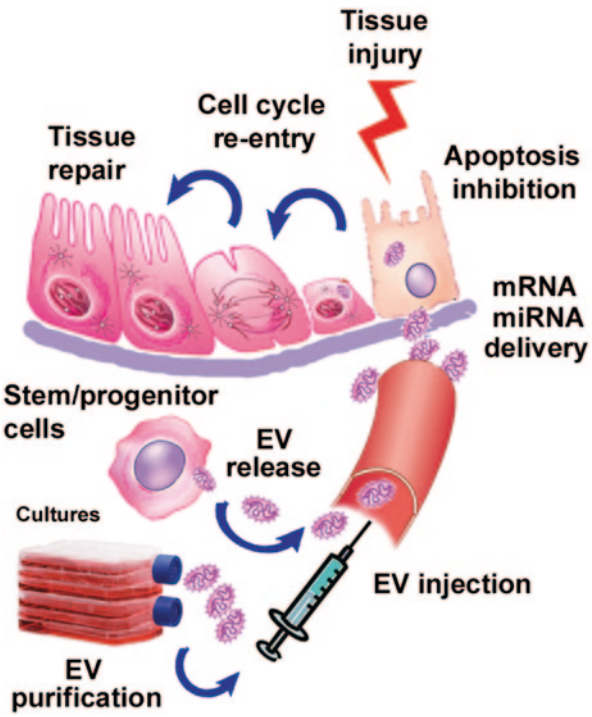

Fig. 13.2 Schematic representation of the potential therapeutic effect of stem/progenitor cell-derived extracellular vesicles (*EVs*). EVs derived from stem/progenitor cells may localize at the site of injury and reprogram tissue-damaged cells by delivering proteins, messenger ribonucleic acid (*mRNA*) and microRNA (*miRNA*) that induce apoptosis inhibition and cell cycle re-entry of these cells, thus, favoring tissue repair. EVs produced in vitro by cultured stem/progenitor cells, once purified from conditioned medium and injected in vivo, may activate regenerative programs in injured tissues

The emerging paradigm that EVs can modify the phenotype of target cells by transferring exRNAs sheds a new light on paracrine/endocrine mechanisms involved in stem cell action, and opens new perspectives for regenerative medicine.

13.4 Therapeutic Potential of Stem Cell-Derived EVs

The paracrine/endocrine hypothesis of stem cell action could modify the therapeutic approach to tissue/organ regeneration. Based on evidence that EVs may transfer genetic information, several studies have investigated whether EVs released from stem cells are able to reproduce the regenerative action of stem cells (Fig. 13.2).

EPC-derived EVs were also found to stimulate neo-angiogenesis *in* vivo, and this property was interpreted as being attributable to EV-mediated transfer of mRNA associated with the PI3K/AKT signaling pathway [18]. Subsequent studies in a model of hind limb ischemia induced by ligation and resection of the left femoral artery in severe combined immunodeficiency (SCID) mice showed that EVs released by EPCs are able to improve neovascularization, to limit injury, and to favor muscle regeneration [59]. Notably, EVs derived from EPCs contained the regulators of angiogenesis, the noncoding RNA molecules: miR 126 and miR 296. To evaluate whether miRNAs were involved in EV-mediated angiogenesis, Dicer

knockdown EPCs were generated in order to obtain EVs that were depleted of miRNAs. miRNA depletion significantly reduced the regenerative potential of EVs in this experimental setting [59]. EPC-derived EVs also showed a renoprotective action in a model of ischemia-reperfusion injury (IRI) [60]. When intravenously injected, labeled EVs localized within peritubular capillaries and entered tubular cells, limiting tissue damage and favoring a prompt recovery from acute kidney injury (AKI) by reducing apoptosis and leukocyte infiltration and enhancing tubular cell proliferation [60]. Interestingly, the progression toward chronic kidney damage was also prevented due to the EPC-derived EV inhibition of capillary rarefaction, glomerulosclerosis, and tubulo-interstitial fibrosis [60]. This biological activity was partially dependent on EV-carried miRNAs which include miRNAs that modulate proliferation, angiogenesis, and apoptosis, as shown by loss of EV activity after nonspecific miRNA-depletion of EVs by Dicer knockdown in EPCs [60]. Vascular protection was, at least partly, attributable to angiomiR 126 and 296, as shown by specific depletion of miR-126 and miR-296, and by the use of antagomirRs [60]. Conversely, the transfer of mRNA may also cause phenotype changes in recipient cells. Our group has demonstrated that adult stem cells, such as MSCs, generated EVs capable of promoting recovery of acute kidney injury [61]. In these experimental settings, the EV-mediated transfer of stem cell-specific mRNA and the transient in vivo synthesis of the correspondent human proteins within the kidneys of SCID mice were observed [61]. EV-encapsulated RNA protects RNA from the physiological concentration of degrading enzymes present in biological fluids. However, when treated with high doses of RNase, it is possible to achieve RNA inactivation [17, 18]. The observation that treatment with high doses of RNase abolished the biological effect of EVs suggests a prominent role of exRNAs in these vesicles. In a lethal model of AKI induced by treatment of SCID mice with cisplatin, the administration of MSC-derived EVs, as part of a therapeutic regimen, not only favored recovery but also significantly improved survival [61]. After localization at the site of tissue injury, EVs delivered transcription regulator exRNAs that modified gene expression in recipient cells and induced upregulation of BCL-XL, BCL2, and BIRC8 antiapoptotic genes and downregulation of CASP1, CASP8, and LTA, genes involved in cell apoptosis [61].

Tomasoni et al. [62] demonstrated that EV-mediated transfer of IGF-1R mRNA from MSC to cisplatin-damaged murine proximal tubular cells induced expression of human IGF-1R and stimulated proliferation, suggesting that the horizontal transfer of the mRNA for IGF-1R enhances tubular cell sensitivity to IGF-1. This observation further supports the powerful renoprotection that a few BM-MSC engrafted in the kidney exert in vivo. However, the effect of EVs not only depends on their cargo but also on the response of the recipient cells, which may vary depending on their functional and metabolic state. For instance, the same EVs may have opposite effects on normal, compared to neoplastic cells, in relation with differentially activated pathways. While EVs produced by human liver stem cells stimulate hepatocyte proliferation and liver regeneration [54], the same EVs inhibit the growth and survival of hepatocellular carcinoma cells [63]. In fact, tumor-suppressive miRNAs carried by EVs, when delivered to tumor cells lacking these miRNAs, induced cell reprogramming with tumor regression [63]. Recently, we also found that EVs

derived from bone marrow MSCs inhibit the growth of different tumors [64]. These EVs that express several mesenchymal markers, such as CD105, CD73, CD44, and CD29, and the exosomal marker LAMP-1, inhibited cell cycle progression and induced apoptosis in several cell lines [64]. When injected into an established tumor in SCID mice, EVs induced regression of the tumor [64].

The potential therapeutic effect of EVs has been studied in several other organs. For example, we have found that human liver stem cells, a cell line of mesenchymal origin with partial hepatic commitment, generated EVs capable of promoting recovery of the liver in 70% hepatectomized rats [54]. Recently, it has been shown that exosomes obtained from human umbilical cord-MSCs reduced fibrosis and lessened hepatic inflammation and collagen deposition in a model of chronic liver injury induced by carbon tetrachloride, by inhibiting the epithelial-to-mesenchymal transition and protecting hepatocytes [65].

Moreover, it has been found that MSCs mediate their cardioprotective paracrine effect by means of EVs. In fact, exosomes purified from MSC-conditioned medium were shown to reduce the infarct size in a mouse model of myocardial ischemia/reperfusion injury [66]. In addition, Lai et al. [67] demonstrated the possibility of a large-scale production of cardioprotective EVs from MSCs obtained by aborted fetal tissues. Recently, Chen et al. [68] established that cardiac progenitor cells can also be used as a source of EVs as a therapeutic vehicle for cardioprotection.

A recent study has shown that human MSC-derived EVs contain cystinosin protein and CTNS mRNA that can be transferred to human cystinotic cells and reduce cystine accumulation in vitro [69]. The observation that EVs may reprogram mutant cells suggests a potential therapeutic application in cystinosis, a rare disease caused by homozygous mutations of the CTNS gene.

13.5 Applications of Exosomes for Drug Delivery

EVs represent potential therapeutic tools for drug delivery and could be biocompatible and effective carriers able to cross biological barriers [70]. There are several potential advantages of using EVs for drug delivery; the knowledge that EVs contain selected patterns of proteins and exRNA indicates that it should be possible to load desirable biologic molecules by manipulating the cells. This implies their incorporation into EVs during biogenesis. It has been shown that transfection of miRNAs or small interfering RNA (siRNA) within the cell of origin is followed by their inclusion and secretion within EVs [42, 63]. Moreover, the group of Gould demonstrated the possibility of specifically targeting genetically engineered proteins by expressing plasma-membrane anchors and an oligomerization domain in the exosomes [38, 71]. In addition, EVs derived from stem/progenitor cells are not immunogenic, and as a large amount of EVs are physiologically present in biological fluids, it is conceivable that exogenously administrated EVs should also be well tolerated. Moreover, EVs can localize in defined tissues or organs, which has been shown, for example, for MSC- and EPC-derived EVs that localize at the site of kidney injury exploiting the surface receptors of the cell of origin [53, 60]. Once localized, EVs are internalized

and deliver their cargo to the target cell. The ability to target specific tissues/organs can be enhanced by manipulating the cell of origin so that EVs express defined molecules/receptors. For example, we generated fibroblast-derived EVs overexpressing CD29, thus enhancing their uptake by hepatoma cells [63]. El Andaloussi et al. generated exosomes expressing the rabies virus glycoprotein (RVG) peptide on their membranes in order to specifically target neurons [72].

EVs have been shown to cross the blood-brain barrier, through their hydrophobic membrane, and they, therefore, represent an ideal tool to deliver drugs to the nervous system. Using exosomes, Alvarez-Erviti et al. [73] demonstrated the delivery of exogenous siRNA, capable of silencing and degrading complementary mRNA by perfect pairing to neurons, microglia, and oligodendrocytes of mouse brain, by systemic injection of exosomes loaded with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA by electroporation. The exosomes were derived from dendritic cells engineered to express the exosomal membrane protein Lamp2b fused to the neuron-specific RVG peptide, and produced a specific gene knockdown of GAPDH in some areas of murine brain. In addition, the authors demonstrated that exosomes transporting beta-secretase 1 (BACE1) siRNA led to BACE1 gene and protein knock-down [72, 73]. BACE1 is the beta secretase enzyme responsible for toxic beta amyloid formation and deposition and is therefore a critical target for treatment of Alzheimer's disease [73]. Exosomes can thus be considered to be natural vectors of genetic material and can also be exploited for the delivery of exogenous siRNA across biological barriers such as the blood-brain barrier.

