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R. Brehm · K. Steger

Regulation of Sertoli Cell and Germ Cell Differentiation

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Regulation of Sertoli Cell and Germ Cell Differentation

With 26 Figures

Springer

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Preface

Unwanted childlessness affects approximately one in six couples worldwide. Although the exact proportion of the predominant cause of the problem remains controversial, according to the World Health Organization (WHO), in nearly 40% of cases the cause can be attributed to the female, in 20% to the male, in 25% to both, and in 15% the cause remains unknown. Based on these figures, the incidence of male factor infertility in the general population is approximately 7%. The majority of these men, approximately 30%, experience irreversible idiopathic infertility and cannot father children without some form of medical intervention.

Male factor infertility, in addition, may be caused by testicular germ cell cancer, which is known to represent the most common cancer among young men, aged 15 to 35 years, in Western industrialized countries. The number of affected men has increased dramatically over the past 50 years. There is now growing evidence that human testicular germ cell cancer originates from fetal germ cells exhibiting an aberrant programme of gene expression, and tumour progression may be favoured by an aberrant Sertoli cell-germ cell communication.

Accepting the challenge of male factor infertility means having to improve our knowledge on the control mechanisms of testis-specific gene expression being involved in the regulation of Sertoli cell and germ cell differentiation. The Human Genome Project demonstrated the testis as one of the tissues of the body with the highest degree of tissue-specific gene expression, currently revealing approximately 23,000 testis expressed UniGenes in the human genome database. Although some of the transcripts represent tissue-specific alternatively spliced isoforms of somatic mRNAs, the majority are unique gene sequences.

Attention must be drawn to the Sertoli cell-germ cell communication, as it is already known that functional Sertoli cells are required for normal spermatogenic progression resulting in the continuous production of numerous fertile spermatozoa which, in turn, is necessary to maintain Sertoli cells in their functional differentiation state. Furthermore, the maturation of spermatogonial stem cells into fertile sperm requires stringent stage-specific sequential gene expression resulting in complete histone-to-protamine exchange followed by a stop of gene expression in haploid spermatids. As a consequence, haploid spermatids exhibit stage-specific but different expression of mRNAs and corresponding proteins due to temporal uncoupling of transcription and translation.

Unfortunately, in vitro, isolated male germ cells do not survive more than approximately 24 h. Co-culture systems with direct contact between germ cells and Sertoli cells, therefore, appear to be mandatory. To date, the most promising in vitro approach is the culture of whole seminiferous tubules displaying minimal disruption of the crucial Sertoli cell–germ cell contact. Targeted mutagenesis in the mouse, in addition, provides a powerful method for the study of control mechanisms that are involved in the regulation of gene expression in the testis and helps to gain new insights into the origins of male infertility. While knockout mice answer the question of what happens when a specific gene is absent, transgenic mice answer the question of which sequences are responsible for the manner in which genes are expressed. Recent developments in transplantation of spermatogonia, in addition, offer an exciting new technology for research in spermatogenesis. The combination of germ cell transplantation with culture and cryopreservation of spermatogonia opens new pathways for genetic engineering. Autologous transfer of spermatogonia might be used as an approach for fertility preservation in oncology patients.

The intention of the present monograph is to shed more light on the regulation of Sertoli cell and germ cell differentiation. Involving knockout and transgenic mouse models, we focus on (1) male factor infertility that might be related to altered maturation of Sertoli cells, (2) male factor infertility that might be due to incorrect histone-to-protamine exchange in haploid spermatids, and (3) progression of testicular germ cell cancer that might be favoured by an aberrant Sertoli cell–germ cell communication.

June 2005 Ralph Brehm, Klaus Steger

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1
Spermatogenesis

1.1
Organization of the Seminiferous Epithelium

Within the seminiferous epithelium of seminiferous tubules, spermatogenesis occurs on the surface of somatic Sertoli cells. Functional Sertoli cells are required for normal spermatogenic progression resulting in the continuous production of numerous fertile spermatozoa which, in turn, is necessary to maintain Sertoli cells in their functional differentiation state. Adjacent Sertoli cells form Sertoli–Sertoli junctional complexes (see Sect. 2.4) dividing the seminiferous epithelium into a basal and an adluminal compartment. During spermatogenesis, germ cells migrate through the Sertoli–Sertoli junctional complexes successively passing through the following three developmental stages (Fig. 1):

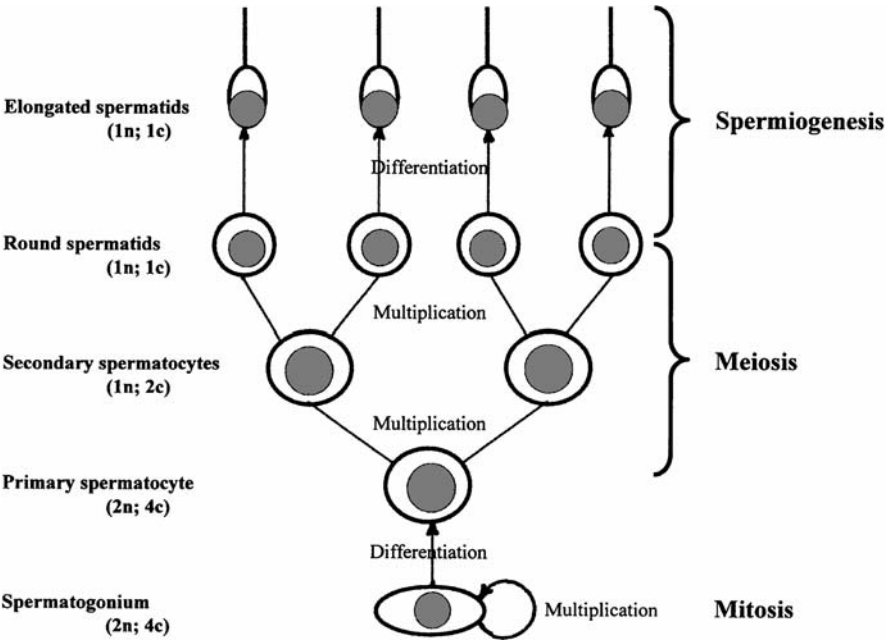


Fig. 1 During spermatogenesis, differentiating germ cells migrate on the surface of the somatic Sertoli cells (not shown) from basal to apical successively passing various developmental stages. Spermatogonia undergo mitosis (1st multiplying phase). Subsequently, spermatogonia differentiate into primary spermatocytes (1st differentiation phase) which enter meiosis (2nd multiplying phase). One primary spermatocyte gives rise to two secondary spermatocytes and each of the two secondary spermatocytes give rise to two round spermatids. Round spermatids then differentiate (2nd differentiation phase) into mature spermatozoa known as spermiogenesis. Maturation of immobile spermatozoa into mobile sperm occurs within the epididymis (not shown)

1. Mitosis: Following mitosis of a spermatogonial stem cell, one spermatogonium is conserved as a spermatogonial stem cell, while the other spermatogonium undergoes further mitoses and, subsequently, enters meiosis.
2. Meiosis: During the first meiotic division, one primary spermatocyte (DNA content: 4c) gives rise to two secondary spermatocytes (DNA content: 2c each). During the second meiotic division, each of the two secondary spermatocytes give rise to two round spermatids (DNA content: 1c each).
3. Spermiogenesis: Round spermatids no longer divide but differentiate into mature spermatozoa undergoing numerous morphological, biochemical, and physiological modifications. Nuclear chromatin condensation, development of the acrosome, and formation of the flagellum occur simultaneously in haploid spermatids.

1.2 Synchronization of Spermatogenesis

During spermatogenesis, germ cells are subjected to permanent proliferation and differentiation processes resulting in the appearance of various germ cell populations each representing a particular phase of germ cell development. A defined arrangement of germ cell populations is called the stage of the seminiferous epithelium. A complete series of changes in stages arranged in the logical sequence of germ cell maturation is called the cycle of the seminiferous epithelium (Fig. 2).

In men, the seminiferous epithelial cycle is divided into six stages (I–VI) (Clermont 1963). Due to the nuclear morphology of the spermatids and the reactivity of spermatid nuclei with periodic acid–Schiff (PAS), spermatid differentiation is further subdivided into eight steps (1–8) (Fig. 3). In mice, the seminiferous epithelial cycle is divided into 12 stages (I–XII) (Russell et al. 1990), while spermatid differentiation is subdivided into 16 steps (1–16) (Fig. 4).

Synchronized germ cell development is due to the presence of intercellular bridges, which are formed by incomplete cytokinesis during the telophase of mitosis and result in an open cytoplasmic continuity between germ cells originating from a common spermatogonial stem cell. It has been demonstrated that transcripts produced by some of the cells of a clone can move through intercellular bridges and, subsequently, will be expressed in all of the cells of the clone (Caldwell and Handel 1991). Haploid spermatids, therefore, represent functional diploid cells.

1.3 Regulation of Spermatogenesis

There are two control mechanisms which are involved in the regulation of spermatogenesis: hormonal and genetic.

Hormonal regulation of spermatogenesis is organized as a control circuit with a negative feed-back mechanism involving the hypothalamus, pituitary gland, and

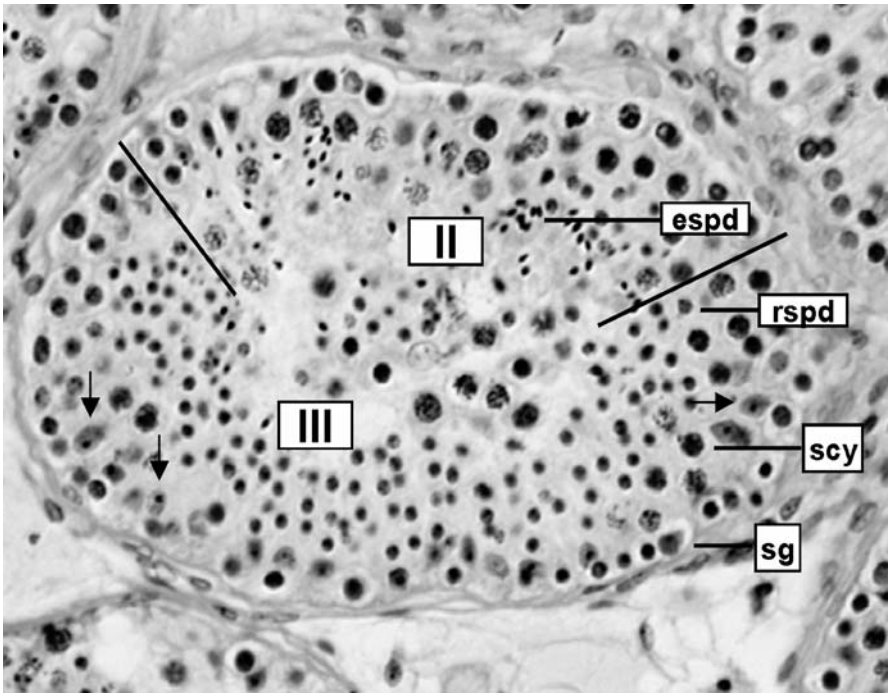
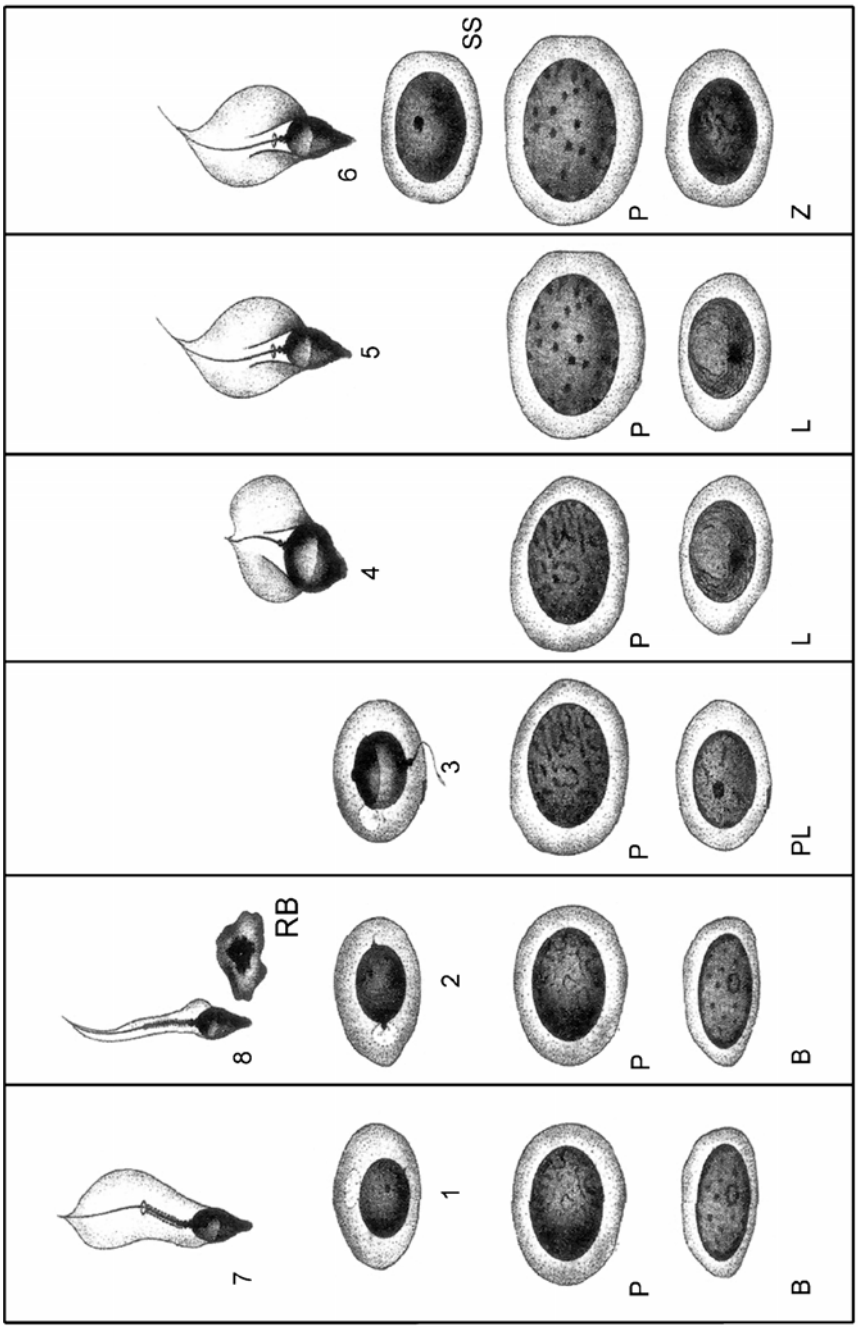


Fig. 2 Seminiferous tubule showing normal human spermatogenesis at stages I–II and III of the seminiferous epithelial cycle (see Fig. 3). *Arrows* point to nuclei of somatic Sertoli cells. Spermatogonia (*sg*), spermatocytes (*scy*), round (*rspd*) and elongated (*espd*) spermatids represent various germ cell populations. Paraffin section with haematoxylin–eosin (H&E) staining. Primary magnification ×40

Fig. 3 The seminiferous epithelial cycle in man is organized in six stages (I–VI). Spermatid differentiation is, in addition, divided into eight steps (1–8). Sertoli cells and A-type spermatogonia are not shown, since these cells are present in each stage of the cycle. *B*, B-type spermatogonia; *PL*, preleptotene spermatocytes; *L*, leptotene spermatocytes; *Z*, zygotene spermatocytes; *P*, pachytene spermatocytes; *SS*, secondary spermatocytes; *RB*, residual body. (Modified from Clermont 1963)