Studies by Akao et al. [74] demonstrated that ex vivo manipulation of the miRNA content of EVs could be an efficient strategy for delivering miRNAs to specific tissues and organs. When transfected in human monocytic leukemia THP-1 cells, the RNA molecules were shed within EVs. After intravenous injection of shed EVs, the level of the transfected miR-143BPs was significantly increased in the serum, tumor, and kidneys of the host animals [75]. Van den Boorn et al. [75] exploited exosomes to efficiently deliver siRNAs to target cells in vivo in mice.

The use of EVs for drug delivery would imply having a reliable, expandable source of cells that generate nonimmunogenic EVs. In this regard, MSCs could be a suitable candidate, but the use of these cells is limited by their senescence after repeated culture passages. In order to circumvent this problem, Chen et al. [76] achieved oncogenic immortalization of human embryonic stem cell-derived MSCs, enabling large-scale production of exosomes for therapeutic use. Of interest, the oncogene was not present in exosomes derived from immortalized MSCs that remained unchanged in quantity and quality.

Conclusions

Taken together, these studies suggest that EVs derived from different stem cell sources retain the biological activity of stem cells and can mimic the therapeutic effects of the cell of origin. However, it is evident that an EV-based therapy does

not replace the injured tissue, but may rather coordinate tissue self-repair and limit the injury. By exploiting membrane receptors expressed by the stem cell of origin, EVs were able to home into the site of injury. In addition, EVs have the potential to interfere with multiple cellular pathways involved in different physiological and pathological processes because they contain a complex array of constituents. Identification of the molecular components accountable for the beneficial action of EVs in different pathologies, along with a better understanding of homing processes and of exRNA containment within EVs may prompt new strategies for producing engineered EVs for therapeutic purposes. The use of EVs instead of stem cells may bypass problems such as maldifferentiation and tumorigenesis that can result from the injection of replicating cells into the host organism. Other potential advantages from using EVs include the possibility of extensive expansion in vitro and of cryopreservation and, regarding the stem cell-derived EVs, the absence of immunogenicity. However, before a clinical application can be envisaged, many problems need to be solved. Firstly, the definition of GMP protocols for large-scale EV production and the evaluation of bio-distribution, pharmacokinetics, biosafety, and effectiveness in different pathological conditions.

References

1. Ratajczak MZ, Kucia M, Jadczyk T, Greco NJ, Wojakowski W, Tendera M et al (2012) Pivotal role of paracrine effects in stem cell therapies in regenerative medicine: can we translate stem cell-secreted paracrine factors and microvesicles into better therapeutic strategies? Leukemia 26:1166–1173
2. Gnecchi M, Danieli P, Cervio E (2012) Mesenchymal stem cell therapy for heart disease. Vascul Pharmacol 57:48–55
3. Sanges D, Lluis F, Cosma MP (2011) Cell-fusion-mediated reprogramming: pluripotency or transdifferentiation? Implications for regenerative medicine. Adv Exp Med Biol 713:137–159
4. Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F et al (2006) Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 20:661–669
5. Timmers L, Lim SK, Arslan F, Armstrong JS, Hoefer IE, Doevendans PA et al (2007) Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. Stem Cell Res 1:129–137
6. Bi B, Schmitt R, Israilova M, Nishio H, Cantley LG (2007) Stromal cells protect against acute tubular injury via an endocrine effect. J Am Soc Nephrol 18:2486–2496
7. Majka M, Janowska-Wieczorek A, Ratajczak J, Ehrenman K, Pietrzkowski Z, Kowalska MA et al (2001) Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. Blood 97:3075–3085
8. Janowska-Wieczorek A, Majka M, Ratajczak J, Ratajczak MZ (2001) Autocrine/paracrine mechanisms in human hematopoiesis. Stem Cells 19:99–107
9. Sahoo S, Klychko E, Thorne T, Misener S, Schults KM, Millay M et al (2011) Exosomes from human CD34+ stem cells mediate their proangiopoietic paracrine activity. Circ Res 109:724–728
10. Lataillade JJ, Clay D, Bourin P, Hérodin F, Dupuy C, Jasmin C et al (2002) Stromal cell-derived factor 1 regulates primitive hematopoiesis by suppressing apoptosis and by promoting G(0)/G(1) transition in CD34(+) cells: evidence for an autocrine/paracrine mechanism. Blood 99:1117–1129

11. Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ (2006) Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia 20:1487–1495
12. Lee Y, El Andaloussi S, Wood MJ (2012) Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. Hum Mol Genet 21(R1):R125–R134
13. Cocucci E, Racchetti G, Meldolesi J (2009) Shedding microvesicles: artefacts no more. Trends Cell Biol 19:43–51
14. Théry C, Ostrowski M, Segura E (2009) Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 9:581–593
15. Mathivanan S, Ji H, Simpson RJ (2010) Exosomes: extracellular organelles important in intercellular communication. J Proteomics 73:1907–1920
16. György B, Szabó TG, Pásztói M, Pál Z, Misják P, Aradi B et al (2011) Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci 68:2667–2688
17. Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P et al (2006) Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. Leukemia 20:847–856
18. Deregibus MC, Cantaluppi V, Calogero R, Lo Iacono M, Tetta C, Biancone L et al (2007) Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. Blood 110:2440–2448
19. Quesenberry PJ, Dooner MS, Aliotta JM (2010) Stem cell plasticity revisited: the continuum marrow model and phenotypic changes mediated by microvesicles. Exp Hematol 38:581–592
20. Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C (2005) Exosomal-like vesicles are present in human blood plasma. Int Immunol 17:879–887
21. Pisitkun T, Shen RF, Knepper MA (2004) Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci U S A 101:13368–13373
22. Xiao H, Wong DT (2012) Proteomic analysis of microvesicles in human saliva by gel electrophoresis with liquid chromatography-mass spectrometry. Anal Chim Acta 723:61–67
23. Lässer C, Alikhani VS, Ekström K, Eldh M, Paredes PT, Bossios A et al (2011) Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. J Transl Med 9:9
24. Admyre C, Grunewald J, Thyberg J, Gripenback S, Tornling G, Eklund A et al (2003) Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid. Eur Respir J 22:578–583
25. Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M et al (2007) Exosomes with immune modulatory features are present in human breast milk. J Immunol 179:1969–1978
26. Street JM, Barran PE, Mackay CL, Weidt S, Balmforth C, Walsh TS et al (2012) Identification and proteomic profiling of exosomes in human cerebrospinal fluid. J Transl Med 10:5
27. Bard MP, Hegmans JP, Hemmes A, Luider TM, Willemsen R, Severijnen LA et al (2004) Proteomic analysis of exosomes isolated from human malignant pleural effusions. Am J Respir Cell Mol Biol 31:114–121
28. Schara K, Jansa V, Sustar V, Dolinar D, Pavlic JI, Lokar M et al (2009) Mechanisms for the formation of membranous nanostructures in cell-to-cell communication. Cell Mol Biol Let 14:636–656
29. Bianco F, Perrotta C, Novellino L, Francolini M, Riganti L, Menna E et al (2009) Acid Sphingomyelinase activity triggers microparticle release from glial cells. EMBO J 28:1043–1054
30. George JN, Thoi LL, McManus LM, Reimann TA (1982) Isolation of human platelet membrane microparticles from plasma and serum. Blood 60:834–840
31. Martínez MC, Tesse A, Zobairi F, Andriantsitohaina R (2005) Shed membrane microparticles from circulating and vascular cells in regulating vascular function. Am J Physiol Heart Circ Physiol 288:H1004–H1009
32. Henne WM, Buchkovich NJ, Emr SD (2011) The ESCRT pathway. Dev Cell 21:77–91
33. Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A et al (2010) Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat Cell Biol 12:19–30
34. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F et al (2008) Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science 319(5867):1244–1247

35. van Niel GC, Saftig P et al (2011) The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. Dev Cell 21:708–721
36. Verweij FJ, Van Eijndhoven MA, Hopmans ES, Vendrig T, Wurdinger T, Cahir-Mc Farland E et al (2011) LMP1 association with CD63 in endosomes and secretion via exosomes limits constitutive NF-kB activation. EMBO J 30:2115–2129
37. Shen B, Fang Y, Wu N, Gould SJ (2011) Biogenesis of the posterior pole is mediated by the exosome/microvesicle protein-sorting pathway. J Biol Chem 286:44162–44176
38. Fang Y, Wu N, Gan X, Yan W, Morrell JC, Gould SJ (2007) Higher-order oligomerization targets plasma membrane proteins and HIV gag to exosomes. PLoS Biol 5:e158
39. Baietti MF, Zhang Z, Mortier E, Melchior A, Degeest G, Geeraerts A et al (2012) Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nat Cell Biol 14:677–685
40. Mathivanan S, Fahner CJ, Reid GE, Simpson RJ (2012) ExoCarta 2012: database of exosomal proteins, RNA and lipids. Nucleic Acids Res 40(Database issue):D1241–D1244
41. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9:654–659
42. Collino F, Deregibus MC, Bruno S, Sterpone L, Aghemo G, Viltono L et al (2010) Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs. PLoS ONE 5:e11803
43. Del Tatto MN, Berz D et al (2011) Marrow cell genetic phenotype change induced by human lung cancer cells. Exp Hematol 39:1072–1080
44. Quesenberry PJ, Aliotta JM (2010) Cellular phenotype switching and microvesicles. Adv Drug Deliv Rev 62:1141–1148
45. Quesenberry PJ, Dooner MS, Goldberg LR, Aliotta JM, Pereira M, Amaral A et al (2012) A new stem cell biology: the continuum and microvesicles. Trans Am Clin Climatol Assoc 123:152–166
46. Chen X, Liang H, Zhang J, Zen K, Zhang CY (2012) Secreted microRNAs: a new form of intercellular communication. Trends Cell Biol 22:125–132
47. Gallo A, Tandon M, Alevizos I, Illei GG (2012) The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. PLoS ONE 7:e30679
48. Wang K, Zhang S, Weber J, Baxter D, Galas DJ (2010) Export of microRNAs and microRNA-protective protein by mammalian cells. Nucleic Acids Res 38:7248–7259
49. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF et al (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A 108:5003–5008
50. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT (2011) MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol 13:423–433
51. Whitehead J, Pandey GK, Kanduri C (2009) Regulation of the mammalian epigenome by long noncoding RNAs. Biochim Biophys Acta 1790:936–947
52. Yuan A, Farber EL, Rapoport AL, Tejada D, Deniskin R, Akhmedov NB et al (2009) Transfer of microRNAs by embryonic stem cell microvesicles. PLoS ONE 4:e4722
53. Bruno S, Grange C, Deregibus MC, Calogero RA, Saviozzi S, Collino F et al (2009) Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. J Am Soc Nephrol 20:1053–1067
54. Herrera MB, Fonsato V, Gatti S, Deregibus MC, Sordi A, Cantarella D et al (2010) Human liver stem cell-derived microvesicles accelerate hepatic regeneration in hepatectomized rats. J Cell Mol Med 14:1605–1618
55. Aliotta JM, Pereira M, Johnson KW, de Paz N, Dooner MS, Puente N et al (2010) Microvesicle entry into marrow cells mediates tissue-specific changes in mRNA by direct delivery of mRNA and induction of transcription. Exp Hematol 38:233–245
56. Krol J, Loedige I, Filipowicz W (2010) The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 11:597–610
57. Höck J, Meister G (2008) The Argonaute protein family. Genome Biol 9:210