Fig. 4 (on page 5) The seminiferous epithelial cycle in mouse is organized in 12 stages (I–XII). Spermatid differentiation is, in addition, divided into 16 steps (1–16). Sertoli cells and A-type spermatogonia are not shown, since these cells are present in each stage of the cycle. *B*, B-type spermatogonia; *PL*, preleptotene spermatocytes; *L*, leptotene spermatocytes; *Z*, zygotene spermatocytes; *P*, pachytene spermatocytes; *D*, spermatocytes in diplotene; *SS*, secondary spermatocytes. (Modified from Russell et al. 1990)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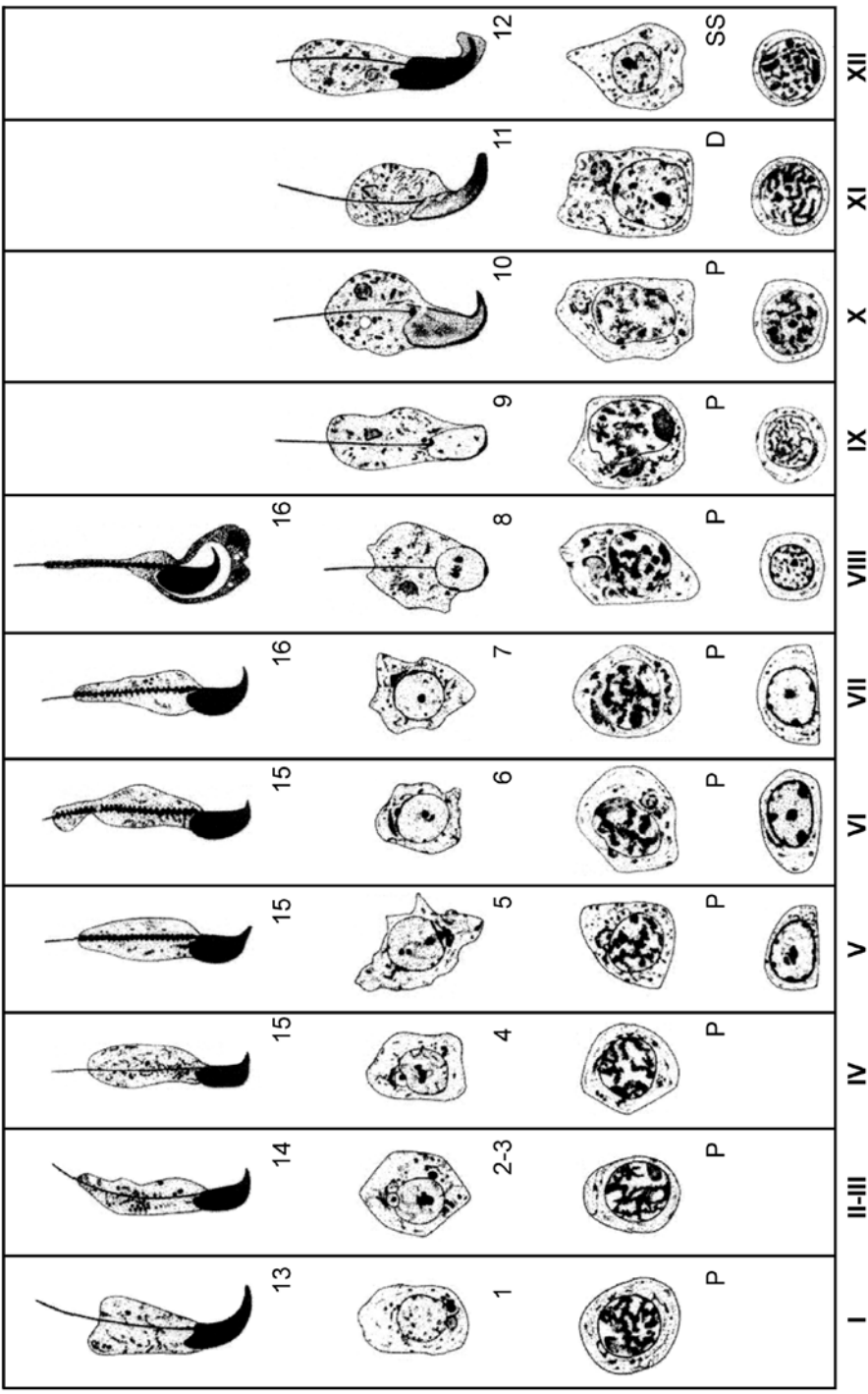
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testis (Behre and Nieschlag 1998). Specific neurons of the hypothalamus synthesize gonadotropin-releasing hormone (GnRH), which induces the production of two hormones within the pituitary, luteinizing hormone (LH) and follicle stimulating hormone (FSH). While a high pulse rate of GnRH release (1 impulse per 1 h) results in the production of LH, a low pulse rate of GnRH release (1 impulse per 2 h) results in the production of FSH. Within the testis, LH causes synthesis of testosterone by intertubular Leydig cells, which negatively influences hormone release in the hypothalamus and pituitary. By contrast, FSH acts on intratubular Sertoli cells. It induces the production of androgen-binding protein (ABP) by means of which testosterone can pass the Sertoli–Sertoli junctional complexes, and also induces the production of activin and inhibin by Sertoli cells which both influence hormone release in the hypothalamus and pituitary (Fig. 5).

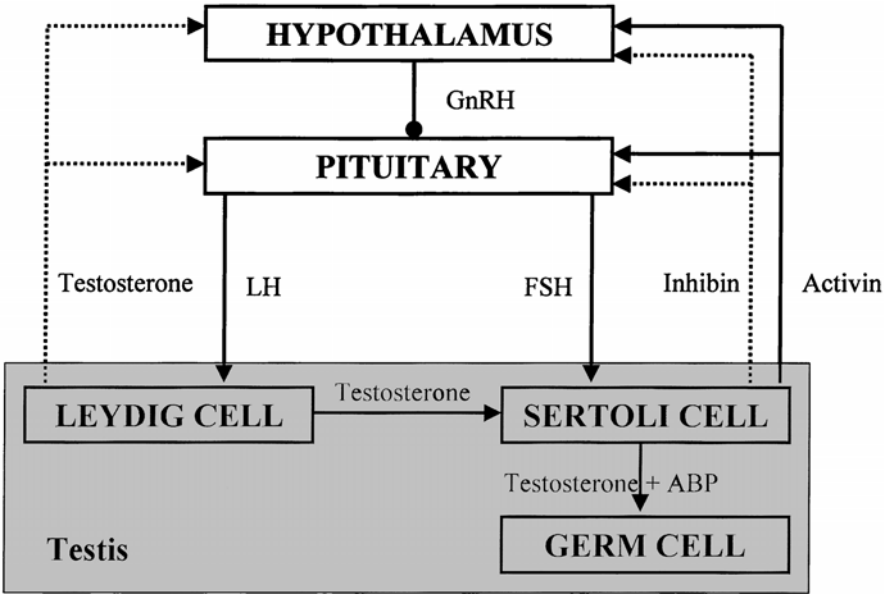


Fig. 5 Hormonal regulation of spermatogenesis occurs via a negative feed-back closed-loop control circuit involving the hypothalamus, anterior lobe of pituitary gland and testis. The hypothalamus, namely neurons of the area praeoptica and nucleus arcuatus, produce gonadotropin-releasing hormone (*GnRH*) which, in the pituitary gland, induces the secretion of both luteinizing hormone (*LH*) and follicle stimulating hormone (*FSH*). Within the testis, LH binds to receptors that are located on Leydig cells and stimulate the production of testosterone. By contrast, FSH binds to receptors that are located on Sertoli cells and stimulate the production of androgen-binding protein (*ABP*). Testosterone binds to ABP and is then able to pass the Sertoli–Sertoli junctional complexes. Sertoli cells, in addition, secrete inhibin and activin which act negatively and positively on both GnRH production by the hypothalamus and LH/FSH secretion by the pituitary gland, respectively. *Solid line*, positive effect; *dotted line*, negative effect

In contrast to hormonal regulation of spermatogenesis, our knowledge on genetic control mechanisms that are involved in the regulation of spermatogenesis is still in its infancy. Sections 2 and 3 will review available data on gene expression in Sertoli cells and germ cells, respectively, including knockout and transgenic mouse models and mutations in man.

2 The Sertoli Cell

2.1 Origin and Maturation of the Sertoli Cell

2.1.1 Origin of the Sertoli Cell

The early embryo contains an indifferent gonad exhibiting no histological signs of sex differentiation in either female or male direction. However, the final sex determination is already established within the zygote lacking or containing a Y chromosome. The first histological sign of male sex differentiation within the indifferent gonad is the appearance of pre-Sertoli cells forming primitive testicular cords. Pre-Sertoli cells play a crucial role for correct testicular development, as these cells (1) accept the primordial germ cells migrating from the yolk sac to the primitive seminiferous cords and prevent these cells from entering meiosis; (2) express the gene for the sex-determining region of the Y chromosome (SRY) (Lovell-Badge 1993; Koopman 1995) initiating the genetic sex determination of the embryo; and (3) secrete the anti-Müllerian hormone (AMH) (Josso et al. 1993) causing regression of the Müllerian ducts initiating the hormonal sex determination of the embryo. Simultaneously, secretion of testosterone by interstitial Leydig cells plays a vital role for downstream masculinization events inducing the differentiation of the Wolffian ducts into the male efferent seminal ducts and the descent of the testes into the scrotum, being a prerequisite for the production of fertile sperm in adulthood (Sharpe 2001).

The origin of the somatic Sertoli cell, however, remains a matter of debate. To date, it is still not known precisely whether these cells form a uniform population or represent various cell types with different origin. A dual origin of Sertoli cells, namely dark cells and light cells, has been suggested for the fetal human testis (Wartenberg 1978). The dark cells, representing meiosis-inducing cells, were assumed to originate from the mesenchymal mesonephros, whereas the light cells, representing meiosis-preventing cells, were supposed to derive from the coelomic epithelium. However, murine Sertoli cells have been demonstrated to originate from the coelomic epithelium (Karl and Capel 1998). Therefore, it may be speculated that human Sertoli cells also derive from epithelial precursor cells. This view is supported by the fact that developing Sertoli cells reveal a transient expression of the epithelial differentiation marker cytokeratin 18 during early ontogenesis

(Rogatsch et al. 1996; Franke et al. 2004). In addition, expression of the epithelial differentiation marker and gap junctional protein connexin 26 has been reported to occur between neighbouring Sertoli cells, corroborating the epithelial origin of these cells in man (Brehm et al. 2002).

2.1.2 The Prepubertal Sertoli Cell

The efficiency of spermatogenesis being reflected by the daily sperm production in adulthood is known to be determined by the total number of functional Sertoli cells (Orth et al. 1988; Sharpe 1994). Since adult Sertoli cells represent postmitotic cells, the final Sertoli cell number, therefore, needs to be generated until the onset of puberty. In man, Sertoli cells are known to exhibit two proliferation periods: a fetal/neonatal period and a peripubertal period (Cortes et al. 1987; Sharpe et al. 2003). However, factors determining the final Sertoli cell number are still far from clear, but should involve both genes and hormones, such as FSH known to stimulate Sertoli cell proliferation, thyroid hormones, growth hormones and various other paracrine growth factors (Jegou and Sharpe 1993).

2.1.3 The Sertoli Cell at Puberty

Puberty represents the most important time period within the life of a somatic Sertoli cell, as this cell develops from an undifferentiated pre-Sertoli cell into a differentiated and functional adult Sertoli cell representing the supporting cell of the developing germ cells. The functional switch from pre-Sertoli cells to Sertoli cells is called maturation or terminal differentiation. As the latter term is, in addition, used to describe the morphological differentiation of pre-Sertoli cells during fetal life, recently, the term functional maturation has been proposed for the functional switch of Sertoli cells during puberty (Sharpe et al. 2003).

Onset of spermatogenesis and presence of differentiating germ cells may represent a possible reason for the induction of the functional maturation of Sertoli cells at the beginning of puberty. However, it is known from both animal models and human patients that absence of germ cells does not necessarily result in a failure of Sertoli cell maturation (Sharpe et al. 2003), but there might be a temporal delay, such as in the formation of Sertoli–Sertoli tight junctions (Means et al. 1976).

The presence of meiotic and postmeiotic germ cells, however, has been demonstrated to have profound effects on the function of mature Sertoli cells, while absence of these germ cell types may lead to secondary changes in Sertoli cell function exhibiting functional resemblance to immature pre-Sertoli cells (Jegou and Sharpe 1993; Sharpe et al. 1993; Boujrad et al. 1995; Guitton et al. 2000) or features of dedifferentiation of adult Sertoli cells (Steger et al. 1996, 1999a; Kliesch et al. 1998; Brehm et al. 2002). Lack of germ cells may, in addition, represent a reflection of underlying abnormalities in Sertoli cells, as there exists a well-known

reciprocal regulation of Sertoli cell and germ cell differentiation (Sharpe et al. 2003).

Whether maturation of Sertoli cells involves a rapid switch from an immature to a mature stage or a step-wise cascade of changes that may occur over a period of time is not yet entirely clear. While some Sertoli cell characteristics, e.g. expression of aromatase, reveal a clear switch at puberty (Palmero et al. 1995; Panno et al. 1995; Turner et al. 2002; Sharpe et al. 2003), others, e.g. expression of AMH, do not show such a sharp demarcation (Gondos and Berndston 1993; Steger et al. 1996, 1999a; Sharpe et al. 2003) suggesting a multistep process. Irrespective of this debate, the analysis of Sertoli cell differentiation markers can be potentially informative, especially in patients in whom disorders of sexual differentiation or testicular development are suspected or diagnosed (see Sect. 2.3).

During puberty, Sertoli cells develop complex morphological interactions with each other and with adjacent germ cells and thus undergo specific maturation processes as indicated by their morphological and metabolic changes, finally resulting in their adult phenotype (Gondos and Berndston 1993; Schulze and Holstein 1993a; Guraya 1998).

Summarized, structural and functional changes from immature pre-Sertoli cells to functional Sertoli cells at puberty involve:

- An increase in cell size and the development of extensive cytoplasmic processes between differentiating germ cells
- A change of the nuclear morphology from a pyramidal to a tripartite form
- Changes in nuclear chromatin condensation and nuclear envelope infoldings
- The development of different and specific cell organelles such as huge amounts of smooth endoplasmic reticulum
- The appearance of numerous filaments and microtubules
- A loss of proliferative activity
- A loss of expression of certain maturation markers (see Sect. 2.3)
- The formation of Sertoli–Sertoli junctional complexes (see Sect. 2.4)

The latter represent the morphological equivalent of the so-called blood–testis barrier (BTB) that segregates spermatogonia and preleptotene spermatocytes in a basal compartment from meiotic spermatocytes and spermatids in an adluminal compartment (Dym and Fawcett 1970; Nistal and Paniagua 1983; Griswold 1995). The creation of the BTB coincides with the ability of developing germ cells to enter meiosis and progress to spermatozoa. Without the physical and metabolic support of the Sertoli cells, germ cell differentiation into mature spermatozoa would not occur (Sharpe 1994). This demonstrates that Sertoli cells and germ cells reveal obligatory morphological and functional interactions starting early in testis development and continue throughout spermatogenesis (Griswold 1998; Griswold and McLean 2002). Since functional maturation of Sertoli cells during puberty is temporally separate from Sertoli cell development and function during fetal life, it is inevitable that whatever happens to Sertoli cells during fetal life will to some extent predetermine their multiple functions in adulthood (Sharpe et al. 2003).

2.1.4 The Adult Sertoli Cell

Within the seminiferous epithelium of the adult testis, Sertoli cells reveal a highly differentiated and complicated morphological structure with observed alterations being indicative of their multiple functions related to both initiation and maintenance of spermatogenesis (Byers et al. 1993; Clermont 1993; Gondos and Berndston 1993; Russell 1993a, b; Guraya 1998) (Fig. 6).

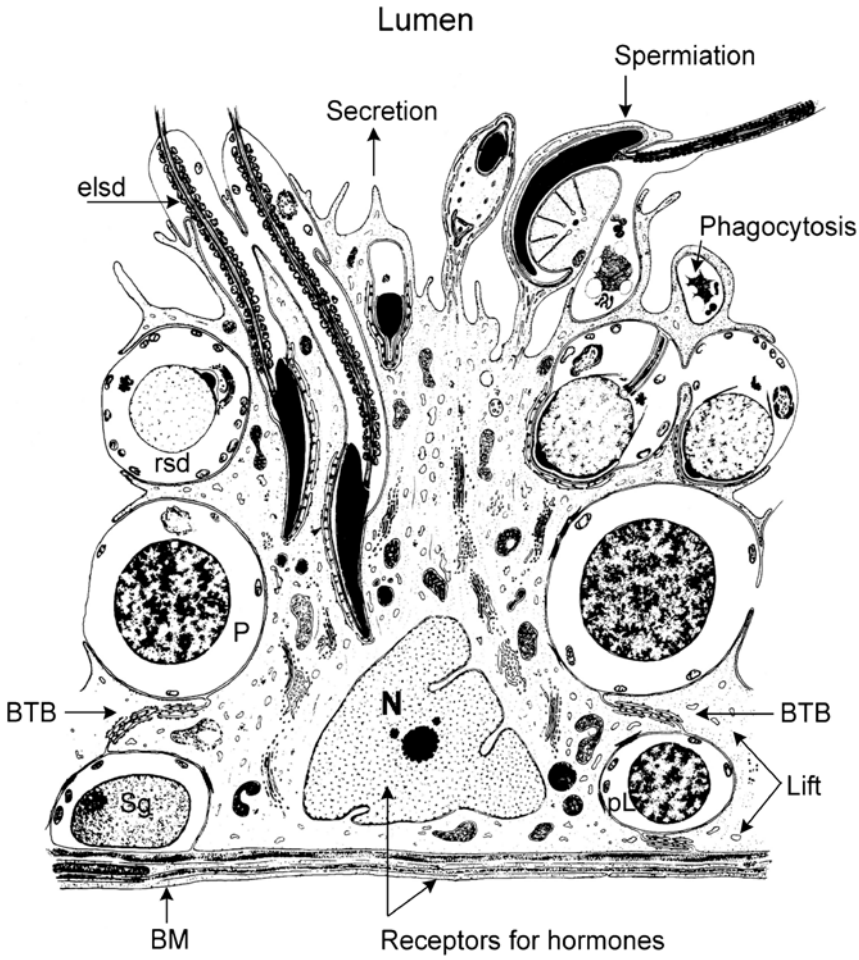


Fig. 6 Diagram showing the morphological structure and different functions of an adult Sertoli cell within the seminiferous epithelium in relationship to the various germ cell populations. *N*, Sertoli cell nucleus; *sg*, spermatogonium; *pl*, preleptotene spermatocyte; *p*, pachytene spermatocyte; *rsd*, round spermatid; *elsd*, elongated spermatid; *BM*, basement membrane; *BTB*, blood–testis barrier. (Modified from Clermont 1993)

Summarized, Sertoli cells:

- Maintain the architecture of the seminiferous epithelium as their cytoplasmic processes extend around the differentiating germ cells
- Are involved in the metabolic exchange with germ cells, as well as in their nutrition and the secretion of tubular fluid into the luminal compartment
- Perform the role of coordination of spermatogenesis in relation to the release of spermatozoa and phagocytosis of residual bodies and degenerating germ cells
- Produce a variety of proteins including AMH and ABP
- Form the Sertoli–Sertoli junctional complexes consisting of tight, adherens, and gap junctions (see Sect. 2.4)
- Are the exclusive targets for FSH, androgens and other hormones within the adult testis, as they are known to express specific receptors
- Form a complex network of specific intercellular junctions with individual adjacent germ cell populations
- Offer early meiotic spermatocytes a lift from the basal to the adluminal compartment of the seminiferous tubules

2.2 Altered Sertoli Cell Differentiation in Testicular Disorders

2.2.1 Histological Phenotypes of Testicular Disorders

Histological phenotypes of testicular disorders are: hypospermatogenesis, spermatogenic arrest, Sertoli cell only (SCO) syndrome and carcinoma in situ (CIS) of the testis (Fig. 7).