58. Eulalio A, Behm-Ansmant I, Izaurralde E (2007) P bodies: at the crossroads of post-transcriptional pathways. Nat Rev Mol Cell Biol 8:9–22
59. Ranghino A, Cantaluppi V, Grange C, Vitillo L, Fop F, Biancone L et al (2012) Endothelial progenitor cell-derived microvesicles improve neovascularization in a murine model of hindlimb ischemia. Int J Immunopathol Pharmacol 25:75–85
60. Cantaluppi V, Gatti S, Medica D, Figliolini F, Bruno S, Deregibus MC et al (2012) Microvesicles derived from endothelial progenitor cells protect the kidney from ischemia-reperfusion injury by microRNA-dependent reprogramming of resident renal cells. Kidney Int 82:412–427
61. Bruno S, Grange C, Collino F, Deregibus MC, Cantaluppi V, Biancone L et al (2012) Microvesicles derived from mesenchymal stem cells enhance survival in a lethal model of acute kidney injury. PLoS ONE 7:e33115
62. Tomasoni S, Longaretti L, Rota C, Morigi M, Conti S, Gotti E et al (2013) Transfer of growth factor receptor mRNA via exosomes unravels the regenerative effect of mesenchymal stem cells. Stem Cells Dev 22:772–780
63. Fonsato V, Collino F, Herrera MB, Cavallari C, Deregibus MC, Cisterna B et al (2012) Human liver stem cell-derived microvesicles inhibit hepatoma growth in SCID mice by delivering antitumor microRNAs. Stem Cells 30:1985–1998
64. Bruno S, Collino F, Deregibus MC, Grange C, Tetta C, Camussi G (2013) Microvesicles derived from human bone marrow mesenchymal stem cells inhibit tumor growth. Stem Cells Dev 22:758–771
65. Li T, Yan Y, Wang B, Qian H, Zhang X, Shen L et al (2013) Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. Stem Cells Dev 22:845–854
66. Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS et al (2010) Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. Stem Cell Res 4:214–222
67. Lai RC, Arslan F, Tan SS, Tan B, Choo A, Lee MM et al (2010) Derivation and characterization of human fetal MSCs: an alternative cell source for large-scale production of cardioprotective microparticles. J Mol Cell Cardiol 48:1215–1224
68. Chen L, Wang Y, Pan Y, Zhang L, Shen C, Qin G et al (2013) Cardiac progenitor-derived exosomes protect ischemic myocardium from acute ischemia/reperfusion injury. Biochem Biophys Res Commun 431:566–571
69. Iglesias DM, El-Kares R, Taranta A, Bellomo F, Emma F, Besouw M et al (2012) Stem cell microvesicles transfer cystinosin to human cystinotic cells and reduce cystine accumulation in vitro. PLoS ONE 7:e42840
70. Kooijmans SA, Vader P, van Dommelen SM, van Solinge WW, Schiffelers RM (2012) Exosome mimetics: a novel class of drug delivery systems. Int J Nanomed 7:1525–1541
71. Shen B, Wu N, Yang JM, Gould SJ (2011) Protein targeting to exosomes/microvesicles by plasma membrane anchors. J Biol Chem 286:14383–14395
72. El Andaloussi S, Lakhal S, Mäger I, Wood MJ (2013) Exosomes for targeted siRNA delivery across biological barriers. Adv Drug Deliv Rev 65:391–397
73. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ (2011) Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol 29:341–345
74. Akao Y, Iio A, Itoh T, Noguchi S, Itoh Y, Ohtsuki Y et al (2011) Microvesicle-mediated RNA molecule delivery system using monocytes/macrophages. Mol Ther 19:395–399
75. van den Boorn JG, Schlee M, Coch C, Hartmann G (2011) SiRNA delivery with exosomes nanoparticles. Nat Biotechnol 29:325–326
76. Chen TS, Arslan F, Yin Y, Tan SS, Lai RC, Choo AB et al (2011) Enabling a robust scalable manufacturing process for therapeutic exosomes through oncogenic immortalization of human ESC-derived MSCs. J Transl Med 9:47

Chapter 14
Extracellular Vesicles and Tissue Organ Regeneration

P. Quesenberry, J. Aliotta, M. Dooner, D. Chatterjee, B. Ramratnam, L. Goldberg, M. Del Tatto, M. Pereira, A. Sorokina and S. Wen

14.1 Introduction

Marrow hematopoietic stem cells appeared to be capable of transforming into nonhematopoietic tissues. These phenomena were termed stem cell plasticity, an area that generated a great deal of unnecessary and destructive controversy [1]. We had demonstrated that engraftment of green fluorescent protein (GFP) expressing marrow cells into lethally irradiated mice resulted in the appearance of GFP + pulmonary epithelial cells in the lungs of the transplanted mice [2]. Subsequent studies showed that when lung tissue was cultured opposite murine marrow cells, the marrow cells expressed the pulmonary-specific messenger ribonucleic acids (mRNAs) surfactants A–D, aquaporin-5 and Clara cell-specific

P. Quesenberry (✉) · J. Aliotta · M. Dooner · D. Chatterjee · B. Ramratnam · L. Goldberg · M. D. Tatto · M. Pereira · A. Sorokina · S. Wen
Department of Medicine, Division of Hematology/Oncology, Rhode Island Hospital and Brown University, Providence, RI 02903, USA
e-mail: pquesenberry@lifespan.org

J. Aliotta
e-mail: jaliotta@lifespan.org

D. Chatterjee
e-mail: Devasis_chatterjee@brown.edu

B. Ramratnam
e-mail: bramratnam@lifespan.org

L. Goldberg
e-mail: lgoldberg@lifespan.org

M. D. Tatto
e-mail: mdeltatto@lifespan.org

A. Sorokina
e-mail: asorokina@lifespan.org

S. Wen
e-mail: swen@lifespan.org

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,*
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_14,

Table 14.1 Results of organ cocultures of murine liver, heart, and brain across from murine marrow cells

Originator tissue	mRNA expressed in target marrow tissue
Lung	Surfactants A-D, aquaporin-5, clara-cell-specific protein
Liver	Albumin, serum amyloid A1
Brain	Glial fibrillary acidic protein, Beta 3 tubulin, neurofilament heavy chain
Heart	Troponin 1, troponin 2, myosin light chain 2

protein [3]. If the lungs were from mice exposed to 500 or 1000 cGy 5 days before harvest, the expression levels were significantly higher. Conditioned media from the lungs reproduced these mRNA inducing effects and it was then demonstrated that the active inducer of lung-specific mRNA in marrow cells was isolated by differential centrifugation (300 g, 10,000 g, and 100,000 g). In the pellet after the 100,000 g spin were extracellular vesicles, which turned out to be the active principle in the observed genetic cell fate change. We demonstrated that surfactant protein was expressed in marrow cells and that marrow cells which expressed lung-specific mRNA showed an increased efficiency in forming pulmonary epithelial cells after infusion into lethally irradiated mice. Using Carboxyfluorescein succinimidyl ester (CFSE) (green fluorescence) and PKH26 (red fluorescence) labeled vesicles and separating marrow cells which were fluorescent, i.e., had taken up the vesicles, from those which were not, we established that uptake of the vesicles was necessary for the genetic changes to occur. The vesicles were found to be replete with mRNA, protein, microRNA (miRNA), and noncoding long RNA. There was also some deoxyribonucleic acid (DNA) present. Adhesion proteins were expressed on their surface. Organ cocultures of murine liver, heart, and brain across from murine marrow cells, but separated from them by a 0.4 μ membrane, showed tissue-specific expression of mRNA. These results are summarized in Table 14.1.

In general, the marrow target cells only expressed the tissue-specific mRNA from the originator tissue.

We have had variable results with RNase incubations of vesicles, but most recently have shown that the induction of lung-specific mRNA in murine marrow by vesicles from irradiated lungs was suppressed by exposure to RNase and pronase. In further studies, we incubated rat lung opposite mouse marrow and then assessed the mouse marrow cells for expression of surfactants B and C [4]. We utilized rat and mouse species-specific primers for surfactants B and C and were able to determine that immediately after coculture the induced mRNA for both surfactants were both rat (transferred mRNA from rat lung) and mouse (induced mRNA from mouse marrow). However, when these marrow cells were established in cytokine supported culture, the rat mRNA rapidly disappeared while the mouse surfactant mRNA persisted out to 12 weeks of culture [5]. Thus, an epigenetic change

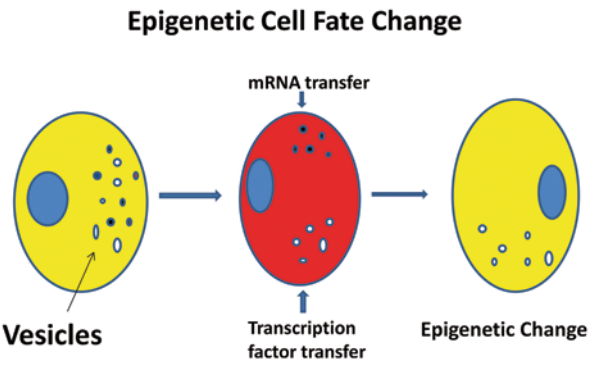

Fig. 14.1 Vesicle-induced cell fate change. Vesicles leave originator cell, enter target cell (*red*), and lead to long-term epigenetic change

was induced which was stable over time and presumably mediated by non-coding RNAs. These phenotypic effects are illustrated in Fig. 14.1.

14.1.1 Vesicle Induction or Repair of Tissue Damage

Professor Camussi has summarized their elegant work on restoration of damaged renal tissue in Chap. 13 in this volume and I will not repeat this [6].