Hypospermatogenesis represents a histological grouping in primary exocrine testicular failure in which all germ cell types are present in some or all seminiferous tubules, but are mildly, moderately or severely reduced in number. While some patients show complete germ cell aplasia in some tubules and complete spermatogenesis in adjacent tubules, other patients reveal an excess number of precursor germ cells in relation to the number of mature spermatids in the seminiferous epithelium. This common histological finding of testicular biopsies taken from non-obstructive azoospermic men is a phenomenon described as mixed atrophy (Sigg 1979; Sigg and Hedinger 1981).

Microdeletions within the azoospermic factor (AZF) c locus of the Y chromosome are known to result in hypospermatogenesis (Ferlin et al. 1999). In addition, inhibin-B which is secreted by Sertoli cells has been reported to represent a sensitive and specific endocrine marker for the presence of hypospermatogenesis (Ballesca et al. 2000; Brugo-Olmedo et al. 2001).

Spermatogenic arrest can be interpreted as a histopathological description of the interruption of normal germ cell maturation at the level of a specific cell type leading from spermatogonia to spermatids. It remains to be elucidated whether

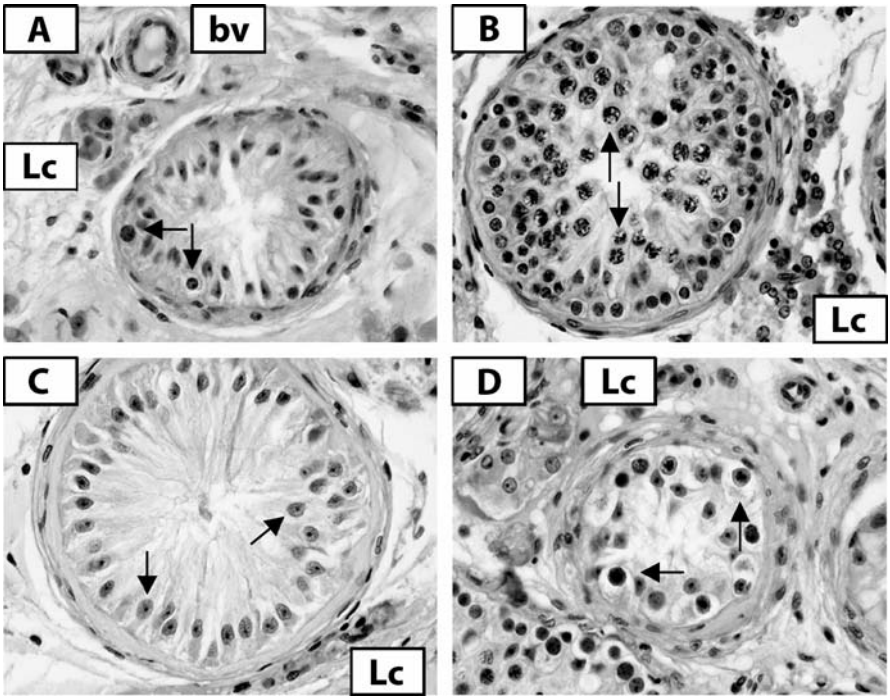


Fig. 7A–D Paraffin sections showing histological phenotypes of testicular disorders, namely spermatogenic arrest at the level of spermatogonia (**A**), primary spermatocytes (**B**), Sertoli cell only (SCO) syndrome (**C**) and carcinoma in situ of the testis (CIS) (**D**). *Arrows* point to spermatogonia (**A**), primary spermatocytes (**B**), Sertoli cell nuclei (**C**) and CIS cells (**D**). Note morphological features of large CIS cells exhibiting a clear and bright cytoplasm and an irregular shaped nucleus with one or more prominent nucleoli. *Lc*, intertubular Leydig cells; *bv*, blood vessels. H&E, ×40

spermatogenic arrest is caused by genetics or by secondary influences. However, microdeletions within the AZF b locus of the Y chromosome are known to result in spermatogenic arrest at the level of spermatocytes (Elliott et al. 1997). Possible secondary factors for spermatogenic arrest may be toxic substances (radiotherapy, chemotherapy, antibiotics), heat or general diseases (Martin-du Pan and Campana 1993) (Fig. 7A, B).

Sertoli cell only (SCO) syndrome, also called germ cell aplasia, is not a manifestation of a single disease, but rather a histopathologic phenotype. It was first described by Del Castillo et al. (1947). Testes from men with Del Castillo's syndrome contain three types of Sertoli cells: mature, dysgenetic, and involuting cells, indicating that SCO syndrome may be due to at least three different aetiologies (Nistal et al. 1990).

Interestingly, histological evaluations of testicular biopsies from infertile men exhibiting seminiferous tubules with SCO characteristics due to hypogonadotropic

hypogonadism, cryptorchidism, oestrogen treatment or chemotherapy reveal four different types of Sertoli cells (Nistal et al. 1990; Schulze and Holstein 1993b):

- Normal adult cells showing an indented nucleus, grossly triangular in shape with a prominent tripartite nucleolus
- Immature cells with round regularly outlined nuclei and immature cytoplasm
- Dysgenetic cells showing immature nuclei and a nearly mature cytoplasm with less developed cytoplasmic organelles
- Involuting cells with very irregularly outlined nuclei and a mature cytoplasm containing abundant lipid droplets, residual bodies and atypical Sertoli–Sertoli junctional complexes

Histologically, SCO syndrome occurs in two manifestations, total or focal. Testes with total SCO syndrome exhibit only Sertoli cells in seminiferous tubules showing reduced diameters. Testes with focal SCO syndrome reveal a variable percentage of seminiferous tubules containing germ cells. However, spermatogenesis is often limited in both quantitative and qualitative terms in these tubules (Silber et al. 1995).

In congenital germ cell aplasia, primordial germ cells either do not migrate from the yolk sac into the future gonads or do not survive in the epithelium of the seminiferous tubule. Microdeletions within the AZF a locus of the Y chromosome are known to result in total SCO syndrome (Foresta et al. 1998; Grimaldi et al. 1998). Furthermore, anti-neoplastic therapy with radiation or chemotherapy may also cause complete loss of germ cells, as well as viral infections, such as mumps orchitis and cryptorchid testes (Nistal et al. 1990).

In man, Sertoli cells are the only cells within the seminiferous epithelium expressing the FSH receptor. In Sertoli cells of SCO tubules, an aberrant accumulation of FSH immunoreactivity has been observed indicating an impairment of Sertoli cell function (Böckers et al. 1994). Furthermore, the majority of patients with hypospermatogenesis display reduced testicular volume and elevated serum FSH levels. The latter exhibit a positive correlation with the degree of seminiferous tubules showing germ cell aplasia (Bergmann et al. 1994) (Fig. 7C).

Carcinoma in situ of the testis (CIS) represents the common non-invasive precursor of most adult testicular germ cell tumours including seminoma, but with the exception of spermatocytic seminoma (Skakkebaek 1972; Rorth et al. 2000). Furthermore, it is the most severe phenotype of the testicular dysgenesis syndrome (Skakkebaek et al. 2001; see Sect. 5) (Fig. 7D).

2.2.2 Altered Sertoli Cell Differentiation: Primary Undifferentiated or Secondary Dedifferentiated?

In man, testicular germ cell cancer, cryptorchidism, hypospadias and some cases of low sperm counts form a syndrome of disorders, the testicular dysgenesis syndrome (TDS), with a proposed common origin in fetal life (Skakkebaek et al.

2001; Hoei-Hansen et al. 2003). In addition, it has been hypothesized that testicular impairment in adults, in general, originates during fetal life as a result of altered Sertoli cell differentiation and/or proliferation (Skakkebaek et al. 2001; Sharpe et al. 2003).

Sertoli cells with immature characteristics are found in adult testes under some pathological conditions usually associated with impaired spermatogenesis (Schulze and Holstein 1993b; Steger et al. 1999a; Hoei-Hansen et al. 2003). Without specific immunohistochemical markers, the distinction would mainly rely on morphological criteria, such as the persistence of small and spindle-shaped nuclei arranged closely in a palisade in immature Sertoli cells with no evidence of formation of a tubular lumen (Paniagua et al. 1990; Nistal et al. 2002). These seminiferous tubules are commonly described in cryptorchid testes, but are also reported in scrotal testes of some patients with Klinefelter's syndrome (Nistal et al. 1982) and impaired spermatogenesis (Regadera et al. 2001). In addition, a reduction in nuclear infoldings has been observed in Sertoli cells associated with spermatogenic arrest and SCO syndrome (Terada and Hatekayama 1991; Bruning et al. 1993; Schulze and Holstein 1993b).

The occurrence of immature Sertoli cells in testes of adult men may either be interpreted as a reflection of failure of maturation (Steger et al. 1999a; Sharpe et al. 2003), or as a re-emergence of an immature phenotype due to dedifferentiation of previously mature Sertoli cells (Steger et al. 1999a; Brehm et al. 2002). In fact, both possibilities may occur which can be demonstrated by two model systems: While Sertoli cells in patients with 5α-reductase deficiency exhibiting SCO syndrome or spermatogenic impairment at the level of spermatogonia represent primary undifferentiated cells (Steger et al. 1999a), elderly men and patients with CIS represent secondary dedifferentiated cells previously localized within seminiferous tubules with normal spermatogenesis (Kliesch et al. 1998).

Therefore, it seems most probable that maturation of Sertoli cells represents a multistep process (Gondos and Berndston 1993; Steger et al. 1999a; Sharpe et al. 2003; Franke et al. 2004). Intrinsic to this explanation is the likelihood that disorders of Sertoli cell maturation may occur at different steps and failure at an early step may prevent or interfere with future steps. It is obvious that failure of a Sertoli cell to mature functionally will presumably render it incapable of supporting the survival and development of various germ cell populations that appear during puberty. This might explain certain observations, such as the mixed patterns of Sertoli cell androgen receptor (AR) expression found in SCO tubules (Regadera et al. 2001). Here, seminiferous tubules with Sertoli cells have been detected exhibiting immature nuclear morphology and not expressing the androgen receptor adjacent to seminiferous tubules containing Sertoli cells with partly differentiated nuclei that express the AR weakly. In addition, different patterns of AMH and cytokeratin 18 expression in SCO tubules were detected indicating that Sertoli cells in SCO syndrome show a mosaic pattern of differentiation (Steger et al. 1996).

Thus, the definition of the temporal sequence of appearance of Sertoli cell markers during prenatal and early postnatal development in Sertoli cells will be

important and helpful for the understanding of the mechanisms underlying their (re)expression in disorders of the adult testis. Since various immunophenotypes reflect distinct differentiation stages, this knowledge will also be important for understanding adult testicular pathology (Steger et al. 1996, 1999a; Sharpe et al. 2003; Franke et al. 2004).

2.3 Differentiation Markers of the Sertoli Cell

Besides the morphological appearance of Sertoli cells, the expression of various proteins can clearly be associated with certain periods of Sertoli cell differentiation and the immunohistochemical study of such differentiation markers can potentially aid the interpretation of distinct phenotypes that may be present (Sharpe et al. 2003). Such studies might indicate whether and when there has been a fundamental failure of Sertoli cell maturation. If Sertoli cell differentiation occurs as a cascade (Gondos and Berndston 1993; Steger et al. 1999a), a better understanding of when various protein markers are expressed in relation to acquisition of Sertoli cell maturation can be used as pointers as to when the maturation process failed. There exist numerous immunohistochemical protein markers to establish the maturation state of Sertoli cells (Fig. 8).

2.3.1 Anti-Müllerian Hormone

Anti-Müllerian hormone (AMH) is present in pre-Sertoli cells from early stages of testicular development to puberty, but not in adult Sertoli cells (Tran et al. 1987; Josso et al. 1993; Rey et al. 1996; Steger et al. 1996, 1999a) and its functional expression in human is reported to start at the 7–8th week of gestation (Gaskell et al. 2004; Franke et al. 2004). Although AMH plays a key role during early development of the internal genitalia by inducing regression of the Müllerian ducts in the male fetus (Josso et al. 1993), serum AMH levels remain high throughout childhood and do not decrease until puberty in response to increasing androgen levels (Rey et al. 1993). AMH expression seems to be downregulated as soon as the androgen receptor (AR) is functionally expressed in adult Sertoli cells and when germ cells enter meiosis (Rajpert-de Meyts et al. 1999). High AMH blood concentrations due to persisting AMH expression in adult Sertoli cells, therefore, may indicate aberrant maturation of Sertoli cells probably due to deficiencies in androgen action. Male mice lacking both AMH and inhibin have been reported to show testicular tumours that develop earlier and grow faster than in mice which solely lack inhibin (Matzuk et al. 1995), suggesting that AMH may act as an autocrine modifier in Sertoli cell tumourigenesis (Yan et al. 2003). No AMH immunoreactivity could be demonstrated in testes of adult patients exhibiting normal spermatogenesis, but a renewal of AMH immunoreactivity was described in Sertoli cells of patients with both spermatogenic arrest at the level of spermatogonia and SCO

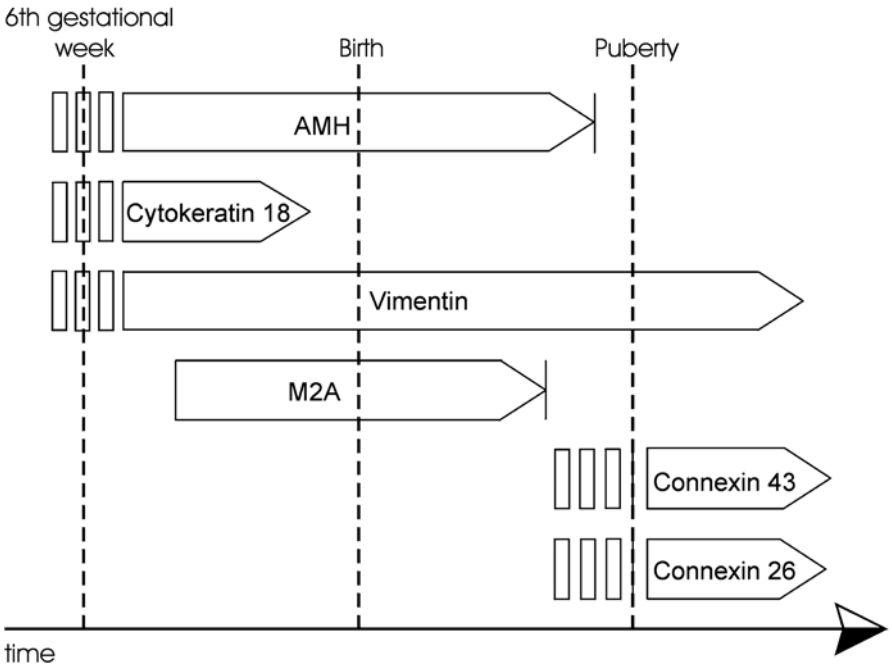


Fig. 8 Diagram showing the temporal expression pattern of Sertoli cell differentiation markers during normal human spermatogenesis. Production of functional AMH starts between the 7th and 8th week of gestation. AMH expression is downregulated around puberty, as soon as the AR becomes functionally active. Cytokeratin 18 can be used as a fetal Sertoli cell marker, as this intermediate filament protein is completely absent from the 20th gestational week onwards. Vimentin is present throughout all phases of life and, therefore, represents a stable and reliable marker for both pre-Sertoli cells and Sertoli cells. By contrast, the M2A antigen is expressed in prepubertal, but not adult Sertoli cells. M2A expression starts at the 14th week of gestation and is downregulated prior to that of AMH. Expression of the gap junctional proteins connexin 26 and connexin 43 is supposed to start around puberty and, therefore, they may be used as pubertal markers

syndrome (Steger et al. 1996, 1999a). In contrast, no AMH was detected in adult patients showing SCO and maturation arrest with and without AZF microdeletions (Blagosklonova et al. 2002). The decrease of AMH expression during puberty and the simultaneous onset of spermatogenesis may reflect the terminal differentiation or functional maturation of Sertoli cells which in most cases follow their normal internal differentiation programme (Rajpert-DeMeyts et al. 1999) (Fig. 9A, B).

2.3.2 Cytokeratin 18

Cytokeratin 18 is an intermediate filament protein which is expressed in epithelial cells including fetal pre-Sertoli cells. In man, cytokeratin 18 serves as a marker for

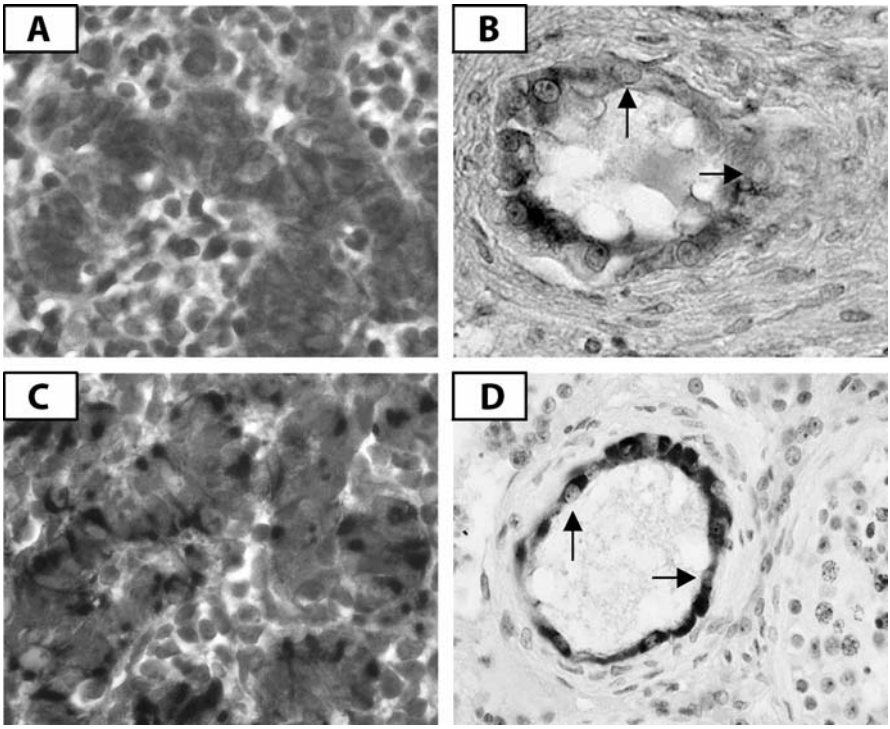


Fig. 9A–D Paraffin sections showing immunohistochemical staining for AMH (**A** and **B**) and cytokeratin 18 (**C** and **D**) in pre-Sertoli cells of the 8th gestational week (**A** and **C**) and in adult Sertoli cells associated with spermatogenic arrest at the level of spermatogonia (*arrows*; **B** and **D**). APAAP method, positive signal red, ABC method, positive signal brown. ×40

fetal Sertoli cells, as it has been demonstrated to be completely absent after the 20th gestational week (Rogatsch et al. 1996; Franke et al. 2004). In addition, cytokeratin 18 was observed under certain pathological circumstances, such as spermatogenic arrest at the level of spermatogonia and SCO syndrome (Miettinen et al. 1985; Stosiek et al. 1990; Bergmann and Kliesch 1994; Steger et al. 1999a; Maymon et al. 2000) and in SCO patients associated with AZF microdeletions (Blagosklonova 2003) (Fig. 9C, D).

2.3.3 Vimentin

Vimentin is an intermediate filament protein which is continuously expressed in Sertoli cells throughout all phases of life and thus provides a stable and reliable marker for these cells (Franke et al. 1979; Paranko et al. 1986; Aumüller et al. 1988; Stosiek et al. 1990; Aumüller et al. 1992; Rogatsch et al. 1996) (Fig. 10A, B).

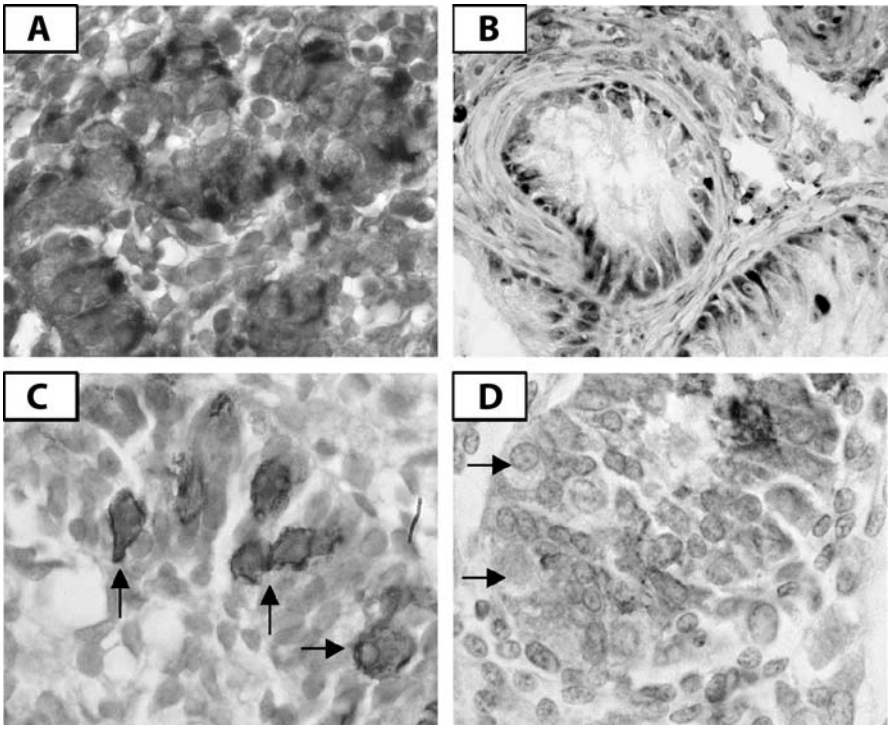


Fig. 10A–D Paraffin sections showing immunohistochemical staining for vimentin (**A** and **B**) in pre-Sertoli cells of the 8th gestational week (**A**) and in adult Sertoli cells associated with Sertoli cell only (SCO) syndrome (**B**). **C**, **D** Immunohistochemical staining for M2A antigen. In a 9-week-old testis (**C**), primordial germ cells (*arrows*) exhibit positive signals, while surrounding pre-Sertoli cells remain negative. By contrast, in a 25-week-old testis (**D**), pre-Sertoli cells are immunopositive, whereas germ cells (*arrows*) are immunonegative. APAAP method, positive signal red, ABC method, positive signal brown. ×40

2.3.4 M2A Antigen

M2A antigen represents an oncofetal marker corresponding to a surface protein which is expressed in fetal gonocytes (Baumal et al. 1989; Jorgensen et al. 1995; Marks et al. 1999; Brehm et al. 2002). This antigen was identified on abnormal germ cells in several testicular germ cell tumours and CIS (Bailey et al. 1986; Marks et al. 1999; Brehm et al. 2002). The M2A antigen is expressed in prepubertal, but not adult Sertoli cells (Bailey et al. 1986; Baumal et al. 1989; Steger et al. 1999a; Blagosklonova et al. 2002; Franke et al. 2004). It was not detected in adult patients in whom various disorders of spermatogenesis were found to be present (Steger et al. 1999a; Blagosklonova et al. 2002). Therefore, it has been proposed that the observed loss of expression of M2A antigen in mature Sertoli cells could be interpreted as an irreversible sign of transition of prepubertal to mature Sertoli cells (Steger et al. 1999) (Fig. 10C, D).

2.3.5 p27Kip1

p27Kip1, a cyclin-dependent kinase inhibitor, plays a role in cell cycle regulation. Normally, its expression is associated with inhibition of proliferation. Accordingly, immunodetection of p27Kip1 coincides with maturation of Sertoli cells in mice (Beumer et al. 1999; Cipriano et al. 2001), rats and humans (Beumer et al. 1999). Failure of maturation of Sertoli cells in rats results in absence of p27Kip1 expression. During prepubertal testicular development, Sertoli cells show active proliferation and, therefore, only occasionally weakly stained Sertoli cells can be observed. By contrast, in the adult testis, the terminally differentiated Sertoli cells are strongly stained for p27Kip1 (Beumer et al. 1999).

2.3.6 Androgen Receptor

AR nuclear expression is a feature of mature Sertoli cells exhibiting variations of expression, according to the stage of the seminiferous epithelial cycle (Suarez-Quian et al. 1999). However, AR expression does not coincide exactly with maturation of Sertoli cells, but first appears before the final maturation of Sertoli cells (Al-Attar et al. 1997; Rajpert-DeMeyts et al. 1999). During fetal and early neonatal life, Sertoli cells do not express AR in man, rat or marmoset monkey (Williams et al. 2001). In man, Sertoli cell nuclear AR expression probably does not occur until close to the onset of puberty (Sharpe et al. 2003) and failure of maturation of Sertoli cells, based on nuclear and general morphology, can be associated with absent or weak AR expression (Regadera et al. 2001). Weak expression might be an indication for maturation failure at a later step than in Sertoli cells that show no expression of AR at all. Additionally, there is some evidence for abnormal differentiation and/or persistent mitotic activity of Sertoli cells in men with AR mutations (Giwercman et al. 2000).

2.3.7 Inhibin-α

Inhibin-α, a gonadal polypeptide hormone secreted by Sertoli cells that inhibits FSH secretion by the anterior pituitary gland, seems to represent one of the most important molecules for the investigation of Sertoli cells. Like vimentin, it can be used as a stable marker for Sertoli cell detection, e.g. in mixed testicular tumours in combination with other specific cell markers. The inhibin-α subunit is known to be expressed in both Sertoli cells and Leydig cells (Vliegen et al. 1993; Toppari et al. 1998; Kommoss et al. 2000). Male mice lacking the α-subunit of the inhibin dimer (Matzuk et al. 1992; Yan et al. 2003) develop Sertoli cell tumours with a 100% penetrance, indicating that inhibin may be secreted as a tumour suppressor. It has been shown further by genetic intercross strategy (breeding of inhibin-α mutant with other knockout mice) that endocrine factors may play a role in Sertoli

cell tumourigenesis by altering the cell cycle machinery of Sertoli cells. Mutant mice lacking GnRH and inhibin do not exhibit gonadal tumours, indicating that gonadotropins (FSH, LH) are essential modifying factors for Sertoli cell tumour development in inhibin-deficient mice (Kumar et al. 1996). Absence of FSH and lack of AR slowed but did not stop tumour growth, whereas absence of AMH or p27 caused earlier onset and more aggressive development of tumour (Yan et al. 2003).

2.3.8 Connexin 26

Connexin 26, a gap junctional protein, can be associated with the junctional complex between Sertoli cells and may also occur between Sertoli cells and basally located germ cells (Brehm et al. 2002; Yu et al. 2003). It has recently been described to be weakly expressed in human mature Sertoli cells in seminiferous tubules with normal spermatogenesis and, like cytokeratin 18, to be upregulated in altered Sertoli cells in the presence of spermatogenic arrest at the level of spermatogonia and CIS (Brehm et al. 2002). Furthermore, connexin 26 can function as an additional marker for dedifferentiating Sertoli cells (Donner et al. 2004) (Fig. 11A, B).

2.3.9 Connexin 43

Connexin 43, another gap junctional protein, which is expressed by Sertoli cells, spermatogonia, spermatocytes and interstitial Leydig cells, represents the predominant testicular connexin. Gap junctions containing connexin 43 seem to be necessary for the regulation of the terminal differentiation of Sertoli cells and the coordinated start of differentiation, proliferation and maturation of normal germ cells (Pelletier and Byers 1992; Pelletier 1995). In man, mink and guinea pig, it seems to be first expressed in Sertoli cells at puberty and can thus be used as a pubertal differentiation marker (Pelletier 1995; Brehm et al. 2002). Alterations of connexin 43 expression in man have been demonstrated to be correlated with various testicular disorders and male infertility, since seminiferous tubules with spermatogenic arrest at the level of spermatogonia and those with SCO display an intratubular downregulation of connexin 43 at the protein level (Steger et al. 1999b; Brehm et al. 2002; Defamie et al. 2003) (Fig. 11C, D).

2.4 Sertoli Cell Intercellular Communication

Intercellular communication between testicular cells is regulated through several mechanisms including paracrine and endocrine communication pathways. These cell–cell interactions involve physical and dynamic organization mediated by distant signals through secreted growth factors and signalling molecules, as well as direct junctions. Within the seminiferous epithelium, communication is mainly

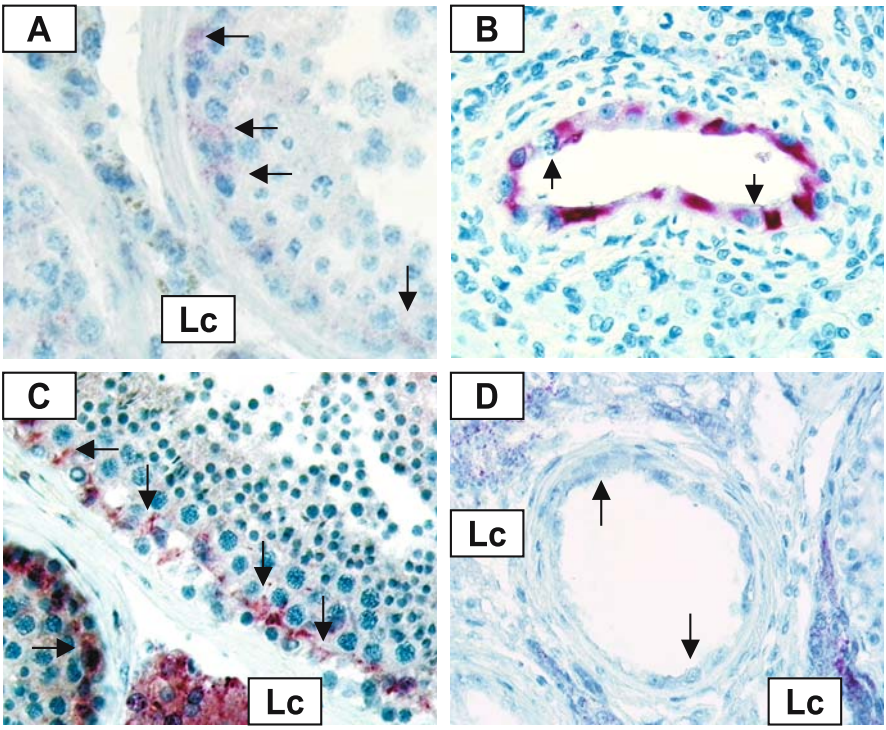


Fig. 11A–D Paraffin sections showing immunohistochemical staining for the gap junctional adherens proteins connexin 26 (**A** and **B**) and connexin 43 (**C** and **D**) during normal human spermatogenesis (**A** and **C**) and associated with spermatogenic arrest at the level of spermatogonia (*arrows*; **B** and **D**). During normal spermatogenesis, connexin 26 exhibits weak signals (*arrows*) between Sertoli cells associated with the Sertoli–Sertoli junctional complexes and between Sertoli cells and basally located germ cells, while connexin 43 reveals strong signals (*arrows*) between adjacent Sertoli cells occurring apical to spermatogonia and basal to primary spermatocytes and along the Sertoli–Sertoli junctional complexes. Sertoli cells associated with spermatogenic arrest at the level of spermatogonia are immunopositive for connexin 26, but not connexin 43. Interstitial Leydig cells (*Lc*) are immunonegative for connexin 26, but immunopositive for connexin 43. APAAP, positive signal *red*. ×40

mediated by Sertoli cells (Byers et al. 1993; Enders et al. 1993; Jegou 1993; Russell 1993b; Sharpe 1993; Risley et al. 2002). Sertoli cells are in contact with each other via the so-called Sertoli–Sertoli junctional complexes consisting of tight, adherens and gap junctions constituting the anatomical basis of the blood–testis barrier (BTB). This barrier is situated near the basal third of the seminiferous epithelium and alterations of this junctional complex may be associated with abnormal germinal development (Dym and Fawcett 1970; Gilula et al. 1976; Pelletier and Byers 1992; Russel 1993b; Enders 1993; Jegou 1993; Cavicchia et al. 1996) (Fig. 12).

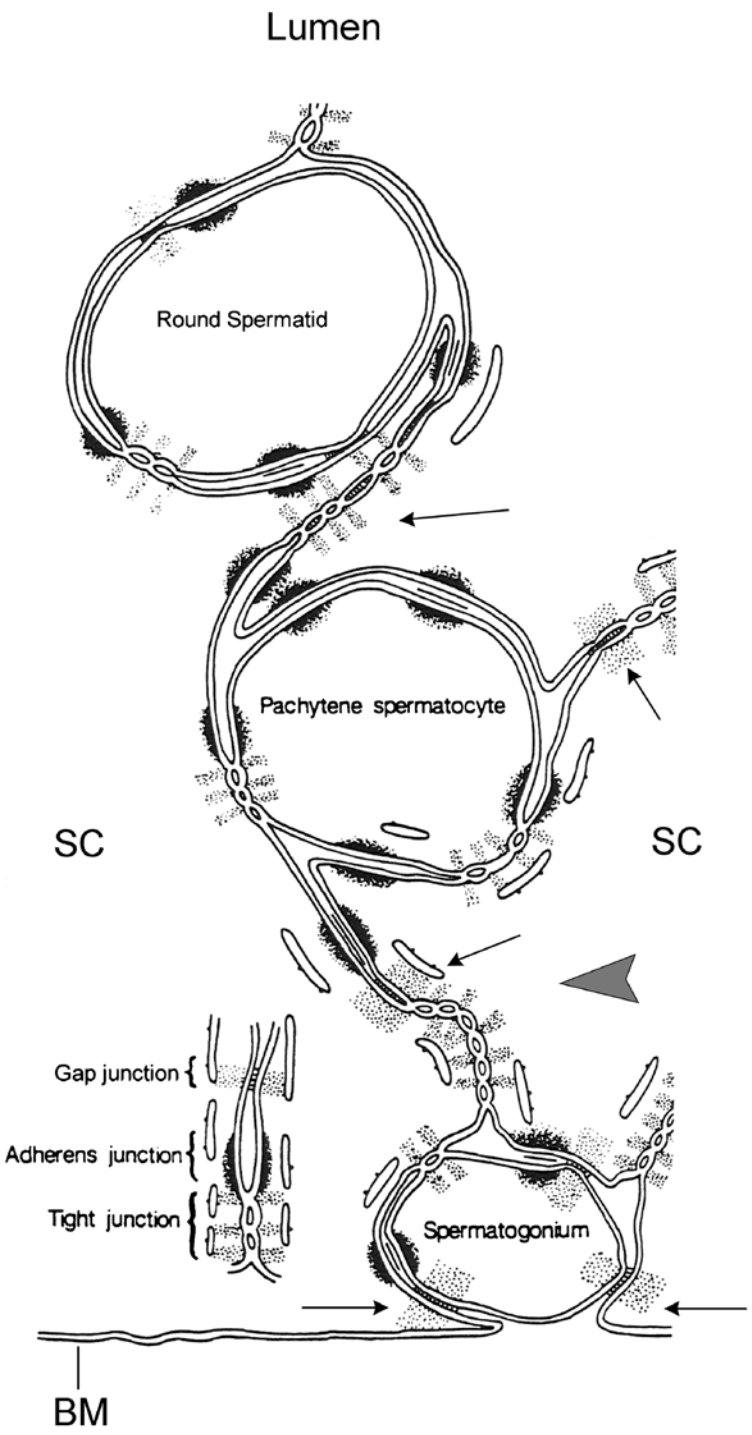
Almost all substances from the systemic circulation are excluded from the epithelium, because of the unique anatomical location of the BTB between Sertoli

cells. For instance, the BTB physically divides the seminiferous epithelium into a basal and an adluminal compartment (Dym and Fawcett 1970; De Kretser and Kerr 1988; Cheng and Mruk 2002; Siu and Cheng 2004). Cytoplasmic processes from the Sertoli cell are tightly associated with germ cells via different anchoring junctions, such as cell–cell actin-based adherens junctions (e.g. ectoplasmic specializations and tubulobulbar complexes, both are testis-specific adherens junctions), and gap junctions. The preleptotene and leptotene spermatocytes, residing in the basal compartment, must pass through the BTB to gain entry into the adluminal compartment where they continue their differentiation and movement to the luminal edge of the epithelium (Russell 1977a; Cheng and Mruk 2002; Siu and Cheng 2004). As such, the opening and closing of the BTB together with the intensive restructuring events in the junctions between Sertoli cells and germ cells must be precisely regulated via coordinated mechanisms thereby facilitating germ cell movement. To complicate this issue further, the relative morphological appearance between tight junctions and adherens junctions stands in sharp contrast to other epithelia, suggesting that the mechanisms involved in the regulation of junction dynamics in other epithelia may not be applicable to the seminiferous epithelium. For instance, tight junctions are closest to the basement membrane at the basolateral region of the Sertoli cell, coexisting side-by-side with adherens junctions, instead of being located at the apical portion of the cell, as demonstrated in other epithelia such as in the epididymal epithelium (Cyr et al. 1999; Siu and Cheng 2004).