We have focused on irradiated or monocrotaline-treated lung and marrow tissue. We showed earlier on that infusion of GFP + marrow cells into lethally or sublethally irradiated mice resulted in the formation of GFP + pulmonary epithelial cells. More recently, we have investigated the impact of vesicles on murine pulmonary hypertension. Monocrotaline administration to mice results in pulmonary hypertension and infusion of normal marrow cells could reverse this phenomena [7]. We have further shown that infusion of vesicles from monocrotaline-treated mice induced pulmonary hypertension in normal mice [8]. Perhaps, of more significance was work indicating that infusion of mesenchymal-stem-cell-derived vesicles into mice with monocrotaline-induced pulmonary hypertension could reverse the pulmonary hypertension [9].

In a similar vein, we have evaluated the capacity of mesenchymal-stem-cell-derived vesicles to reverse marrow stem cell irradiation toxicity. Exposure of irradiated murine marrow (100 or 500 cGy) to either human or murine mesenchymal-stem-cell-derived vesicles resulted in a partial reversal of radiation toxicity in in vitro culture systems and in vivo.

Additional work on the impact of Tylenol on liver vesicles has indicated that Tylenol injury induces increased vesicle release.

14.1.2 Vesicles and Cancer

Vesicles can affect the phenotype of cancer cells and normal cells depending upon the originator source. When human prostate or lung cancer cells were cocultured across from human marrow cells, the human marrow cells variably expressed prostate or lung mRNA [10, 11]. This was due to cancer vesicle interactions with the marrow cells. In cell line experiments, malignant-prostate- or breast-cancer-derived vesicles induced chemo resistance and anchorage independent growth, neoplastic characteristics in the normal cell line cells. Conversely, if normal breast or prostate cell vesicles were interacted with malignant cells, there was a reversal of anchorage independent growth and chemo resistance [12].

The importance of the injury state of an originator tissue was demonstrated in studies showing that the genetic alteration of murine marrow cells varied with the cell cycle status of the marrow cells and whether the originator lung was subjected to irradiation or not [13]. In these studies, murine Lineage depleted, Sca-1 + (Lin-/Sca-1 +) marrow were cultured with interleukin 3 (IL-3), IL-6, IL-11, and stem cell factor (cytokine-cultured cells), removed at 0 h (cell cycle phase G0/G1), 24 (late G1/early S), and 48 (late S/early G2/M), and cocultured with lung tissue, lung conditioned media (LCM), or lung-derived vesicles from mice exposed to irradiation or not exposed. Alternatively, Lin-/Sca-1+ cells were separated into G0/G1 and S/G2/M cell cycle phase populations by fluorescence-activated cell sorting (FACS) and used in coculture. Separately, lung-derived vesicles from irradiated and nonirradiated mice were analyzed for the presence of adhesion proteins. Peak pulmonary epithelial cell-specific mRNA expression was seen in G0/G1 cytokine-cultured cells cocultured with irradiated lung and in late G1/early S cells cocultured with nonirradiated lung. A similar pattern was seen in cytokine-cultured Lin-/Sca-1 cells cocultured with LCM and or lung-derived vesicles, and when FACS-separated Lin-/Sca-1 cells were used in coculture. Cells and lung-derived vesicles expressed adhesion proteins which differed with irradiation exposure and cell cycle phase. This indicated a mechanism for possible mechanism for vesicle entry. These data demonstrate that microvesicle modification of progenitor/stem cells is influenced by cell cycle and the treatment of the originator lung tissue.

The concept of different vesicle effects depending on the nature of the originator tissue is presented in Fig. 14.2.

The stability of the functional effects of lung-derived vesicles was found to be preserved at 4 or −20 °C with 1 % DMSO.

14.1.3 Translational Potential of Vesicles

The above suggests that extracellular vesicles might have significant therapeutic potential in cancer or various tissue injuries. Reversal of irradiation injury to marrow stem cells by mesenchymal-stem-cell-derived vesicles could have a prominent role in therapeutics of bioterrorism victims or in the setting of chemoradiotherapy

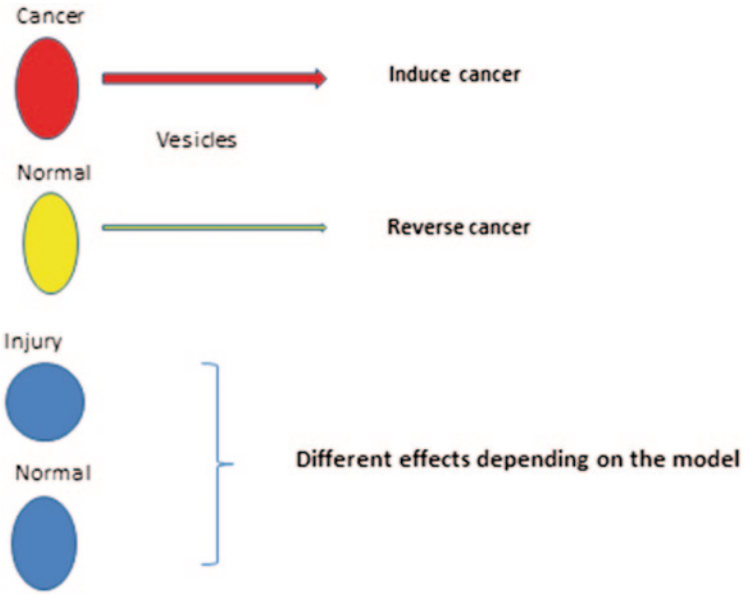

Fig. 14.2 Vesicles effects on cell fate determined by nature of originator cell

while various renal injuries could be approached in a similar fashion. Vesicles could be characterized for their effects and the stored for use in various therapeutic settings. Phase 1 trials are envisioned in the near future.

References

1. Quesenberry PJ, Dooner G, Dooner M, Abedi M (2005) Developmental biology: ignoratio elenchi: red herrings in stem cell research. Science 308(5725):1121–1122
2. Aliotta JM, Keaney P, Passero M, Dooner MS, Pimentel J, Greer D, Demers D, Foster B, Peterson A, Dooner G, Theise ND, Abedi M, Colvin GA, Quesenberry PJ (2006) Bone marrow production of lung cells: the impact of G-CSF, cardiotoxin, graded doses of irradiation, and subpopulation phenotype. Exp Hematol 34(2):230–241
3. Aliotta JM, Sanchez-Guijo FM, Dooner GJ, Johnson KW, Dooner MS, Greer KA et al (2007 Sep) Alteration of marrow cell gene expression, protein production, and engraftment into lung by lung-derived microvesicles: a novel mechanism for phenotype modulation. Stem Cells 25(9):2245–2256
4. Aliotta JM, Pereira M, Johnson KW, de Paz N, Dooner MS, Puente N et al (2010 Mar) Microvesicle entry into marrow cells mediates tissue-specific changes in mRNA by direct delivery of mRNA and induction of transcription. Exp Hematol 38(3):233–245
5. Aliotta J, Pereira M, Li M, Amaral A, Sorokina A, Dooner M, Sears E, Brilliant K, Ramratnam B, Hixson D, Quesenberry P (2012) Stable Cell Fate Changes in Marrow Cells induced by Lung derived microvesicles. J Extracell Vesicles. doi: 10.3402/jev.v1i0.18163

6. Biancone L, Bruno S, Deregibus MC, Tetta C, Camussi G (2012) Therapeutic potential of mesenchymal stem cell-derived microvesicles. Nephrol Dial Transplant 27(8):3037–3042. doi: 10.1093/ndt/gfs168
7. Aliotta JM, Keaney PJ, Warburton RR, Del Tatto M, Dooner MS, Passero MA, Quesenberry PJ, Klinger JR (2009) Marrow cell infusion attenuates vascular remodeling in a murine model of monocrotaline-induced pulmonary hypertension. Stem Cells Dev 18(5):773–782. doi: 10.1089/scd.2008.0237
8. Aliotta JM[1], Pereira M, Amaral A, Sorokina A, Igbinoba Z, Hasslinger A, El-Bizri R, Rounds SI, Quesenberry PJ, Klinger JR (2013) Induction of pulmonary hypertensive changes by extracellular vesicles from monocrotaline-treated mice. Cardiovasc Res Dec 1;100(3):354-362. doi: 10.1093/cvr/cvt184. Epub 2013 Jul 18
9. Aliotta JM, Pereira M, Quesenberry PJ, Klinger JR (2014) Extracellular vesicles from mesenchymal stem cells prevent monocrotaline-induced right ventricular hypertrophy and pulmonary vascular remodeling in mice. Abstract presented at the American Thoracic Society Conference. May, 2014. San Diego, CA
10. Renzulli JF 2nd, Del Tatto M, Dooner G, Aliotta J, Goldstein L, Dooner M, Colvin G, Chatterjee D, Quesenberry P (2010) Microvesicle induction of prostate specific gene expression in normal human bone marrow cells. J Urol 184(5):2165–2171. doi: 10.1016/j.juro.2010.06.119. Epub 2010 Sep 18
11. Del Tatto M, Ng T, Aliotta JM, Colvin GA, Dooner MS, Berz D, Dooner GJ, Papa EF, Hixson DC, Ramratnam B, Aswad BI, Sears EH, Reagan J, Quesenberry PJ (2011) Marrow cell genetic phenotype change induced by human lung cancer cells. Exp Hematol 39(11):1072–1080. doi: 10.1016/j.exphem.2011.08.008. Epub 2011 Aug 22
12. Panagopoulos K, Cross-Knorr S, Dillard C, Pantazatos D, Del Tatto M, Mills D, Goldstein L, Renzulli J, Quesenberry P, Chatterjee D (2013) Reversal of chemosensitivity and induction of cell malignancy of a non-malignant prostate cancer cell line upon extracellular vesicle exposure. Mol Cancer Oct 8;12(1):118
13. Aliotta JM, Lee D, Puente N, Faradyan S, Sears EH, Amaral A, Goldberg L, Dooner MS, Pereira M, Quesenberry PJ (2012) Progenitor/stem cell fate determination: interactive dynamics of cell cycle and microvesicles. Stem Cells Dev 21(10):1627–1638. doi: 10.1089/scd.2011.0550. Epub 2012 Feb 15