Gap junctions, in general, are formed between adjacent cells that assemble between homologous and heterologous cell types in nearly all epithelia (Bruzzone et al. 1996). They are composed of aggregations of membrane channels localized in the plasma membranes directly linking their cytoplasm. A gap junction channel consists of two hemichannels, called connexons, contributed separately by each of the two participating cells. Each connexon is again formed by the hexameric assembly of protein subunits known as connexins (Fig. 13). The connexin family consists of at least 20 members in humans and 19 members in rodents (Bennett et al. 1991; White et al. 1995; Bruzzone et al. 1996; Willecke et al. 2002).

Gap junctions form intercellular pathways for the direct exchange of small molecules (<1 kDa) such as peptides, second messengers, ions (e.g. Ca^{2+}) and nucleotides and constitute the basis for direct intercellular communication mediating metabolic coupling and coordinated responses of coupled cells to hormones

Fig. 12 Diagram showing the distribution pattern of tight, adherens and gap junctions within the seminiferous epithelium during normal spermatogenesis. Sertoli cells form gap junctions with different populations of germ cells and are in contact with each other via the Sertoli–Sertoli junctional complexes constituting the anatomical basis of the blood–testis barrier. Note locations of gap junctions (*arrows*) and the Sertoli–Sertoli junctional complex (*arrowhead*). *Sc*, Sertoli cell cytoplasm; *BM*, basement membrane. (Modified from Pelletier and Byers 1992)


Lumen
Round Spermatid
Pachytene spermatocyte
SC
SC
Gap junction
Adherens junction
Tight junction
Spermatogonium
BM

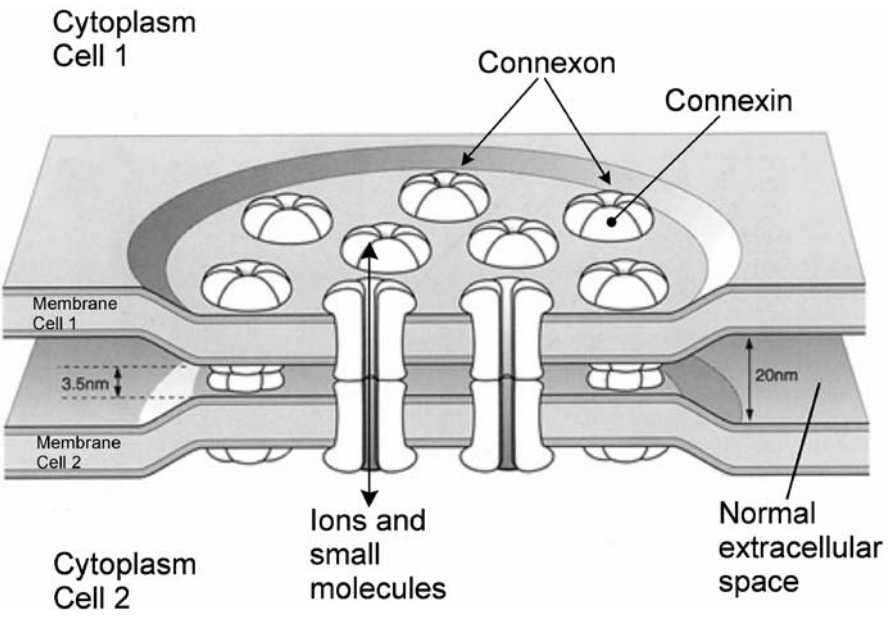


Fig. 13 Diagram showing composition of a gap junctional plaque. Gap junctions are composed of aggregations of membrane channels localized in the plasma membrane of adjacent cells directly linking their cytoplasm. A single gap junction channel consists of two hemichannels, called connexons, contributed separately by each of the two participating cells and each connexon is formed by the hexameric assembly of protein subunits known as connexins. At theses sites the extracellular space between adjacent cells is reduced to only 3.5 nm. (Modified from Kandel et al. 1995)

and growth factors (Risley 2000). The flux of material between cells via gap junction channels is known as gap junctional intercellular communication (Trosko and Ruch 1998). Gap junctions in various normal tissues are thought to be involved in the regulation of development, cell differentiation, cell proliferation and maintenance of tissue homeostasis, but they are also involved in the regulation of cellular growth, expression of the neoplastic phenotype and seem to participate in oncogenic transformation processes (Yamasaki 1990a,b; Bennett et al. 1991).

The occurrence of gap junctions in the testis of different species has been firmly established by various morphological, immunocytochemical and functional assays (Enders 1993; Tan et al. 1996; Risley et al. 2002). Within the seminiferous epithelium, gap junctions can be detected between Sertoli cells as junctional member of the Sertoli–Sertoli junctional complexes, as well as between Sertoli cells and different generations of germ cells. Probably founded upon this omnipotent gap junctional cell-to-cell communication, a complex network of direct cellular interactions between peritubular cells and Sertoli cells, and between Sertoli cells and spermatogenic cells, plays an important role for the initiation and maintenance of normal spermatogenesis and for the functional differentiation of germ cells

and Sertoli cells (Jegou 1993; Griswold 1995; Risley 2000, 2002). Furthermore, gap junctions could play a key role in testicular response to different hormones by means of releasing a wave of second messengers from hormonally activated cells (e.g. Sertoli cells and peritubular cells) to adjacent cells (e.g. germ cells) which are probably non-activated or non-responsive to hormones. Furthermore, the gap junctional protein connexin 43 is supposed to play a role in the regulated and coordinated formation of the BTB at puberty (Pelletier 1995).

In rodent testis, six connexin isoforms have been demonstrated by immunohistochemistry and/or Western blot analysis so far. Connexin 37 was solely found in endothelia of blood vessels (Tan et al. 1996), while Sertoli cells express connexin 26, connexin 32, connexin 33 and connexin 43 (Risley et al. 1992; Pelletier 1995; Tan et al. 1996; Batias et al. 1999, 2000; Bravo-Moreno et al. 2001; Perez-Armendariz et al. 2001; Decrouy et al. 2003; Fiorini et al. 2004). Intertubular Leydig cells are solely immunopositive for connexin 43 (Risley et al. 1992; Perez-Armendariz et al. 1994; Pelletier 1995; Tan et al. 1996; Batias et al. 1999, 2000; Segretain et al. 2003), as are peritubular cells (Risley et al. 1992; Risley 2000). Germ cells are positive for connexin 31 (spermatogonia, spermatocytes and elongated spermatids) and connexin 43 (spermatogonia and spermatocytes) (Mok et al. 1999; Batias et al. 2000; Decrouy et al. 2003). In addition, connexin 43 has been identified in cultured Sertoli cells (Lablack et al. 1998; Defamie et al. 2001).

Thus, amongst testicular connexins, connexin 43 seems to be the predominant one and its expression was shown to be dependent on testis maturation and the stage of the seminiferous epithelial cycle (Risley et al. 1992; Pelletier 1995; Tan et al. 1996; Batias et al. 1999, 2000), whereas connexin 26 appeared to be expressed independent of the stage of the seminiferous epithelium (Risley et al. 1992). While connexin 43 has mainly been detected in the basal compartment of the seminiferous tubules (Risley et al. 1992), connexin 26 was observed solely in cells (spermatids and/or Sertoli cells) in the apical region of rodent seminiferous epithelium (Zhang and Nicholson 1989; Haefliger et al. 1992; Risley et al. 1992; Tan et al. 1996).

Applying in situ hybridization, recent investigations in adult rodent testis revealed stage-dependent signals in different cells located in the basal compartment of the seminiferous epithelium. The highest amount of connexin 43 mRNA was detected in the cytoplasm of Sertoli cells, Leydig cells and to a lesser extent in spermatogonia and spermatocytes, indicating that these cells are able to assemble connexin 43 into gap junctions with Sertoli cells (Batias et al. 2000). In addition, RT-PCR analyses with rat testis homogenate showed that even 11 connexin mRNAs were present in total RNA from seminiferous tubules and ten of these connexin mRNAs were present in polysomes and presumably translated. Nine connexin mRNAs were found in germ cells, eight in Sertoli cells and five in peritubular cells (Risley 2000). Furthermore, comparison of connexin mRNAs in postnatal rat tubules and isolated cell types within fetal and prenatal testes suggests that connexin gene expression becomes even more complex with the development of spermatogenetic cells, especially meiotic cells and with the onset of spermatogenesis at puberty (Risley et al. 2000).

In adult men, connexin 43 protein expression was investigated in the testis both with normal spermatogenesis and associated with different forms of spermatogenic impairment (Steger et al. 1999b; Brehm et al. 2002; Defamie et al. 2003; Roger et al. 2004). Like in rodent testis, connexin 43 immunoreactivity was generally present between interstitial Leydig cells corresponding to the extensive gap junctions of this cell type known from electron microscopy and electrophysiological investigations (Perez-Armendariz et al. 1994; Varanda and de Carvalho 1994). Within the normal seminiferous epithelium, connexin 43 immunoreactivity was localized between adjacent Sertoli cells occurring apical to spermatogonia and basal to primary spermatocytes and along the Sertoli–Sertoli junctional complexes, whereas the immunopositive signal for connexin 43 was drastically reduced or absent during stages II and III of the seminiferous epithelial cycle, suggesting a stage-dependent Sertoli cell function (Steger et al. 1999b; Brehm et al. 2002; Defamie et al. 2003).

The importance of connexin 43 to gametogenesis is further indicated by severe depletion of germ cells in prenatal male and female mice lacking the connexin 43 gene (Juneja et al. 1999). Postnatal proliferation of spermatogonia is also impaired in connexin 43-null mutants (Roscoe et al. 2001). Insertion of connexin 32 or connexin 40 coding regions into connexin 43 coding region of connexin $43^{-/-}$ mice restored oogenesis and other deficiencies caused by connexin 43 deletion (Reaume et al. 1995), but spermatogonial amplification and spermatogenesis remained defective (Plum et al. 2000) indicating its unique intratubular role in the testis. Thus, expression of connexin 43 may be an essential component of communication pathways starting in early embryogenesis in rodents and possibly with onset of spermatogenesis at puberty concomitant with functional maturation and terminal differentiation of Sertoli cells (Pelletier 1995; Steger et al. 1999b; Brehm et al. 2002).

Some information is available concerning the expression of gap junctions and connexin 43 in seminiferous tubules of pathologic human testes (Nagano and Suzuki 1976; Bigliardi and Vegni-Talluri 1977; Schleiermacher 1980; Wilgenbus et al. 1992; Cavicchia et al. 1996; Steger et al. 1999b; Brehm et al. 2002; Defamie et al. 2003; Roger et al. 2004). Early morphological examinations showed alterations of gap junctions in pathological testes. By means of freeze fracture, no gap junctions were detected in feminized human testis (Nagano and Suzuki 1976) and the presence of atypical testicular gap junctions was observed in infertile patients (Bigliardi and Vegni-Talluri 1977). Gap junction-like cell membrane specializations were rare in hypospermatogenic and aspermatogenic testis (Schleiermacher 1980). In mutant mice deficient for the retinoid X receptor β, which exhibited abnormal spermatogenesis due to altered Sertoli cell function, connexin 43 was markedly reduced in the seminiferous epithelium, assuming that retinoids and perhaps similar substances are able to regulate connexin 43 expression (Batias et al. 2000). In mice with impaired spermatogenesis, the level of connexin 43 immunoreactivity was found to be reduced or absent, indicating that normal germ cells are able to influence connexin 43 expression and gap junction formation associated with Sertoli cells (Batias et al. 1999).

Seminiferous tubules showing spermatogenic arrest at the level of spermatogonia, those with SCO syndrome and infiltrated with CIS cells, display a downregulation of connexin 43 in humans (Steger et al. 1999b; Brehm et al. 2002; Defamie et al. 2003). Finally, alterations of connexin 43 expression have been correlated to various testicular disorders and male infertility including CIS and testicular germ cell tumours (Juneja et al. 1999; Steger et al. 1999b; Plum et al. 2000; Roscoe et al. 2001; Brehm et al. 2002; Defamie et al. 2003; Roger et al. 2004).

2.5 Knockout and Transgenic Mouse Models and Mutations in Man

Constitutive knockout mice contain the same artificially introduced specific mutation in every cell abolishing the activity of this preselected gene. This animal model, therefore, answers the question of what happens when a specific gene is absent. Since the phenotype of a knockout mouse provides important clues about the normal role of a specific gene, one major application of this technology is the modelling of human diseases caused by the loss of a specific gene function, e.g. cystic fibrosis, beta-thalassaemia and various forms of cancer (Barinaga 1992; Paszty 1997).

After constructing a mutant from a preselected target gene, the knockout is achieved by removing the functional copy of the gene for the mutated version in mouse embryonic stem cells. Mice that derived from these modified embryonic stem cells carry the same mutation in every cell. As one goal of conventional knockout technology is to knockout both alleles of a gene from all cells, an additional round of breeding is required to produce mice that are homozygous for the mutation. In traditional constitutive knockout mice, the mutation is present throughout development and in all cells of the adult. Although the knockout technology is highly advantageous for both biomedical research and drug development, it reveals also a number of limitations. Due to developmental defects, many knockout mice die in the course of embryogenesis and even before the investigator has the chance to use this animal model for experimentation. Even if a mouse survives, several mouse models have somewhat different physical, physiological and phenotypic traits than their human counterparts. Although the p53 gene is known to be implicated in as many as half of all human cancers, p53 knockout mice develop a completely different range of tumours than do humans. In particular, mice develop lymphomas and sarcomas, whereas humans tend to develop epithelial cell-derived cancers (Philipp-Staheli et al. 2004). Therefore, it cannot be assumed that a particular gene will exhibit identical function in both mouse and human and, thus, it limits the utility of knockout mice as models of human diseases.

Recently, new technologies have been developed which allow for the production of conditional knockouts, also known as tissue-specific gene targeting. The purpose of conditional knockouts is to delete a specific gene solely in a particular organ, cell type or stage of development. Researchers use this technique to knock out certain portions of specific genes at particular times when those genes

seem to be important. Conditional knockout mice are often superior to constitutive knockout mice, since they display a longer life expectancy. Furthermore, conditional knockout methods are more precise.

There are different possibilities to create conditional knockout mice; however, the most widely used method that allows mutations to be induced at different stages of development or in selected cell types in vivo is the Cre/loxP recombinase system (Nagy 2000).

Cre is a 38-kDa recombinase protein from the bacteriophage P1 which mediates site-specific recombination between loxP sites (Hamilton and Abremski 1984; Sauer 1993; 2002). This is a 34-bp consensus sequence consisting of a core spacer sequence of 8 bp and two 13 bp palindromic flanking sequences. Either one Cre molecule binds per inverted repeat, or two Cre molecules line up at one loxP site, while the recombination occurs in the asymmetric spacer region. These 8 bp are also responsible for the directionality of the site (Fig. 14). Two loxP sequences in opposite orientation to each other invert the intervening piece of DNA, two sites in direct orientation dictate excision of the intervening DNA between the sites leaving one loxP site behind. This precise removal of DNA can be used to eliminate an endogenous gene or transgene, which makes the Cre/loxP system an interesting tool for tissue-specific knockout of genes that cannot be investigated in differentiated tissues because of their early embryonic lethality in mice with conventional knockouts. It can also be used for the removal of a transgene which has been overexpressed in a specific tissue at a certain time point to study the invert effect of downregulation of the transgene in a time course experiment.