Chapter 15
Stem Cells in Infection and Sepsis

Tomasz Skirecki, Grażyna Hoser and Jerzy Kawiak

15.1 The Continuous Challenge of Sepsis for Clinicians and Researchers: A New Niche for Stem Cell Research

Sepsis, severe sepsis, and septic shock are the main noncardiological causes of death in intensive care units with mortality reaching 76 % depending on the country [30]. The core pathophysiologic mechanism in the development of sepsis is the hyperactivation of the host systemic inflammatory response and coagulation evoked by overwhelming infection. These inflammatory and procoagulant mediators contribute to endothelial damage, hypotension, microvascular thrombosis, and subsequent organ ischemia and failure. Inflammatory cascades in the lung microvessels lead to respiratory failure. Similarly, renal and liver failures develop often. However, exaggerated activation of immune response is accompanied by progressing reprogramming of the immune response which leads to the state of "immunoparalysis." Nowadays, most deaths due to sepsis occur in this later phase of immunoparalysis. This clinical state is characterized by secondary and opportunistic infections, reactivation of latent viruses and anergy [25]. In spite of a few decades of the intensive research in the field of sepsis, no specific treatment regimens have successfully passed clinical trial. So far, the only effective rules of the management of sepsis are captured in the Surviving Sepsis Guidelines, which include among others: early administration of antibiotics, hemodynamic management, prophylaxis of gastric ulcers, venous thrombosis, and protective mechanical ventilation [15]. Not only is sepsis a complicated syndrome itself but also it is often secondary to other severe conditions like trauma, burns, or major surgeries. Altogether, these factors contribute to the extremely complex pathophysiology of sepsis and explain why the development of new therapies remains challenging. Tissue injury and multiorgan failure which occurs during the septic shock is one reason explaining why the use of

J. Kawiak (✉) · T. Skirecki · G. Hoser
Laboratory of Flow Cytometry, The Centre of Medical Postgraduate Education, Marymoncka 99/103, 01-813 Warsaw, Poland
e-mail: jkawiak@ibib.waw.pl

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,* Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4_15,

stem cells can be a tempting approach in the management of sepsis-induced tissue damages. The growing body of evidence suggests that different types of stem cells, i.e., hematopoietic, endothelial, and mesenchymal, express receptors for pathogens associated patterns (toll-like receptors)and pro- and anti-inflammatory cytokines and can interact with cells of the immune system via paracrine mechanisms. In the light of the abovementioned capacities supported by the encouraging results of animal trials, sepsis can be the next target for stem cell therapies.

15.2 Hematopoetic Stem Cells

The immune response to infection is a process that consumes immune cells by trapping in the site of infection and inducing their death. A further consequence of a systemic infection, such as sepsis is the reprogramming of the immune cells and some of the features of this phenomenon include the activation of pro-apoptotic and pro-anergic pathways resulting in the loss of the cells of adaptive immunity [59]. Homeostasis of the blood cells can be maintained by the activation of the hematopoietic progenitor cells and hematopoietic stem cells (HSCs) residing in their bone marrow niches. The rapid response of bone marrow residing precursor cells is often observed at the bedside as a high frequency of immature myeloid cells in the blood smears. Such hematological response to acute severe infection is often called an emergency hematopoiesis [65]. Previously, this process was ascribed only to the progenitor cells activated by inflammatory cytokines. Nowadays, it becomes clear that also primitive HSCs are awaken from the dormancy by (a) indirect activation by inflammatory cytokines, (b) direct ligation of pathogen associated molecular patterns (PAMPs), and (c) signals from cells creating the bone marrow niche. Furthermore, HSCs are mobilized from their niches and participate in the immune response.

Early HSCs in contrast to more mature progenitor cells do not express receptors for lineage-specific cytokines, but express receptors for major inflammatory cytokines: tumor necrosis factor α (TNFα) and interferons (IFNs, both type I α/β and type II γ). These cytokines play crucial role in the early phase of sepsis and their systemic generation mediates many detrimental events in the development of organ failure in septic shock [23, 36]. The effects of stimulation of HSCs by IFNs are not straightforward. IFNγ was primarily found to be an inhibitor of hematopoiesis as it was shown to reduce colony formation by human CD34 + cells and induce apoptosis in these cells [55, 56]. However, other studies revealed that IFNγ signaling can activate and promote survival and colony forming by the human HSCs in the in vitro studies [10]. Moreover, in the model of *Mycobacterium avium* murine infection the long-term repopulating HSCs were extensively proliferating which was an IFNγ-mediated effect [5]. Stimulation of mice with recombinant IFNγ evoked similar effect and the expanded HSCs population was functional. Interestingly, baseline IFNγ signaling also has a role in the maintenance of HSCs proliferation [5]. Even more relevant model of acute infection by *Erlichia muris* confirms the activatory effects of IFNγ on the proliferation of HSCs, although HSCs, expanded during this

infection, showed reduced engraftment capabilities [35]. IFNα is also a potent direct activator of the proliferation of quiescent HSCs. This effect is mediated via phosphorylation of signal transducer and activator of transcription 1(STAT1) and AKT1 signaling. Interestingly, even the interferon-α/β receptor (IFNAR)-/- HSCs are able to proliferate in response to IFNα if they are surrounded by sufficient number of wild-type cells which suggests more complex role of this cytokine. Of importance, IFNα-treated HSCs in an acute manner do not show impairment in the competitive transplantation assays [19]. Another sepsis-related cytokine that has its receptors (p55: TNFRSF1A and p75: TNFRSF1B) on the HSCs is TNFα. Similar to IFNs, the effects of TNFα on HSCs are complex and vary in different experimental settings. This cytokine was found to comprise the repopulating function of human CD34+CD38 HSCs [18] and murine Lin-Sca1+c-kit+cells [8]. The inhibition of HSCs was mediated by both receptors and additionally by the inducible expression of Fas receptor. On the contrary, TNFα stimulation of umbilical cord blood and peripheral blood HSCs mediated via the TNFRSF1A receptor had protective effect on these cells by decreasing apoptosis rates, improving the engraftment in NOD. SCID mice and stimulation of myeloid progenitors expansion [41]. The positive role of TNFα stimulation on the function of HSCs was also showed by utilization of Tnfrsf1a-/- mice which have increased number of hematopoietic progenitors, but their functionality is decreased [49]. In summary, these pleiotropic effects of these cytokines of HSCs function suggest their participation in the trafficking of the emergency hematopoiesis. The correlation between decreased production of TNFα and IFNs in the later phase of sepsis can contribute to the failure of effective fight with infection by insufficient stimulation of HSCs.

Direct sensing of pathogens by cells is possible by the recognition of PAMPs by toll-like receptors (TLR), a group of pattern recognition receptors. This family includes 13 types of receptors in mouse and human recognizing for viral, bacterial and fungal antigens as well as endogenous damage-associated molecular patterns released by injured cells [1]. Thus, TLR signaling takes central place in the pathophysiology of sepsis and mediates production of inflammatory mediators [52]. Surprisingly, TLRs have been found on murine HSCs (TLR2, 4, 9) and on human CD34+cells (TLR1, 2, 4, 7, 8) [42, 58]. Ligation of TLR4 complex with adaptor proteins (CD14, Myd88) by lipopolysaccharide (LPS) on murine HSCs drives differentiation of myeloid progenitors and dendritic cells while it inhibits B cell development. Concordantly, TLR1/2 activation directly stimulates human HSCs to differentiation into myeloid cells [14]. Activation of TLR7/8 pathways in human CD34 + cells leads to their differentiation into dendritic cells [57]. However, it was also reported that LPS stimulated murine HSCs were more effective in the transplantation assay than proliferating control HSCs and were not lineage-biased [66]. As a low number of HSCs steadily circulate between bone marrow, blood, and lymphoid tissues, the presence of TLRs enables their direct response to infection and boosting of local immunity by giving rise to myeloid cells. Moreover, systemic inflammation has an impact on the mobilization of HSCs [29]. The plasma concentration of sphingosine-1 phosphate (S1P, a major plasma chemoattractant for HSCs) may be rapidly raised after activation of complement cascade what occurs in the course of sepsis. Formation of the membrane-attack complex leads to the

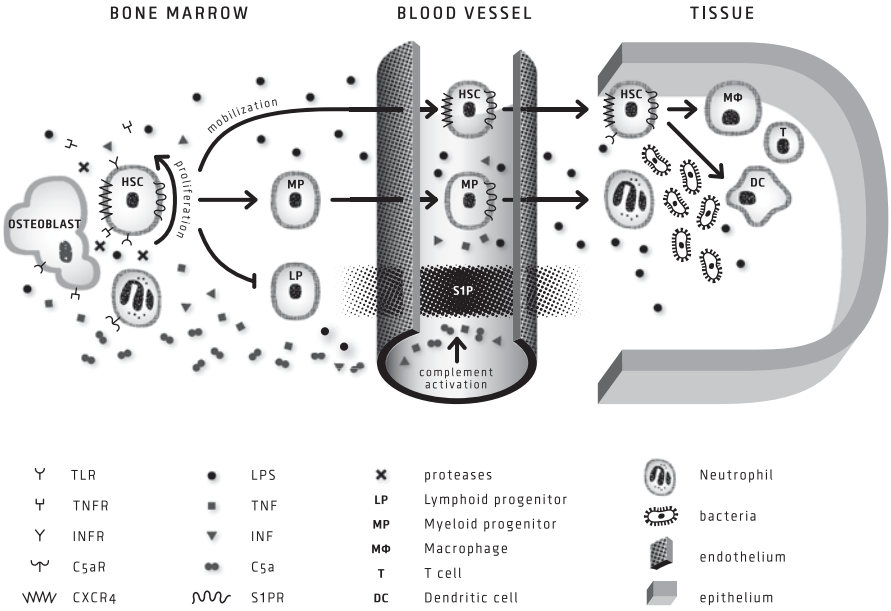

Fig. 15.1 The hematopoietic stem cells (*HSC*) reside in bone marrow niche composed of cells like mesenchymal stromal cells and osteoblast and the SDF-1–CXCR4 axis is one of the major mechanisms of their retention. Direct effects of tumor necrosis factor (*TNF*), interferons (*IFNs*), and pathogen associated molecular patterns (*PAMPs*) include proliferation of quiescent *HSCs* and their differentiation towards myeloid progenitors. During infection, activated complement proteins (C5a) bind to their receptors on the bone-marrow neutrophils which triggers release of proteases cleaving the stromal cell-derived factor 1 (*SDF-1*) axis. Also, ligation of toll-like receptors (*TLRs*) by osteoblasts downregulates their expression of *SDF-1*. Systemic activation of complement cascade leads to the production of sphingosine-1 phosphate (*S1P*) in the blood which is a strong plasma chemoattractant for *HSCs*. *HSCs* and myeloid progenitors are mobilized to the blood and then localize in the infectious foci due to the gradient of *SDF-1*. At the site of infection, *PAMPs* downregulate *S1P* receptors and enhances responsiveness of *HSCs* to *SDF-1*, altogether promoting capture of these cells in the inflamed tissue where they boost the immune response by differentiation toward monocytes or dendritic cells and releasing cytokines and growth factors

release of S1P from erythrocytes and establishment of dynamic gradient of this molecule mobilizing HSCs from bone marrow [48]. Stimulation of TLR4 downregulates the S1P receptor while it also increases responsiveness for stromal cell-derived factor 1 (SDF-1). This interplay promotes retention of HSCs in injured and inflamed tissues where they can participate in immunity and regeneration by differentiation and paracrine effects [37, 39] (Fig. 15.1). Such conception is supported by the observation that during human sepsis the number of circulating early HSCs is decreased [61].

Considering the emergency hematopoiesis raises a question whether effector cells produced under such conditions are fully functional. Experiments with pretreatment of human HSCs with TLR2 agonist prior to their macrophage differentiation show that such macrophages are more tolerant to the stimulation of TLR2 (reduced production of TNFα, interleukin 6 (IL6), IL1, and reactive oxygen species

(ROS). This phenomenon may partially explain the depressed immune response in the later phase of sepsis [59]. The more clinically relevant studies applying models of sepsis like injection of the heat-killed *E. coli* [70], *P. aeruginosa* [50] or polymicrobial sepsis (cecal ligation and puncture) [53] all showed expansion of the HSCs compartment. However, some differences are observed between the models. For instance, in polymicrobial sepsis both long-term and short-term HSCs expanded while in *P. aeruginosa* sepsis only long-term HSCs expanded, but the cells were not functional. Although TLR4 signaling was necessary for the effects of purified LPS on HSCs, the bacterial infections stimulated HSCs even in the absence of MyD88and TRIF molecules which abrogates TLR signaling. The egress of immature myeloid cell from the bone marrow sinusoids stimulates proliferation of HSCs [53]. The discrepancy between various models of infection reflects alterations in the patient's immune response in sepsis relying on the type of primary infection [24]. Altogether, it can be concluded that systemic infection has a direct impact on the proliferation and maturation of the HSCs. This impact can depend on the etiology of the infection and is shaped by an interplay between the milieu of HSCs niche and signals from a direct recognition of PAMPs. In the light of the inefficiency of immune system occurring during sepsis, the HSCs may become a new target for therapies improving the patient's defense capacity.

15.3 Endothelial Progenitor Cells

The endothelium is one of the major targets in the development of septic shock. Endothelial cells express TLRs by which they directly sense pathogens and express receptors for inflammatory cytokines. Upon septic activation, the cells undergo structural (swallowing, detachment) and functional changes (procoagulant state, adhesion, production of inflammatory mediators, loss of vasomotor tone) and finally they may undergo injury and apoptosis [3, 46].

Endothelial progenitor cells (EPCs) originating from the bone marrow are of interest in the sepsis research, as they are responsible for the neovascularization after ischemia and the repair of injured vascular endothelium in a process called re-endothelialization [4]. It is important to bear in mind that the term EPCs includes heterogeneous population of precursor cells including at least: CD34+CD133+VEGFR-2+hemangioblast-derived cells, CD34-CD133+VEGFR-2+cells derived from bone marrow multipotent adult stem cells and CD14+CD45+VEGFR-2+monocytic precursors [27]. EPCs are mobilized during vascular injury or tissue hypoxia and localize at the site of the damaged vasculature, in a processes partially mediated by the vascular endothelial growth factor (VEGF) [22]. VEGF and SDF-1 are among target gene for the Hypoxia Inducible Factor-1 (HIF-1) and the level of expression of this factor was recently found to be increased in the human shock states, but the exact role of this finding is yet to be investigated [67]. Although EPCs incorporate into damaged endothelium, their significance is likely due to the release of paracrine proangiogenic factors [26]. The number of circulating EPCs in septic shock patients is increased comparing to healthy control

and other critically ill patients, and this is accompanied by the higher serum concentration of pro-angiogenic factors. However, the proliferative capacity of septic EPCs is diminished and inversely correlates with the disease severity and mortality [13, 45]. These clinical observations are supported by some experimental data showing that endotoxemia injures EPCs by reducing their clonogenic potential [21]. The impairment of EPCs functionality is widely described in the cardiovascular diseases and the cardiological risk factors (hypertension, diabetes, smoking, homocysteine levels, aging) are known to affect these cells. Growing body of evidence links the oxidative stress evoked under such circumstances with the depressed EPCs functions [9, 32]. Furthermore, some inflammatory mediators have a direct harmful effect on the EPCs function, e.g., C-reactive protein and TNFα [54, 63]. It is of interest that hyperglycemia, a situation often present in septic shock patients, also reduces the potential of EPC in the bone marrow [34]. However, IL-1β, which is generally a pro-inflammatory cytokine, may via activation of phosphatidylinositol-3-kinase (PI3K)-Akt stimulate proliferation and migration of EPCs [69]. The potential role of EPCs in the regeneration of damaged vasculature was studied by transplantation of autologous EPCs in the endotoxin-induced organ injury and this procedure was found to ameliorate the lung injury, decrease cytokine concentrations, and improve outcomes [20, 38]. The effects of EPCs are related with their migration to injured tissue and downregulation of endothelin and iNOS. Protective action of EPCs was shown to be enhanced by combining their transplantation with statins [31]. Even though the results of the abovementioned studies are encouraging, all of them were performed with EPCs from healthy animal, obtained either in autologous manner before the injury or from healthy allogeneic donors. Such conditions are not likely to be applicable in clinical settings and thus the stimulation and/or application of the patient's own EPCs (altered by the septic state) should be studied. Current knowledge on the complex alterations of EPCs in sepsis is not satisfying as the persistent injury of microvasculature in sepsis plays a pivotal role in the progress of shock leading to death.

15.4 Mesenchymal Stromal Cells

Due to their immunoregulatory properties (summarized in Fig. 15.2), mesenchymal stromal cells (MSCs) have been extensively investigated in the context of potential application in sepsis therapy. Originally isolated from bone marrow, MSCs are found in most tissues as they are derived from pericytes [12]. This widespread localization of MSCs combined with their unique capabilities sheds new lights on their role in the pathophysiology of inflammation and particularly sepsis.

Original observations concerning the immunomodulatory capacity of MSCs came from studies in which MSCs inhibited T cells activation and proliferation following introduction of alloantigenes [16]. Since these observations, numerous studies have been performed on the influence of MSCs on the adaptive and innate immune system (Fig. 15.1). MSCs can suppress the activity of CD8 lymphocytes by inhibiting their proliferation and increase the relative proportion of CD4 regulatory lymphocytes [2]. Also, B cells function and proliferation may be supported by

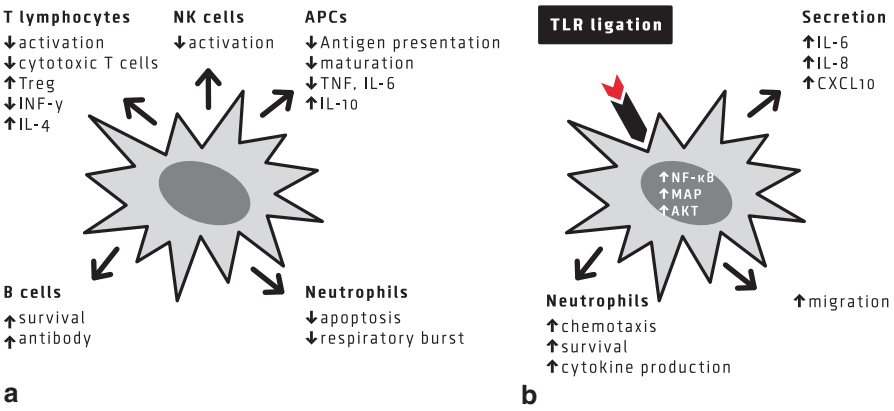

Fig. 15.2 The pleiotropic interaction between mesenchymal stem cells (*MSCs*) and the immune system. **a** Unstimulated *MSCs*. **b** Simplified response of the *MSCs* to ligation of *TLR*. *Treg* regulatory T cells, *NK* natural killer cells, *APCs* antigen presenting cells, *TLR* toll-like receptor

the direct interaction with MSCs [11]. Moreover, MSCs exert a suppressive effect on the NK cells [62]. All these effects are mediated by cell-contact dependent and independent mechanisms through the release of soluble factors. The list of potential mediators is long and includes TGF-β, PGE2, IDO, IL-10, and others. In the experimental model of sepsis, it was found that beneficial effect of exogenous MSCs is partially mediated by the prostaglandin E2-induced production of IL-10 by macrophages [43]. In the coculture experiments, cell contact between MSCs and macrophages was required to stimulate IL-10 production following endotoxin stimulation. Importantly, MSCs can prevent neutrophil apoptosis and degranulation in culture without inhibiting their phagocytic or chemotactic capacities [47]. When the expression of the TLRs was found on the surface of MSCs it became apparent that these cells play even more complex role in the in vivo immune response. Liotta et al. [33] found that ligation of TLR3 and TLR4 on bone marrow MSCs leads to the activation of NF-κB followed by the production of IL-6, IL-8, and chemokine CXCL10. Such activated MSCs show decreased ability to suppress proliferation of T cells. Further data show that TLR activation on MSCs can induce the migration and recruitment of the innate immune cells, as evaluated by human neutrophil chemotaxis assay and by the analysis of immune effectors retrieved from Matrigel-embedded MSCs injected into mice after in vitro preactivation with TLR ligands [51]. Similar observations were reported for LPS activated MSCs from parotid gland [6]. LPS-primed MSCs recruite neutrophils that show prolonged lifespan and increased expression of proinflammatory cytokines. Also, TLR3 and TLR4 ligation enhances immunosuppressive phenotype of the bone marrow MSCs [44]. This effect mediated by TLRs is dependent on the production of kynurenines by the tryptophan-degrading enzyme indoleamine-2,3-dioxygenase (IDO1) while the induction of IDO1 by TLR is mediated by the IFN-β autocrine signaling loop.

MSCs were also tested in the experimental therapies of sepsis and sepsis-induced acute lung injury (ALI). Intravenously or intratracheally administered MSCs

attenuate the sepsis-induced lung inflammation, injury and edema. The use of MSCs also decreases systemic inflammatory cytokines and increases survival of treated animals. MSCs also increase the number of CD4+CD25+FoxP3+regulatory T cells in the bronchoalveolar compartment which are able to ameliorate the inflammatory process [64]. MSCs localize in the inflamed lungs but neither integrate into them nor differentiate to lung cells [28, 68]. Administration of MSCs after the onset of murine sepsis triggered by cecal ligation and puncture (CLP) reduces mortality in numerous mechanisms. Injection of MSCs reduce serum concentration of cytokines (IL-6, IL-1b, IL-10, KC, JE, CCL5), diminish injury of kidneys and lungs, and reduce bacterial load by enhancement of phagocytes function. On the molecular level, the exogenous MSCs act by "normalizing" the expression pattern of inflammation-related genes. Genes related with the expression of IL-1, IL-6, IL-10, CD14 are downregulated while genes related with phagocytosis (like Fc receptors) are upregulated. However, the protective effects of MSCs in sepsis are mediated by the regulation of networks of gene sets modulating inflammatory and metabolic pathways [17, 40].