The general strategy for creating a conditional knockout mouse is as follows: Two mouse lines are required for the conditional gene deletion. Firstly, a conventional transgenic mouse line with Cre targeted to a specific tissue or cell type and, secondly, a mouse strain that embodies a target gene (endogenous gene or transgene) flanked by two loxP sites in a direct orientation, the so-called floxed gene. Recombination, namely excision and consequently inactivation of the target gene, occurs only in those cells expressing the Cre recombinase. Hence, the target gene remains active in all cells and tissues which do not express Cre (Fig. 15).

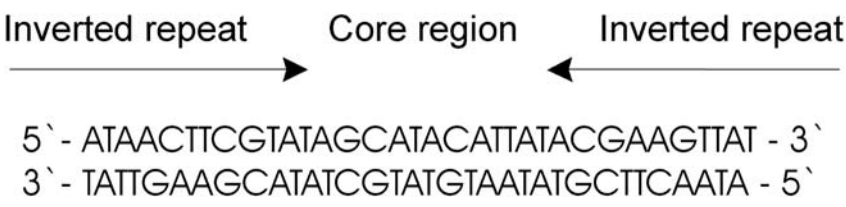


Fig. 14 The loxP sequence consists of two 13-bp inverted repeats and an 8-bp asymmetrical core spacer region, which determines the orientation of the site. The recombination reaction is initiated by Cre binding specifically to the inverted repeat sequences at the loxP sites

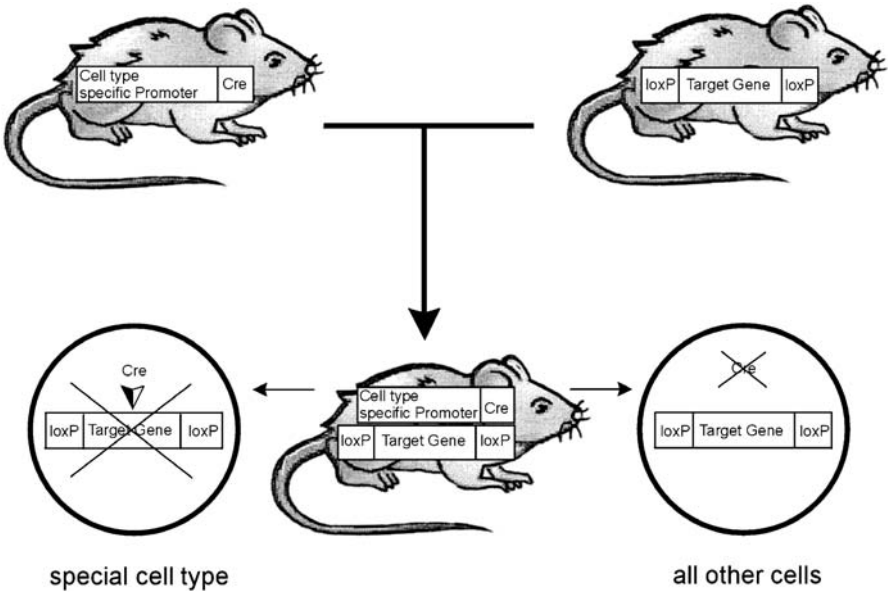


Fig. 15 Example of Cre/loxP mouse breeding. Transgenic mice containing a gene surrounded by loxP sites are mated with transgenic mice that express the Cre gene under the control of a cell type-specific promoter. The resulting mice have both the Cre gene and the loxP flanked gene. In tissues without a Cre gene, the target gene will be present and functions normally. However, in the cells where Cre protein is expressed, the target gene of interest will be specifically deleted

2.5.1 Anti-Müllerian Hormone Mutants

Gene knockout experiments in mice confirmed that AMH is necessary and sufficient for regression of the Müllerian ducts (Behringer et al. 1994). In male mice lacking a functional AMH gene, the Müllerian ducts remained. Although testes of these mice develop normally, descend normally and produce spermatozoa, many male individuals remain sterile. It appears that persistence and development of the Müllerian ducts into adulthood interferes with sperm transport out of the testes. The testes also exhibited hyperplasia of interstitial Leydig cells and, in one case, even neoplasia, suggesting that AMH acts as a negative regulator of Leydig cell proliferation and may also be active in adults to prevent proliferation of these testosterone-secreting cells.

By eliminating the Wolffian ducts and using mice with the androgen insensitivity syndrome (testicular feminization, Tfm), double mutants lacking both the AMH and AR have been generated (Behringer et al. 1994). It has been demonstrated that complete morphogenesis of the oviduct requires the elimination of the Wolffian ducts. XY Tfm/AMH double mutants develop like females showing an uterus, coiled oviducts and without male reproductive organs except undescended dys-

functional testes. In addition, AMH has been demonstrated to induce mesonephric cell migration in XX gonads, whereas AMH-deficient embryos exhibited no abnormalities during early stages of testicular development (Ross et al. 2003). In $AMH^{-/-}$ gonads at 11.5 days postcoitum (dpc), germ cell migration, as well as early morphological and vascular development, was not impaired. In addition, expression of Sox9, an early marker of Sertoli cell differentiation, was not disrupted in gonads at 12.5 dpc, suggesting that AMH is not required for theses processes in XY gonads. Therefore, if AMH plays a physiological role during normal testicular morphogenesis, it may be redundant with other factors (Ross et al. 2003). Deficiency of either the AMH or the AMH receptor causes the male pseudohermaphroditism syndrome, also referred to as the persistence of the Müllerian duct syndrome (PMDS). This syndrome is similar to that seen in the AMH knockout mouse except that in approximately 70% of the PMDS patients the testes do not descend properly (Guerrier et al. 1989; Carre-Eusebe et al. 1992; Imbeaud et al. 1996).

2.6 Cytokeratin 18 Mutants

Given the fact that both cytokeratin 8 and cytokeratin 18 are coexpressed and represent subunits of the same intermediate filament group throughout embryonic development and many adult epithelia (Jackson et al. 1980), it might be assumed that corresponding knockout mice exhibit a similar phenotype. However, midgestational death accompanied by liver haemorrhage or colorectal hyperplasia in adults was detected in cytokeratin 8 knockout mice (Baribault et al. 1993, 1994), whereas cytokeratin 18-deficient mice were found to be viable, fertile and exhibit a normal life expectancy. In young cytokeratin 18-null mice, hepatocytes were completely devoid of keratin filaments and old mice developed a distinctive liver pathology with abnormal hepatocytes containing cytokeratin 8-positive aggregates (Magin et al. 1998). So far, mutations of human cytokeratin 18 have been associated with cryptogenic and non-cryptogenic cirrhosis and other forms of liver disease (Ku et al. 1997, 2003), but not with reproductive disorders.

2.7 Vimentin Mutants

Homozygous vimentin knockout mice develop and reproduce without any obvious phenotype, showing that an important developmental and cell-specific structure that is known to be an integral part of the cytoskeleton can be eliminated without apparent effects on mouse reproduction and development (Colucci-Guyon et al. 1994). However, the first evidence for an abnormal phenotype in these mutant animals was documented for a subset of astrocytes, wherein the lack of vimentin network precluded the formation of an organized glial fibrillary acidic protein (GFAP) network (Galou et al. 1996). Furthermore, a cerebellar defect and an impaired motor coordination has been observed in mice lacking vimentin (Colucci-Guyon et al.

1999), as well as deficiencies in the modulation of vascular tuning (Terzi et al. 1997) and the mechanotransduction of shear stress (Henrion et al. 1997). Finally, $Vim^{-/-}$ mice demonstrated that this intermediate filament is not associated with growth and differentiation of teratocarcinomas (Langa et al. 2000). So far, no mutations of vimentin intermediate filament influencing reproduction have been detected in humans.

2.8 p27Kip1 Mutants

Knockout of the p27Kip gene resulted in multiple organ hyperplasia in immune, nervous and reproductive systems. Mice are viable and larger than normal littermates, but exhibit increased cellularity of all tissues. The thymus and spleen were found particularly enlarged. Nullizygous adult mice reveal a shortened lifespan due to the growth of benign intermediate lobe pituitary tumours indicating that loss of this cyclin-dependent kinase inhibitor may contribute to carcinogenesis and tumour progression. Interestingly, female mice are infertile with a follicular phase ovulatory block. Large doses of exogenous gonadotropin induced ovulation, but both implantation and intrauterine embryonic development were impaired. In addition, p27Kip1 knockout mice have twofold larger testes than their wild-type littermates (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996; Beumer et al. 1999). Mutations of the p27Kip1 gene are rare in human tumours (Ponce-Castaneda et al. 1995; Ferrando et al. 1996; Spirin et al. 1996; Takeuchi et al. 1998).

In testes of p27Kip1-deficient mice, aberrations in the spermatogenic process were observed (Beumer et al. 1999). It was found that its expression in adult Sertoli cells may be important in the regulation of proliferation or apoptosis of undifferentiated spermatogonia and the start of the meiotic prophase. On the one hand, an increase in the number of A spermatogonia was found, on the other hand, abnormal (pre)leptotene spermatocytes were observed, some of which probably entering mitotic division instead of entering meiotic prophase. These results indicate that p27Kip1 may have a role in the regulation of spermatogonial proliferation, apoptosis and/or the onset of meiotic prophase in preleptotene spermatocytes. However, as p27Kip1 is only expressed in Sertoli cells, its role in both spermatogonia and preleptotene spermatocytes is supposed to be indirect (Beumer et al. 1999). In the adult testis, all Sertoli cells are terminally differentiated and stain for p27Kip1 in both mouse and human. Although this suggests that p27Kip1 plays a role in the quiescence of adult Sertoli cells, in $p27Kip1^{-/-}$ mice, no signs of altered Sertoli cell proliferation have been observed, suggesting that the role of p27Kip1 in the arrest of proliferation and cell division of adult somatic Sertoli cells may be redundant (Beumer et al. 1999). However, the increased testis size in p27Kip1 knockout mice may be caused by increased prepubertal Sertoli cell proliferation (Beumer et al. 1999).

The human p27Kip1 gene is localized within the 12p12–13.1 locus (Pietenpol et al. 1995; Ponce-Castaneda et al. 1995), which is often the subject of multiplication

and rearrangements in testicular tumours (Sandberg et al. 1996). Another hypothesis on the origin of germ cell tumours is that they have a spermatocyte origin (Chaganti et al. 1994). As in the p27Kip1 knockout testis, preleptotene spermatocytes that attempt to enter mitotic division can be seen, indicating that p27Kip1, via somatic Sertoli cells, could play a role in the development of spermatocytes just at the time that they may be vulnerable to oncogenic transformation. Therefore, p27Kip1 might be an additional factor in male germ cell tumourigenesis (Beumer et al. 1999).

2.9 Androgen Receptor Mutants

Generation and characterization of male AR knockout (ARKO) mice confirmed the necessity of AR signalling for male phenotype development (Yeh et al. 2002; Matsumoto et al. 2003). Male ARKO mice displayed feminized external genitalia and reduced body weight. Internally, the accessory sex organs were absent and the testes were cryptorchid and appeared to contain a reduced number of germ cells and seminiferous tubules with decreased diameter than the wild-type AR testis. In contrast to the normal compliment of germ cells in various stages of maturation including late stage elongated spermatids observed in wild-type animals, some tubules in the ARKO sections lacked germ cells, while others contained only a few germ cells revealing spermatogenic arrest at the level of spermatocytes (Yeh et al. 2002). In Tfm mice, a strain lacking a functional AR in all cell types, Leydig cells develop through an initial fetal stage and are later replaced by an adult population in the adult mouse (Vergouwen et al. 1991). Furthermore, it has been demonstrated that fetal Leydig cells develop and function normally, whereas adult Leydig cells fail to mature properly (O'Shaughnessy et al. 2002).

Evidence for roles of the AR relevant to male gonadal development and spermatogenesis has been generated through the study of AR-deficient mice (O'Shaughnessy et al. 2002; Yeh et al. 2002); however, due to the developmental roles of the AR in establishing the male phenotype, the disruption of the AR throughout an experimental animal does not allow the study of AR function specifically in cell types intimately involved in the spermatogenic process. To determine whether it is the early developmental consequence of loss of AR signalling that causes the spermatogenic defects in AR-deficient mice or a combination of these developmental abnormalities and the dysregulation of AR function in specific testis cell types, disruption of AR function exclusive to somatic Sertoli cells has been studied (De Gendt et al. 2004). Using the Cre/loxP technology, a Sertoli cell-selective knockout (SCARKO) of the AR was generated. It has been demonstrated that, in contrast to constitutive AR knockout mice, SCARKOs exhibited normal urogenital tracts and normally descended testes. However, from puberty onwards, these conditional knockout mice displayed a spermatogenic arrest at the spermatocyte/spermatid stage, indicating that cell autonomous action of the AR in Sertoli cells seems to be an absolute requirement for androgens to maintain complete spermatogenesis.

Complete AR knockout mice displayed a complete androgen insensitivity syndrome phenotype as testes were found to be abdominally located and germ cell development was severely disrupted (De Gendt et al. 2004).

In humans, it is known that defects in male sexual differentiation in 46,XY individuals show an X-linked pattern of inheritance. The AR gene is located on the X chromosome at Xq11–12 and was reported in families with severe hypospadias, infertility and gynaecomastia (Reifenstein et al. 1947). The end-organ resistance to androgens has been designated as androgen insensitivity syndrome and has to be differentiated from other forms of male pseudohermaphroditism, such as 17β-hydroxysteroid dehydrogenase type 3 deficiency, Leydig cell hypoplasia due to inactivating LH receptor mutations or 5α-reductase type 2 deficiency (Wilson et al. 1974, 1993; Brinkmann 2001). Moreover, different types of mutations have been detected in DNA of individuals with androgen insensitivity syndrome. It is generally accepted that defects in the AR gene can prevent the normal development of both internal and external male structures in 46,XY individuals, and information on the molecular structure of the human AR gene has facilitated the study of molecular defects associated with androgen insensitivity. Due to the X-linked character of the syndrome, only 46,XY individuals are affected, while in female carriers, only sporadic reports are available on delayed menarche (Sai et al. 1990). The variation in clinical phenotypes provides the opportunity to correlate a mutation in the AR structure with the impairment of a specific physiological function.

Two main clinical features of androgen insensitivity syndrome can be discriminated: complete androgen insensitivity syndrome (CAIS) and the partial androgen insensitivity syndrome (PAIS). The main phenotypic characteristics of individuals with CAIS are female external genitalia, a short blind ending vagina, absence of Wolffian duct-derived structures like epididymides, vasa deferentia and seminal vesicles, the absence of a prostate, the absence of pubic and axillary hair and the development of gynaecomastia (Quigley et al. 1995; Boehmer et al. 2001). Müllerian duct-derived structures are usually absent, because AMH action is normally due to the presence of both testes in the abdomen or in the inguinal canals. In the PAIS, several phenotypes are found, ranging from individuals with predominantly female appearance (e.g. external female genitalia and pubic hair at puberty or with mild clitoromegaly and some fusion of the labia) to persons with ambiguous genitalia or individuals with a predominantly male phenotype known as Reifenstein syndrome (Quigley et al. 1995; Boehmer et al. 2001). Patients from this last group can present a micropenis, perineal hypospadias and cryptorchidism. Within PAIS individuals, Wolffian duct-derived structures can be partially to fully developed depending on the biochemical phenotype of the AR mutation. At puberty, elevated LH, testosterone, and estradiol levels are observed, but in general, the degree of feminization is less than in individuals with CAIS. Furthermore, two additional pathological situations are associated with abnormal AR structure and function: spinal and bulbar muscular atrophy and prostate cancer (La Spada et al. 1991; Newmark et al. 1992).

2.10 Inhibin-α Mutants

Generation of knockout mice lacking the α-subunit of inhibin revealed that both male and female inhibin-deficient mice develop sex cord stromal tumours of the Sertoli cell/granulosa cell lineage, demonstrating the supposed tumour-suppressing functions of inhibin in the gonads (Matzuk et al. 1992; Yan et al. 2003). Inhibin-deficient mice die eventually of a severe, progressive, cancer cachexia-like wasting syndrome (Matzuk et al. 1994). In addition, these mutant mice show elevated serum levels of FSH, estradiol and activins A and B as tumour progresses (Matzuk et al. 1994, 1996). Interestingly, gonadectomized inhibin-deficient male and female animals develop adrenal cortical tumours with a 100% penetrance and eventually die from a similar, but delayed wasting syndrome (Matzuk et al. 1992).

Male mice lacking both inhibin and AMH develop earlier testicular tumours. Leydig cell neoplasia is observed as early as 1 week of age and tumours grow faster, but are less haemorrhagic (Matzuk et al. 1995; Mishina et al. 1996). These results suggest that AMH may act as an autocrine-modifying factor in Sertoli cell tumourigenesis.

In testicular tumours of inhibin-deficient mice, the expression levels of p27 are decreased, implicating that the deregulation of this cell cycle regulator may contribute to tumour formation (Cipriano et al. 2001). By crossing p27 mutant and inhibin mutant mice, double mutants lacking both tumour suppressors have been generated (Yan et al. 2003). These double knockout males died earlier than inhibin$^{-/-}$ single mutants, and Sertoli cell tumours could be observed as early as 2 weeks of age compared with 4 weeks in inhibin single mutant males. These data demonstrated that p27 may be interpreted as an inhibitory modifier of testicular tumourigenesis in the absence of inhibin (Cipriano et al. 2001; Burns et al. 2003).

Generation of double mutant mice deficient in both inhibin and the AR by intercrossing the Tfm mice with inhibin-α mutant mice (Shou et al. 1997) revealed that androgen signalling was not required for testicular tumour development in inhibin-deficient mice, but could play a regulatory role in testicular tumour progression. Two thirds of the double mutant males displayed less haemorrhagic testicular tumours and half of these mice survived longer than 17 weeks (compared to 12 weeks in males lacking inhibin-α alone). In man, it has been shown that mutations of the inhibin-α gene may play a role in ovarian dysfunction (Shelling et al. 2000; Marozzi et al. 2002).

2.11 Connexin Mutants

For the elucidation of the role and contribution of a specific connexin protein to the function of gap junctions in molecular physiology during embryonic development and/or in the adult organism in vivo, different connexin knockout, knockin and transgenic mouse models have been generated (Willecke et al. 2002).

The first targeted connexin defect described led to altered cardiac morphology and perinatal death in connexin 43-deficient mice (Reaume et al. 1995). Obstruction of the right ventricular outflow tract of the heart prevented regular blood flow into the lungs and hence limited the blood oxygeneration (Reaume et al. 1995; Willecke et al. 2002). The observed lethal heart dysmorphogenesis is thought to be largely caused by a lack of connexin 43 in cardiac neural crest cells (Huang et al. 1998). Targeted ablation of the connexin 26 gene led to embryonic death on embryonal day 11 (Gabriel et al. 1998). In the labyrinth region of the mouse placenta, two adjacent cell layers are connected by connexin 26 and separate embryonic from maternal blood vessels. It is supposed that connexin 26-containing channels are involved in the diffusion of glucose phosphate and other metabolites derived from the nutrients in maternal blood to embryonic blood and/or removal of waste products from the embryo to the mother (Gabriel et al. 1998; Willecke et al. 2002).

In order to circumvent perinatal lethality and pleiotropic effects of the general connexin 43 deficiency and to investigate the role of connexin 43 specifically in endothelial cells, a mouse line was generated that carries the floxed connexin 43 coding region flanked by loxP recognition sites for the Cre recombinase (Theis et al. 2000, 2001; Willecke et al. 2002). Endothelial cell-specific deletion was then achieved by crossing these floxed mice with mice harbouring the Cre transgene under control of endothelial cell-specific TIE2 transcriptional elements (Schlaeger et al. 1997; Theis et al. 2001). It was shown that mice lacking connexin 43 in endothelium did not exhibit changes in blood pressure (Theis et al. 2001).

Connexins may also play a specific functional role in the development of germ cells. Deletion of the connexin 37 coding region, for example, led to a defect in oocyte development before meiotic competence was reached and no transfer of lucifer yellow to granulosa cells of the surrounding follicle cells was observed when this substance was microinjected into connexin $37^{-/-}$ oocytes (Simon et al. 1997). The importance of connexin 43 to gametogenesis and proper development of the gonads is indicated by severe depletion of germ cells in prenatal male and female mice lacking the connexin 43 gene (Juneja et al. 1999). Postnatal proliferation of spermatogonia is also impaired in connexin 43-null mutants (Roscoe et al. 2001). Insertion of connexin 32 or connexin 40 coding regions into connexin 43 coding region of connexin $43^{-/-}$ mice restored oogenesis and other deficiencies caused by connexin 43 deletion (Reaume et al. 1995), but spermatogonial amplification and spermatogenesis remained defective (Plum et al. 2000).

In addition to the generation and characterization of targeted connexin defects in the mouse, phenotypic alterations in identified human connexin mutants may be helpful to offer further insights into the molecular physiology of gap junctions. So far, many different mutations have been identified in the human connexin 32 gene causing Charcot-Marie-Tooth disease (X-type) (CMTX), an inherited demyelination disorder of the peripheral nervous system characterized by progressive wasting of distal muscles in the limbs (Bergoffen et al. 1993; Willecke et al. 2002).

Interestingly, CMTX patients do not exhibit abnormalities in other organs, like the liver, pancreas or brain, where connexin 32 is also expressed. It remains to be elucidated whether defects described in connexin 32-deficient mice, such as decreased glycogen degradation or increased tumourigenesis and carcinogenesis (Temme et al. 1997, 2001; King and Lampe 2004), can be functionally compensated by other connexins that are coexpressed in the corresponding human organs and cell types.

Mutations in the human connexin 26 gene are known to cause the most frequent non-syndromic sensorineural hearing defect in humans (Kelsell et al. 1997). The corresponding conditional connexin 26-deficient mice also show severe hearing impairment and may thus be used as an animal model (Cohen-Salmon et al. 2002) of the human inherited disease. Some human connexin 26 patients exhibit, in addition, alterations in the epidermis (Richard et al. 1998), where connexin 26 can be found to be expressed in keratinocytes.

For connexin 43, Britz-Cunningham et al. (1995) reported single nucleotide exchanges in PCR-amplified coding DNA from the original hearts of young children who suffered from the visceroatrial heterotaxia syndrome and had undergone heart transplantation. Transfection of the mutated connexin 43 coding DNA into a mouse cell line defective in gap junctional communication yielded differences in dye transfer compared to wild-type connexin 43. The authors suggested that impaired junctional permeabilities of connexin 43 channels in the original hearts of these patients could have caused the symptoms of the disease (Britz-Cunningham et al. 1995; Willecke et al. 2002). However, no connexin 43 mutations were found in other cases of visceroatrial heterotaxia (Gebbia et al. 1996; Penman-Splitt et al. 1997). In addition, mutations of connexin 43 have been detected at advanced stages of colon sporadic adenocarcinomas, indicating that mutational alterations are involved in progression of human colon cancer towards malignancy (Dubina et al. 2002), and connexin 43 mutations may be a possible cause of the pleiotropic phenotype of oculodentodigital dysplasia (Paznekas et al. 2003).

3
The Differentiation of Male Germ Cells

3.1
The Regulation of Gene Expression During Prespermatogenesis

Wartenberg (1981) and Hilscher (1991) classified fetal germ cells on the basis of histological criteria and their expected temporal sequence of appearance in the developing testis as follows:

- Between the 6th and 8th gestational week, primordial germ cells (PGCs) migrate from the yolk sac to the primitive seminiferous cords and are then called gonocytes. These small cells present an oval nucleus and pale cytoplasm and can be observed between the 8th and 12th gestational week.

- Proliferating M-prespermatogonia possess a round nucleus and can be observed between the 13th and 16th gestational week.
- T_1-prespermatogonia do not proliferate and are present between the 17th and 23rd gestational week. They are large cells with round nuclei and prominent nucleoli and clear cytoplasm.
- Proliferating T_2-prespermatogonia possess a round nucleus with nucleolus and can be observed between the 25th and 35th gestational week.

Applying immunohistochemistry with OCT-4, c-KIT and MAGE-A4, Gaskell et al. (2004) defined three germ cell populations within the fetal testis, namely gonocytes, intermediate germ cells and prespermatogonia. Subsequently, Franke et al. (2004) demonstrated that M2A antigen, germ cell alkaline phosphatase (GCAP) and somatic angiotensin converting enzyme (sACE) represent characteristic features of specific stages of fetal germ cell differentiation. While M2A antigen is expressed in PGCs, M- and T_1-prespermatogonia, GCAP and sACE is solely present in M- and T_1-prespermatogonia. In addition, GCAP-positive germ cells have already been reported in testes of neonates and infants (Armstrong et al. 1991; Jorgensen et al. 1993). None of these markers can be observed in T_2-prespermatogonia. Results in man are in line with previous data in the mouse (Hofmann and Millan 1993).

The non-uniform entrance of male germ cells in proliferation and subsequent maturation may explain both the varying proportions of immunoreactive germ cells during the course of testicular development and the continuous increase of those germ cells being negative for all of the applied markers towards the end of testicular development. These data may, in addition, be important in the understanding of the pathology of human germ cell tumours, since neoplastic germ cells of virtually all intertubular germ cell neoplasia (IGCN) or carcinoma in situ (CIS) are known to express M2A antigen, GCAP and sACE (Jacobsen and Norgaard-Pedersen 1984; Burke and Mostofi 1988; Bailey et al. 1991; Giwercman et al. 1991; Franke et al. 2000). Interestingly, all of these markers are associated with the differentiation stage of M- and T_1-prespermatogonia, but not with that of PGCs or T_2-prespermatogonia. Transformed germ cells of IGCN or CIS thus resemble, molecularly, prespermatogonia and, cytologically, T_1-prespermatogonia. This is consistent with the current concept that neoplastic transformation takes place in germ cells persisting at a fetal stage of differentiation (Jorgensen et al. 1995; Skakkebaek et al. 1998), but may not necessarily support the conclusion that transformation already occurs during early embryogenesis, since germ cells exhibiting features of M- and T_1-prespermatogonia could be observed during the whole prenatal testicular development and even in childhood. Similarities between fetal and neoplastic germ cells, therefore, may inform on the differentiation stage of a single progenitor cell rather than the time where its neoplastic transformation into IGCN occurs. Data suggest that the expression of these fetal markers is confined to distinct stages of germ cell differentiation in the otherwise not strictly time-related human prespermatogenesis.

3.2
The Regulation of Gene Expression During Spermatogenesis

3.2.1
Sequential Gene Expression of Nucleoproteins and the Outstanding Role of Haploid Spermatids

Germ cells are remarkably different from somatic cells: They undergo meiosis, represent a haploid genome and give rise to totipotent diploid zygotes. Therefore, it is crucial that the male germ cell genome is free of errors. To ensure accurate transmission of the genome, male germ cells have evolved a specialized transcription machinery. The increased expression of transcription factors and polymerases in round spermatids (Schmidt et al. 1997) reflects the greater need for the transcription machinery to fulfil the requirement for the rapid and large amount of transcription in preparation for chromatin condensation which is followed by a complete stop of gene expression in elongating spermatids. In addition, there are many testis-specialized protein homologues that serve as components of both the transcription and the translation machinery, especially in haploid spermatids.

The differentiation of spermatogonial stem cells into fertile sperm requires stringent temporal and stage-specific gene expression. This becomes evident when studying the sequential nucleoprotein expression in developing germ cells resulting in histone-to-protamine exchange in haploid spermatids (Steger 1999). First, part of the somatic histones are replaced by testis-specific histones. Subsequently, transition proteins occur together with testis-specific histones. Finally, both histones and transition proteins are replaced by protamine-1 and protamine-2. In mice, transcripts of testis-specific histone H1t are expressed in mid and late pachytene spermatocytes, while the corresponding proteins are present from mid pachytene spermatocytes to elongated spermatids (Drabent et al. 1996). Transcripts of transition proteins and protamines are expressed in spermatids of step 7–13 and 7–15, respectively. However, the corresponding proteins are synthesized, with temporal delay, in spermatids of step 12–14 and 13–16, respectively (Mali et al. 1989; Yelick et al. 1989; Alfonso and Kistler 1993) (Figs. 16, 17).

In spermatogonia and spermatocytes, like in somatic cells, the DNA double helix is wound around nucleosomes, histone octamers consisting of two molecules of H2A, H2B, H3, and H4 each. Histone H1 is localized between the nucleosomes. Histone-bound DNA is further coiled into solenoids with six nucleosomes per turn (Finch and Klug 1976). The highly conserved nature of histone proteins and the various possibilities of posttranslational modifications, such as acetylation, phosphorylation, methylation and ubiquitination (Bradbury 1992), which can alter the charges, conformation and strength of binding to DNA, reinforce the fundamental regulatory role of histone modifications on chromatin structure.

There are two classes of enzymes which are involved in determining the state of histone acetylation, histone acetyl transferases (HATs) and histone deacetylases (HDACs) (Davie 1998). Substrates for these enzymes include amino groups of lysine residues located in the amino-terminal tails of core histones reducing the

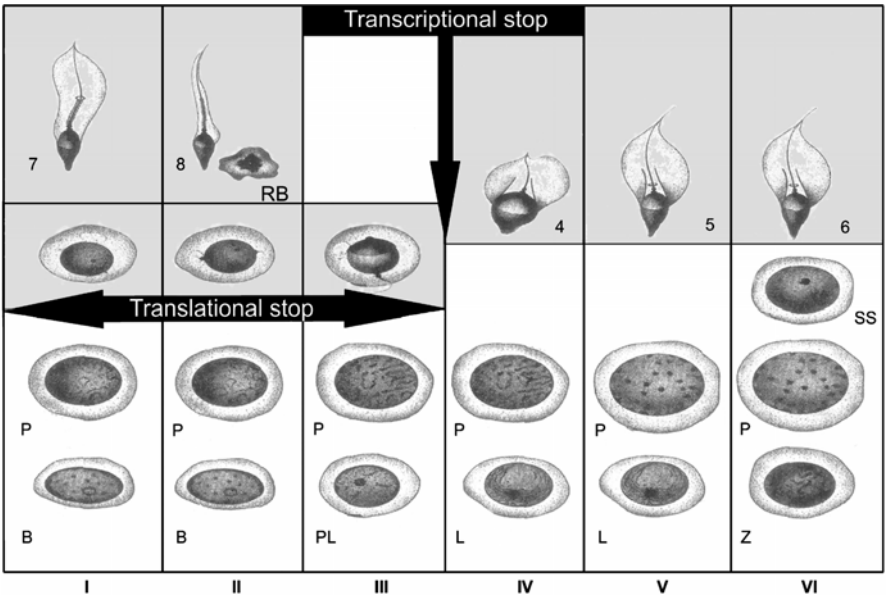


Fig. 16 One characteristic of haploid spermatids is the temporal uncoupling of transcription and translation. As a consequence, transcripts and proteins from a specific gene exhibit different but stage-specific expression patterns. For protamine-1 and protamine-2, mRNA (*bright shadow*) is expressed in the cytoplasm of step 1 to step 3 spermatids, while the corresponding protein (*dark shadow*) is synthesized, with temporal delay, in the nucleus of step 4 to step 8 spermatids. The translational stop is due to the binding of protein repressors to both the 3′-untranslated region (UTR) and the poly-A tail of the transcripts (silent mRNA), whereas the transcriptional stop is caused by a protamine–DNA interaction followed by a strong chromatin condensation. *B*, B-type spermatogonia; *PL*, preleptotene spermatocytes; *L*, leptotene spermatocytes; *Z*, zygotene spermatocytes; *P*, pachytene spermatocytes; *SS*, secondary spermatocytes; RB, residual body

electrostatic alteration between histones and DNA. Subsequently, the basic nature of histones is somewhat neutralized, which decreases their affinity for DNA within the nucleosomes facilitating histone removal (Meistrich et al. 1992). Recently, a detailed analysis of waves of histone acetylation and deacetylation during mouse spermatogenesis has been reported demonstrating a strong decrease in histone deacetylases in elongating spermatids, the stage of spermiogenesis where histone-to-protamine exchange takes place (Hazzouri et al. 2000). Although phosphorylation is not as well understood as acetylation, recent studies suggest a coordinate pattern of histone modification. In vitro, phosphorylation of Ser-10 in histone H3 has been demonstrated to promote acetylation on nearby Lys-14, suggesting promoter-specific regulation by a kinase/acetyltransferase enzyme pair (Lo et al. 2000).

The role of transition proteins is still unclear and may, in addition, be different between transition protein-1 and -2. It has been reported that, in vitro, transi-

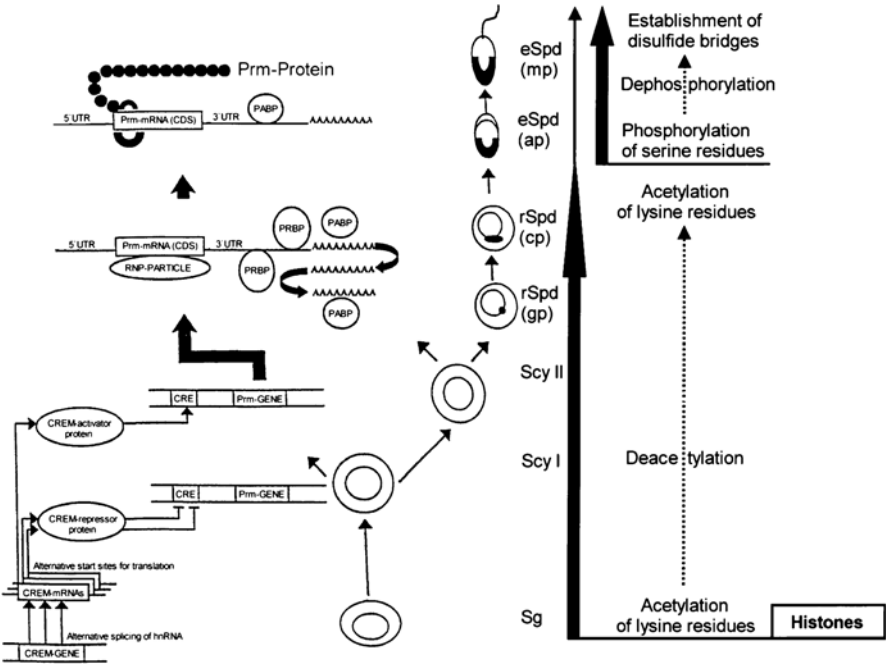


Fig. 17 Histone-to-protamine exchange during spermatogenesis. *Sg*, spermatogonia; *Scy I*, primary spermatocytes; *Scy II*, secondary spermatocytes; *rSpd*, round spermatids; *eSpd*, elongated spermatids; *gp*, Golgi phase; *cp*, cap phase; *ap*, acrosome phase; *mp*, maturation phase; *Prm*, protamine; *CRE*, cAMP-responsive element; *CREM*, cAMP-responsive element modulator; *CDS*, coding sequence; *RNP*, ribonucleoprotein; *UTR*, untranslated region; *PABP*, poly-A binding protein; *PRBP*, protamine mRNA binding protein

tion protein-1 decreases (Singh and Rao 1987) and transition protein-2 increases (Kundu and Rao 1995) the melting temperature of DNA, causing an decrease or increase of the DNA–histone interactions, respectively. Since the frequency of DNA strand breaks becomes less prominent as the level of transition protein-1 increases, this protein has, by contrast, been suggested to enhance local DNA–DNA interactions by neutralizing the negative charges of the phosphate backbone (Levesque et al. 1998). Recently, transition protein-1 has been demonstrated to stimulate resealing of DNA single strand breaks in vitro and be involved in DNA repair processes in vivo (Caron et al. 2001). It is assumed that transition protein-1 acts as an alignment factor holding the broken DNA ends together until an as-yet unidentified ligase bridges the gap.