Conclusions and Therapeutic Perspectives

Although the investigations of the role of stem cells in the pathophysiology of sepsis and their potent therapeutic applications do not have a long record, there is an increasing body of evidence indicating the significance of these cells in sepsis.It should be highlighted that sepsis evokes extremely complex and overlapping responses which challenges the studies of the particular cell types.Direct effects of bacterial compounds, hypercytokinemia (also referred as cytokine storm), activation of complement cascade and coagulation systems, and release of damage-associated patterns from injured cells contribute to the septic milieu and change dynamically. Having appreciated the complexity of these interactions, one should carefully interpret the effects of single septic-related stimuli on the stem cells. The function of stem cells as guardians counteracting the deleterious effects of sepsis is unraveled, but the abovementioned evidence suggests such role for the hematopoietic, endothelial, and mesenchymal stem cells. Such role of endogenous stem cells may provide a new target of therapies enhancing their protective capacities. Moreover, the unique capacities of stem cells may become a basis for the transplantation of exogenous (either auto- or allogenic) cells or their derivatives (such as microvesicles). The encouraging trails with MSCs performed in animal models are likely to be soon transmitted to the clinical settings. First positive trials with exogenous EPCs in the endotoxemic mouse are also reported [21]. Adoptive transfer of murine CD34+hematopoietic progenitor cells decreases mortality and improves late immune response by generation of competent macrophages and rebalancing the immune response [7]. Stimulation and mobilization of HSCs and EPCs by G-CSF in septic shock patients is also possible [60]. However, the introduction of successful stem-cell-based therapies in sepsis requires careful and profound understanding of their mechanisms of action under inflammatory conditions.

Acknowledgements The authors would like to thank Zofia Herbich for her help in preparation of the figures. Supported by EU Structural Funds, "Innovative methods of stem cells applications in medicine," Innovative Economy Operational Programme, POIG 01.02−00-109/09.

References

1. Aderem A, Ulevitch RJ (2000) Toll-like receptors in the induction of the innate immune response. Nature 406(6797):782–787. doi:10.1038/35021228
2. Aggarwal S, Pittenger MF (2005) Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105(4):1815–1822. doi:2004−04-1559 [pii]
3. Aird WC (2003) The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. Blood 101(10):3765–3777. doi:10.1182/blood-2002−06–1887
4. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM (1999) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 85(3):221–228
5. Baldridge MT, King KY, Boles NC, Weksberg DC, Goodell MA (2010) Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. Nature 465(7299):793–797. doi:10.1038/nature09135
6. Brandau S, Jakob M, Hemeda H, Bruderek K, Janeschik S, Bootz F, Lang S (2010) Tissue-resident mesenchymal stem cells attract peripheral blood neutrophils and enhance their inflammatory activity in response to microbial challenge. J Leukoc Biol 88(5):1005–1015. doi:10.1189/jlb.0410207
7. Brudecki L, Ferguson DA, Yin D, Lesage GD, McCall CE, El Gazzar M (2012) Hematopoietic stem-progenitor cells restore immunoreactivity and improve survival in late sepsis. Infect Immun 80(2):602–611. doi:10.1128/IAI.05480−11
8. Bryder D, Ramsfjell V, Dybedal I, Theilgaard-Monch K, Hogerkorp CM, Adolfsson J, Borge OJ, Jacobsen SE (2001) Self-renewal of multipotent long-term repopulating hematopoietic stem cells is negatively regulated by Fas and tumor necrosis factor receptor activation. J Exp Med 194(7):941–952
9. Case J, Ingram DA, Haneline LS (2008) Oxidative stress impairs endothelial progenitor cell function. Antioxid Redox Signal 10(11):1895–1907. doi:10.1089/ars.2008.2118
10. Caux C, Moreau I, Saeland S, Banchereau J (1992) Interferon-gamma enhances factor-dependent myeloid proliferation of human CD34+ hematopoietic progenitor cells. Blood 79(10):2628–2635
11. 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. doi:2005−07-2657 [pii]
12. Corselli M, Chen CW, Crisan M, Lazzari L, Peault B (2010) Perivascular ancestors of adult multipotent stem cells. Arterioscler Thromb Vasc Biol 30(6):1104–1109. doi:10.1161/ATVBAHA.109.191643
13. Cribbs SK, Sutcliffe DJ, Taylor WR, Rojas M, Easley KA, Tang L, Brigham KL, Martin GS (2012) Circulating endothelial progenitor cells inversely associate with organ dysfunction in sepsis. Intensive Care Med 38(3):429–436. doi:10.1007/s00134-012-2480-9
14. De Luca K, Frances-Duvert V, Asensio MJ, Ihsani R, Debien E, Taillardet M, Verhoeyen E, Bella C, Lantheaume S, Genestier L, Defrance T (2009) The TLR1/2 agonist PAM(3)CSK(4) instructs commitment of human hematopoietic stem cells to a myeloid cell fate. Leukemia 23(11):2063–2074. doi:10.1038/leu.2009.155
15. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, Sevransky JE, Sprung CL, Douglas IS, Jaeschke R, Osborn TM, Nunnally ME, Townsend SR, Reinhart K, Kleinpell RM, Angus DC, Deutschman CS, Machado FR, Rubenfeld GD, Webb S, Beale RJ, Vincent J-L, Moreno R (2013) Surviving sepsis campaign: international guidelines for management

of severe sepsis and septic shock, 2012. Intensive Care Med 39(2):165–228. doi:10.1007/s00134-012-2769-8
16. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM (2002) Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99(10):3838–3843
17. dos Santos CC, Murthy S, Hu P, Shan Y, Haitsma JJ, Mei SH, Stewart DJ, Liles WC (2012) Network analysis of transcriptional responses induced by mesenchymal stem cell treatment of experimental sepsis. Am J Pathol 181(5):1681–1692. doi:10.1016/j.ajpath.2012.08.009
18. Dybedal I, Bryder D, Fossum A, Rusten LS, Jacobsen SE (2001) Tumor necrosis factor (TNF)-mediated activation of the p55 TNF receptor negatively regulates maintenance of cycling reconstituting human hematopoietic stem cells. Blood 98(6):1782–1791
19. Essers MA, Offner S, Blanco-Bose WE, Waibler Z, Kalinke U, Duchosal MA, Trumpp A (2009) IFN-alpha activates dormant haematopoietic stem cells in vivo. Nature 458(7240):904–908. doi:10.1038/nature07815
20. Gao X, Chen W, Liang Z, Chen L (2011) Autotransplantation of circulating endothelial progenitor cells protects against lipopolysaccharide-induced acute lung injury in rabbit. Int Immunopharmacol 11 (10):1584–1590. doi:10.1016/j.intimp. 2011.05.019
21. Ghaly T, Rabadi MM, Weber M, Rabadi SM, Bank M, Grom JM, Fallon JT, Goligorsky MS, Ratliff BB (2011) Hydrogel-embedded endothelial progenitor cells evade LPS and mitigate endotoxemia. Am J Physiol Renal Physiol 301(4):F802–812. doi:10.1152/ajprenal.00124.2011
22. Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, Rafii S (2001) Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. Circ Res 88(2):167–174
23. Hatherill M, Tibby SM, Turner C, Ratnavel N, Murdoch IA (2000) Procalcitonin and cytokine levels: relationship to organ failure and mortality in pediatric septic shock. Crit Care Med 28(7):2591–2594
24. Hoser GA, Skirecki T, Zlotorowicz M, Zielinska-Borkowska U, Kawiak J (2012) Absolute counts of peripheral blood leukocyte subpopulations in intraabdominal sepsis and pneumonia-derived sepsis: a pilot study. Folia Histochem Cytobiol 50(3):420–426. doi:10.5603/19751
25. Hotchkiss RS, Monneret G, Payen D (2013) Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. Lancet Infect Dis 13 (3):260–268. doi:10.1016/S1473-3099(13)70001-X
26. Jujo K, Ii M, Losordo DW (2008) Endothelial progenitor cells in neovascularization of infarcted myocardium. J Mol Cell Cardiol 45(4):530–544. doi:10.1016/j.yjmcc.2008.08.003
27. Khakoo AY, Finkel T (2005) Endothelial progenitor cells. Annu Rev Med 56:79–101. doi:10.1146/annurev.med.56.090203.104149
28. Kim ES, Chang YS, Choi SJ, Kim JK, Yoo HS, Ahn SY, Sung DK, Kim SY, Park YR, Park WS (2011) Intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells attenuates Escherichia coli-induced acute lung injury in mice. Respir Res 12:108. doi:10.1186/1465-9921-12-108
29. Lee H, Ratajczak MZ (2009) Innate immunity: a key player in the mobilization of hematopoietic stem/progenitor cells. Arch Immunol Ther Exp (Warsz) 57(4):269–278. doi:10.1007/s00005-009-0037-6
30. Levy MM, Artigas A, Phillips GS, Rhodes A, Beale R, Osborn T, Vincent J-L, Townsend S, Lemeshow S, Dellinger RP (2012) Outcomes of the Surviving Sepsis Campaign in intensive care units in the USA and Europe: a prospective cohort study. The Lancet Infect Dis 12(12):919–924. doi:10.1016/s1473-3099(12)70239−6
31. Li H (2013) Repair of lipopolysaccharide-induced acute lung injury in mice by endothelial progenitor cells, alone and in combination with simvastatin. CHEST J 144(3):876. doi:10.1378/chest.12-2429
32. Lin CP, Lin FY, Huang PH, Chen YL, Chen WC, Chen HY, Huang YC, Liao WL, Huang HC, Liu PL, Chen YH (2013) Endothelial progenitor cell dysfunction in cardiovascular

diseases: role of reactive oxygen species and inflammation. Biomed Res Int 2013:845037. doi:10.1155/2013/845037
33. Liotta F, Angeli R, Cosmi L, Fili L, Manuelli C, Frosali F, Mazzinghi B, Maggi L, Pasini A, Lisi V, Santarlasci V, Consoloni L, Angelotti ML, Romagnani P, Parronchi P, Krampera M, Maggi E, Romagnani S, Annunziato F (2008) Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing Notch signaling. Stem Cells 26(1):279–289. doi:2007−0454 [pii]
34. Loomans CJ, van Haperen R, Duijs JM, Verseyden C, de Crom R, Leenen PJ, Drexhage HA, de Boer HC, de Koning EJ, Rabelink TJ, Staal FJ, van Zonneveld AJ (2009) Differentiation of bone marrow-derived endothelial progenitor cells is shifted into a proinflammatory phenotype by hyperglycemia. Mol Med 15(5–6):152–159. doi:10.2119/molmed.2009.00032
35. MacNamara KC, Jones M, Martin O, Winslow GM (2011) Transient activation of hematopoietic stem and progenitor cells by IFN-gamma during acute bacterial infection. PLoS One 6(12):e28669. doi:10.1371/journal.pone.0028669
36. Mahieu T, Libert C (2007) Should we inhibit type I interferons in sepsis? Infect Immun 75(1):22–29. doi:IAI.00829−06 [pii]
37. Majka M, Janowska-Wieczorek A, Ratajczak J, Ehrenman K, Pietrzkowski Z, Kowalska MA, Gewirtz AM, Emerson SG, Ratajczak MZ (2001) Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. Blood 97(10):3075–3085
38. Mao M, Wang SN, Lv XJ, Wang Y, Xu JC (2010) Intravenous delivery of bone marrow-derived endothelial progenitor cells improves survival and attenuates lipopolysaccharide-induced lung injury in rats. Shock 34(2):196–204. doi:10.1097/SHK.0b013e3181d49457
39. Massberg S, Schaerli P, Knezevic-Maramica I, Kollnberger M, Tubo N, Moseman EA, Huff IV, Junt T, Wagers AJ, Mazo IB, von Andrian UH (2007) Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. Cell 131(5):994–1008. doi:S0092-8674(07)01356−6 [pii]
40. Mei SH, Haitsma JJ, Dos Santos CC, Deng Y, Lai PF, Slutsky AS, Liles WC, Stewart DJ (2010) Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. Am J Respir Crit Care Med 182(8):1047–1057. doi:10.1164/rccm.201001−0010OC
41. Mizrahi K, Stein J, Yaniv I, Kaplan O, Askenasy N (2013) TNF-alpha has tropic rather than apoptotic activity in human hematopoietic progenitors: involvement of TNF receptor-1 and caspase-8. Stem Cells 31(1):156–166. doi:10.1002/stem.1259
42. Nagai Y, Garrett KP, Ohta S, Bahrun U, Kouro T, Akira S, Takatsu K, Kincade PW (2006) Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. Immunity 24(6):801–812. doi:S1074-7613(06)00266−4 [pii]
43. Nemeth K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, Hu X, Jelinek I, Star RA, Mezey E (2009) Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat Med 15(1):42–49. doi:10.1038/nm.1905
44. Opitz CA, Litzenburger UM, Lutz C, Lanz TV, Tritschler I, Koppel A, Tolosa E, Hoberg M, Anderl J, Aicher WK, Weller M, Wick W, Platten M (2009) Toll-like receptor engagement enhances the immunosuppressive properties of human bone marrow-derived mesenchymal stem cells by inducing indoleamine-2,3-dioxygenase-1 via interferon-beta and protein kinase R. Stem Cells 27(4):909–919. doi:10.1002/stem.7
45. Patschan SA, Patschan D, Temme J, Korsten P, Wessels JT, Koziolek M, Henze E, Muller GA (2011) Endothelial progenitor cells (EPC) in sepsis with acute renal dysfunction (ARD). Crit Care 15(2):R94. doi:10.1186/cc10100
46. Peters K, Unger RE, Brunner J, Kirkpatrick CJ (2003) Molecular basis of endothelial dysfunction in sepsis. Cardiovasc Res 60 (1):49–57. doi:S0008636303003973 [pii]

47. Raffaghello L, Bianchi G, Bertolotto M, Montecucco F, Busca A, Dallegri F, Ottonello L, Pistoia V (2008) Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. Stem Cells 26(1):151–162. doi:2007−0416 [pii]
48. Ratajczak MZ, Lee H, Wysoczynski M, Wan W, Marlicz W, Laughlin MJ, Kucia M, Janowska-Wieczorek A, Ratajczak J (2010) Novel insight into stem cell mobilization-plasma sphingosine-1-phosphate is a major chemoattractant that directs the egress of hematopoietic stem progenitor cells from the bone marrow and its level in peripheral blood increases during mobilization due to activation of complement cascade/membrane attack complex. Leukemia 24(5):976–985. doi:10.1038/leu.2010.53
49. Rebel VI, Hartnett S, Hill GR, Lazo-Kallanian SB, Ferrara JL, Sieff CA (1999) Essential role for the p55 tumor necrosis factor receptor in regulating hematopoiesis at a stem cell level. J Exp Med 190(10):1493–1504
50. Rodriguez S, Chora A, Goumnerov B, Mumaw C, Goebel WS, Fernandez L, Baydoun H, HogenEsch H, Dombkowski DM, Karlewicz CA, Rice S, Rahme LG, Carlesso N (2009) Dysfunctional expansion of hematopoietic stem cells and block of myeloid differentiation in lethal sepsis. Blood 114(19):4064–4076. doi:10.1182/blood-2009−04–214916
51. Romieu-Mourez R, Francois M, Boivin MN, Bouchentouf M, Spaner DE, Galipeau J (2009) Cytokine modulation of TLR expression and activation in mesenchymal stromal cells leads to a proinflammatory phenotype. J Immunol 182(12):7963–7973. doi:10.4049/jimmunol.0803864
52. Salomao R, Martins PS, Brunialti MK, Fernandes Mda L, Martos LS, Mendes ME, Gomes NE, Rigato O (2008) TLR signaling pathway in patients with sepsis. Shock 30(Suppl 1):73–77. doi:10.1097/SHK.0b013e318181af2a
53. Scumpia PO, Kelly-Scumpia KM, Delano MJ, Weinstein JS, Cuenca AG, Al-Quran S, Bovio I, Akira S, Kumagai Y, Moldawer LL (2010) Cutting edge: bacterial infection induces hematopoietic stem and progenitor cell expansion in the absence of TLR signaling. J Immunol 184(5):2247–2251. doi:10.4049/jimmunol.0903652
54. Seeger FH, Haendeler J, Walter DH, Rochwalsky U, Reinhold J, Urbich C, Rossig L, Corbaz A, Chvatchko Y, Zeiher AM, Dimmeler S (2005) p38 mitogen-activated protein kinase downregulates endothelial progenitor cells. Circulation 111(9):1184–1191. doi:111/9/1184 [pii]
55. Selleri C, Maciejewski JP, Sato T, Young NS (1996) Interferon-gamma constitutively expressed in the stromal microenvironment of human marrow cultures mediates potent hematopoietic inhibition. Blood 87(10):4149–4157
56. Selleri C, Sato T, Anderson S, Young NS, Maciejewski JP (1995) Interferon-gamma and tumor necrosis factor-alpha suppress both early and late stages of hematopoiesis and induce programmed cell death. J Cell Physiol 165(3):538–546. doi:10.1002/jcp. 1041650312
57. Sioud M, Floisand Y (2007) TLR agonists induce the differentiation of human bone marrow CD34+progenitors into CD11c+CD80/86+DC capable of inducing a Th1-type response. Eur J Immunol 37(10):2834–2846. doi:10.1002/eji.200737112
58. Sioud M, Floisand Y, Forfang L, Lund-Johansen F (2006) Signaling through toll-like receptor 7/8 induces the differentiation of human bone marrow CD34+progenitor cells along the myeloid lineage. J Mol Biol 364(5):945–954. doi:S0022-2836(06)01278−2 pii]
59. Skirecki T, Borkowska-Zielińska U, Złotorowicz M, Hoser G (2012) Sepsis immunopathology: perspectives of monitoring and modulation of the immune disturbances. Arch Immunol Ther Exp (Warsz) 60(2):123–135. doi:10.1007/s00005-012-0166-1
60. Skirecki T, Kawiak J, Hoser G, Tarnowski W, Zlotorowicz M, Zielinska-Borkowska U (2013) Effect of granulocyte colony-stimulating factor on circulating immune and stem cells in septic shock: revisit the basics and consider giving it another chance. Am J Respir Crit Care Med 187(2):217–219. doi:187/2/217 [pii]
61. Skirecki T, Zielińska-Borkowska U, Złotorowicz M, Kawiak J, Hoser G (2012) Mobilization of hematopoietic and nonhematopoietic stem cell subpopulations in sepsis: a preliminary report. Crit Care 16(Suppl 1):P8. doi:10.1186/cc10615
62. Spaggiari GM, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L (2008) Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine

production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood 111(3):1327–1333. doi:blood-2007−02-074997 [pii]
63. Suh W, Kim KL, Choi JH, Lee YS, Lee JY, Kim JM, Jang HS, Shin IS, Lee JS, Byun J, Jeon ES, Kim DK (2004) C-reactive protein impairs angiogenic functions and decreases the secretion of arteriogenic chemo-cytokines in human endothelial progenitor cells. Biochem Biophys Res Commun 321(1):65–71. doi:10.1016/j.bbrc.2004.06.107
64. Sun J, Han ZB, Liao W, Yang SG, Yang Z, Yu J, Meng L, Wu R, Han ZC (2011) Intrapulmonary delivery of human umbilical cord mesenchymal stem cells attenuates acute lung injury by expanding CD4+CD25+Forkhead Boxp3 (FOXP3)+regulatory T cells and balancing anti- and pro-inflammatory factors. Cell Physiol Biochem 27(5):587–596. doi:10.1159/000329980
65. Takizawa H, Boettcher S, Manz MG (2012) Demand-adapted regulation of early hematopoiesis in infection and inflammation. Blood 119(13):2991–3002. doi:10.1182/blood-2011−12–380113
66. Takizawa H, Regoes RR, Boddupalli CS, Bonhoeffer S, Manz MG (2011) Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation. J Exp Med 208(2):273–284. doi:10.1084/jem.20101643
67. Textoris J, Beaufils N, Quintana G, Ben Lassoued A, Zieleskiewicz L, Wiramus S, Blasco V, Lesavre N, Martin C, Gabert J, Leone M (2012) Hypoxia-inducible factor (HIF1alpha) gene expression in human shock states. Crit Care 16(4):R120. doi:10.1186/cc11414
68. Xu J, Woods CR, Mora AL, Joodi R, Brigham KL, Iyer S, Rojas M (2007) Prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice. Am J Physiol Lung Cell Mol Physiol 293(1):L131–L141. doi:00431.2006 [pii]
69. Yang L, Guo XG, Du CQ, Yang JX, Jiang DM, Li B, Zhou WJ, Zhang FR (2012) Interleukin-1 beta increases activity of human endothelial progenitor cells: involvement of PI3K-Akt signaling pathway. Inflammation 35(4):1242–1250. doi:10.1007/s10753-012-9434-9
70. Zhang P, Nelson S, Bagby GJ, Siggins R 2nd, Shellito JE, Welsh DA (2008) The lineage-c-Kit+Sca-1+cell response to Escherichia coli bacteremia in Balb/c mice. Stem Cells 26(7):1778–1786. doi:10.1634/stemcells.2007−1027

Index

M. Z. Ratajczak (ed.), *Adult Stem Cell Therapies: Alternatives to Plasticity,*
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4939-1001-4,