Protamines are the nucleoproteins of elongated spermatids and mature spermatozoa exhibiting several characteristic features:

– In mice, protamine-1 is synthesized as a mature protein of 50 amino acids, whereas protamine-2 is generated from a precursor of 106 amino acids (Yelick et al. 1987).

- Both protamine-1 and protamine-2 are highly basic proteins resulting from an unusually high content of arginine residues. The lack of conservation suggests that the overall basicity of these proteins is more important to their function than a particular amino acid sequence (Balhorn et al. 1984).
- Protamines can be modified by the addition of phosphate groups to serine residues. While serine-arginine (SR) protein-specific kinase-1 phosphorylates Ser-10 and, to a lesser extent, Ser-8 of protamine-1 (Papoutsopoulou et al. 1999), Ca^{2+}-dependent protein kinase-IV (Camk4) phosphorylates Ser-14 of protamine-2 (Wu et al. 2000). Protamine phosphorylation is assumed to facilitate the correct binding of protamines to DNA, while subsequent dephosphorylation increases the positive charge and attraction for DNA and thus is associated with an increase in sperm chromatin condensation.
- Protamines contain cysteine residues which set up disulfide linkages making the sperm nucleus a highly insoluble and chemically stable structure (Bedford and Calvin 1991).
- Protamines bind zinc through cysteine and histidine side chains and thus could be considered as zinc finger proteins representing one C2H2 motif (Reinicke and Chevaillier 1991).

Protamines bind lengthwise within the minor groove of the DNA double helix with their central polyarginine segment crosslinking and neutralizing the phosphodiester backbone of the DNA. These DNA–protamine complexes of one DNA strand fit exactly into the major grooves of a parallel DNA strand and are packed side by side in a linear array within the sperm nucleus (Balhorn 1982). In addition to DNA binding, protamine molecules interact with other protamine molecules by forming disulfide bonds between cysteine residues, thereby facilitating DNA compaction (Ward and Coffey 1991). Ward (1993) created a model for the packing of the entire haploid genome into the sperm nucleus in which DNA loop domains are packed as doughnuts attached to the sperm nuclear matrix. Protamine-bound DNA is coiled into large concentric circles that collapse into a doughnut in which the DNA–protamine complexes are tightly packed together by van der Waals forces.

In man, the replacement of histones appears in haploid spermatids and is only about 85% complete (Gatewood et al. 1987). The human genes for protamine-1 and protamine-2 are both mapped to chromosome 16p13.3 (Domenjoud et al. 1990), encoding a 50-amino acid protein and a 54-amino acid protein, respectively, which are both rich in arginine and cysteine. The protamine-1 specific sequence of alanine-arginine-tyrosine-arginine-cysteine is localized in the N-terminal region. Protamine-2 contains some strongly conserved sequences, such as histidine-arginine-arginine, located at irregular intervals, while clusters of 3–4 arginine residues are regularly distributed along the molecule cysteine. While protamine-1 is synthesized as a mature protein, protamine-2 is synthesized as a precursor.

Protamine–DNA interactions result in chromatin condensation causing cessation of transcription in elongating spermatids. This occurs at a time when many

proteins need to be synthesized and assembled for the complete condensation of the chromatin, the development of the acrosome, and the formation of the flagellum. It is evident that precise temporal regulation of gene expression via transcriptional and translational control mechanisms is of fundamental importance to ensure complete differentiation of round spermatids into mature spermatozoa.

Histone acetylation and phosphorylation have already been mentioned to be involved in histone-to-protamine exchange. In addition, methylation of histones is highly significant to genetic regulation (Bradbury 1992). Addition of each methyl group eliminates one positive charge and reduces the strength of the electrostatic attraction of histones to DNA resulting in conversion of genes from an inactive to active form. DNA methylation of specific cytosines following a guanine in the sequence CpG (p represents the phosphate group connecting C and G nucleotides) is performed by the enzyme DNA (cytosine-5)-methyltransferase (DNA MTase). In sperm, almost all available CpGs are fully methylated. The very few unmethylated CpGs occur in the promoter regions of genes, such as histones, which become active at very early embryonic steps following fertilization. This suggests that methyl groups may be removed from CpGs in 5′ flanking control regions well before genes actually become active in transcription.

3.2.2
Transcriptional Regulation of Gene Expression in Haploid Spermatids

Within the promoter region of protamine-1 and protamine-2, a sequence of 113 nucleotides (Zambrowicz et al. 1993) and 859 nucleotides (Stewart et al. 1988), respectively, have been demonstrated to be sufficient for specific gene expression in haploid spermatids. Furthermore, mutational analyses revealed two regulatory sequences for protamine-2 transcription, namely 5′ACAATCAATCAGG3′ at position −84/−72 and 5′CCGACAAGGTCACAG3′ at position −64/−48 (Yiu and Hecht 1997). The latter sequence contains the core motif 5′AGGTCA3′ recognized by orphan nuclear receptors (Enmark and Gustafsson 1996). Removal or alteration of one of these two sequences is followed by a significant reduction in the protamine-2 transcription, while binding of the protamine activating factor-1 (PAF-1) to site 1 and of the Y-box protein p48/p52 to site 2 induces a more than fivefold increase of protamine-2 transcription (Yiu and Hecht 1997). Y-box proteins (the name refers to a promoter element conserved in MHC class II genes with the sequence 5′CTGATTGGCCAA3′ containing an inverted CCAAT box) comprise a family of transcription factors which are general modifiers of gene activity. The consequence of their binding to promoter elements depends on the interaction with other tissue-specific regulatory proteins. Several genes, identified as functioning specifically in germ cells, have consensus Y-box sequences in their promoters, e.g. mouse protamine-1 at positions −226 and −100 (Zambrowicz et al. 1993) and mouse protamine-2 at positions −489, −178 and −72 (Yiu and Hecht 1997).

The promoter region of the genes for protamine-1 and protamine-2 contain both the TATA-box and the CRE-box (Oliva and Dixon 1991). The cAMP-responsive element (CRE)-box consists of the eight-nucleotide palindromic sequence 5′TGACGTCA3′ (Roesler et al. 1988) and is invariably present at position −57/−48 (Oliva and Dixon 1991).

Haploid spermatids have been demonstrated to contain roughly 1,000-fold more TATA-binding protein (TBP)-mRNA than somatic cells (Schmidt et al. 1997). The appearance of TBP overexpressing spermatids is accompanied by an increase in whole organ levels of total RNA. Quantitative analyses revealed that testis-specific overaccumulation of TBP-mRNA is caused by both modest upregulation of the somatic TBP promoter and, in addition, recruitment of at least two major and three minor testis-specific promoters.

Owing to alternative exon splicing, expression of the cAMP-responsive element modulator (CREM) gene results in the production of functionally different CREM proteins with either activating or repressing potential on target gene expression (Delmas et al. 1992) (Fig. 18). While CREM activator transcripts are characterized by the presence of the kinase-inducible domain (KID), at least one of the transactivation domains, and one of the DNA-binding domains, classical CREM repressor transcripts consist of the KID domain and one of the DNA-binding domains, but lack a transactivation domain (Walker et al. 1994). CREM is activated via phosphorylation of serine-117 by protein kinase K endogenous to germ cells (Walker and Habener 1996). Recent data, however, suggest a phosphorylation-independent mechanism of CREM activation in germ cells by activator of CREM in the testis (ACT) (Fimia et al. 1999).

Fig. 18 The CREM gene consists of 14 exons encoding different functional domains (Masquilier et al. 1993; Walker and Habener 1996; Daniel et al. 2000; Behr and Weinbauer 2001; Gellersen et al. 2002). Exons E and F encode the kinase-inducible domain (*KID*), being rich in serine residues which can be phosphorylated and thereby activated by several kinases. Exons C and G encode the transactivation domains Q1 and Q2 flanking the KID and being essential for protein–protein interactions. Exons H and Ia/Ib, respectively, encode the two alternatively used C-terminally located basic leucine zipper (*bZIP*) DNA-binding domains (*DBD Ia/Ib*). The bZIP domain itself consists of the basic region for protein–DNA interaction and the leucine zipper region for dimerization being essential for binding to the cAMP-responsive element (*CRE*). Owing to alternative exon splicing, CREM gene expression results in the production of functionally different CREM proteins with either activating or repressing potential on target gene expression. While CREM activator transcripts are characterized by the presence of the KID, at least one of the transactivation domains, and one of the DNA-binding domains, classical CREM repressor transcripts consist of the KID and one of the DNA-binding domains, but lack a transactivation domain

3.2.3
Translational Regulation of Gene Expression in Haploid Spermatids

Contrary to transcriptional regulation involving the binding of transcription factors within the promoter region of potential target genes, specific interactions of cytoplasmic protein repressors with the 3′-UTR of mRNAs play a key role in the regulation of translation and polyadenylation of transcripts in haploid spermatids. In a classical experiment, Braun et al. (1989) demonstrated that the 3′ most 62-nucleotide element of mouse protamine-1 mRNA 3′-UTR is sufficient for proper temporal and stage-specific translation of a reporter transgene, human growth hormone (hGH). Subsequently, it has been suggested that translational repression in round spermatids is achieved by the binding of sequence-specific RNA-binding proteins to this 62-nucleotide element followed by the assembly of mRNAs into ribonucleoprotein (RNP) particles, whereas translation in elongating spermatids is achieved by covalent modification of the RNP complex and release of translatable protamine-1 mRNA. The 62-nucleotide element contains both the polyadenylation signal 5′AAUAAA3′ 12 nucleotides upstream of the poly-A tail and a conserved 17-nucleotide motif just 5′ to the polyadenylation signal. The 17-nucleotide motif, in addition, is present in the 3′-UTR of protamine-2 mRNA. Here, this sequence has been denoted Z-box (Kwon and Hecht 1993). Surprisingly, the 62-nucleotide element does not contain sequences that have been identified to bind RNA-binding proteins, such as the Y-box. The 17-nucleotide motif within the 62-nucleotide element involves the conserved 8-nucleotide motif 5′GCCACCUG3′. This 8-nucleotide motif is present in the 3′-UTRs of protamine-1 mRNA and protamine-2 mRNA of both mouse and man, as well as mouse transition protein-1 mRNA but not human transition protein-2 mRNA. The absence of this conserved 8-nucleotide motif is proposed to be the reason for insufficient storage of transition protein-2 mRNA in man (Schlüter et al. 1993). RNA-binding proteins that have, so far, been identified can be divided into three groups, according to their RNA-binding domain:

- Repressor proteins of the first group, such as poly-A binding protein (PABP) (Hornstein et al. 1999), reveal a 90-amino acids ribonucleoprotein (RNP) motif, also known as RNA recognition motif (RRM) being present in one to four copies within the protein repressor. Within the RNP motif, the two most conserved sequences are known as RNP1 and RNP2 exhibiting the consensus sequences KGYGFVHF and NLYVKN, respectively (Adam et al. 1986).
- Repressor proteins of the second group, such as MSY2 (Gu et al. 1998), are members of the Y-box protein family containing a cold shock domain (CSD). In analogy to RNP1 and RNP2, the two most conserved sequences within the CSD are called RNP1-like and RNP2-like motifs exhibiting the consensus sequences NGYGFINR and DVFVHQ, respectively (Grauman and Marahiel 1998). Binding depends on phosphorylation (Herbert and Hecht 1999). The 18-kDa RNA-binding protein has been demonstrated to bind to the Y-box element within the 3′-UTR of both protamine-1 mRNA and protamine-2 mRNA (Kwon and Hecht 1993). The 48/52-kDa Y-box protein representing the murine homologue of

the *Xenopus* 54/56-kDa mRNA-binding protein FRGY2/mRNP$_{3+4}$ (Kwon et al. 1993) has been renamed mouse Y-box protein-2 (MSY2) (Oko et al. 1996). While pachytene spermatocytes contain primarily the 52-kDa subunit, round spermatids contain predominantly the 48-kDa subunit, suggesting that the 48/52-kDa Y-box protein may have temporally distinct regulatory functions (Kwon et al. 1993). Indeed, Y-box proteins are believed to be specific DNA-binding proteins and non-specific RNA-binding proteins at the same time. Therefore, they have the potential to regulate gene expression either as a transcription factor or as a modulator repressing translation of accumulating mRNAs (Matsumoto and Wolffe 1998).

– Protein repressors of the third group, such as protamine-1 RNA-binding protein (PRBP) (Lee et al. 1996), contain the 21-amino acids RNA-binding motif (RBM) GTGPSKKAAKHKAAEVALKHL.

Primary transcripts are normally cleaved within 30 nucleotides downstream of the polyadenylation signal. In pachytene spermatocytes and round spermatids, however, this cleavage is rapidly followed by the addition of a poly-A tail of about 180 adenine residues. Polyadenylated mRNAs are sequestered in RNP particles. Translation, subsequently, takes place in elongated spermatids after polyadenylated mRNAs undergo a partial poly-A shortening by deadenylation. In mice, the length of the poly-A tail on transition protein and protamine mRNAs has been reported to correlate with the translational activity of the transcripts (Kleene 1996; Cataldo et al. 1999). Both stability and translation of the transcripts are mediated by the interaction with a poly-A binding protein (PABP). As has been demonstrated in in vitro studies, polyadenylated mRNAs are degraded faster when PABP is absent. It is assumed that PABP migrates from the poly-A tail to AU-rich elements within the 3′-UTR leaving the poly-A tail naked and vulnerable to degradation (Bernstein et al. 1989). In mice, there exist at least four PABPs. The best characterized PABP is the 70-kDa PABP1 (Jackson and Standart 1990). PABP1 exhibits four RRMs and is associated with mRNAs containing poly-A tails ranging from 30 to 180 adenine residues, confirming that PABP1 binds to both active and stored forms of the same mRNA. PABP1-mRNA is expressed at a level being at least tenfold higher in testes than in somatic tissues.

4
Idiopathic Male Infertility: Impaired Histone-to-Protamine Exchange?

Haploid spermatids are known to play a key role in the production of fertile spermatozoa. In round spermatids, somatic and testis-specific histones occur together with transition proteins. In elongating spermatids, both histones and transition proteins are removed from the chromatin and are replaced by protamines. Protamine–DNA interactions result in chromatin condensation causing cessation of transcription in elongating spermatids (Steger 1999, 2001). Due to temporal uncoupling of transcription and translation in haploid spermatids, gene

expression results in a different but stage-specific expression of mRNA (in round spermatids) and corresponding protein (in elongating spermatids). As no further genetic information will be added after the transcriptional stop, the protein content in spermatozoa can be predicted by analysing the mRNA equipment of their progenitor cells, namely round spermatids in testicular biopsies (Steger et al. 2001, 2003).

A comprehensive review on genetically modified mice resulting in specific arrests of spermatogenesis is given by de Rooij and de Boer (2003). Furthermore, we point to reviews from Scherthan (2003) and Wolgemuth (2003) dealing with male infertility due to meiotic defects. Here, we will focus on spermiogenesis.

Interestingly, male mice lacking genes which are thought to play an essential role in spermatogenesis, such as testis-specific histone H1t (Lin et al. 2000), are fertile and reproduce like wild-type mice. This phenomenon may, at least in part, be explained by redundancy, since gene expression of other H1 subtypes has been demonstrated to be enhanced during spermatogenesis creating a normal H1-to-nucleosome ratio and presumably compensating for H1t functions in H1t-deficient mice (Drabent et al. 2000). A similar situation seems to appear in mice lacking the gene for transition protein-1 (Yu et al. 2000) and transition protein-2 (Adham et al. 2001), exhibiting elevated levels of transition protein-2 and transition protein-1, respectively. Although sperm motility is severely reduced, approximately 40% of these mice are fertile. In addition, even transition protein-1 and -2 double mutant mice (Shirley et al. 2004) and mice lacking three spermatid-specific genes, namely testis-specific histone H1t, transition protein-2 and proacrosin (Nayernia et al. 2003), remain fertile.

By contrast, both protamine-1 and protamine-2 are essential for the production of structurally and functionally intact sperm. It has been demonstrated that haploinsufficiency caused by a mutation in one allele of protamine-1 or protamine-2 prevents genetic transmission of both mutant and wild-type alleles (Cho et al. 2001). Interestingly, in sperm from protamine-1 chimeras, solely protamine-1 was reduced, whereas in sperm from protamine-2 chimeras, both protamine-1 and mature protamine-2 were reduced, with the reduction being greater for protamine-1 than for mature protamine-2. Even premature translation of protamine-1 mRNA causes precocious chromatin condensation and arrests spermatid differentiation resulting in male infertility (Lee et al. 1995). Translational repression of protamine-1 mRNA involves binding of protamine-1 RNA-binding protein (PRBP) to the 3′-UTR of protamine-1 mRNA. Male mice carrying a targeted disruption of the Tarbp2 gene encoding PRBP are infertile due to delayed replacement of transition proteins (Zhong et al. 1999).

Male mice lacking the genes for transcription factors TATA-binding protein-related factor (TRF) (Zhang et al. 2001) or cAMP-responsive element modulator (CREM) (Blendy et al. 1996; Nantel et al. 1996) binding to the TATA-box and the CRE-box, respectively, known to be present in the promoter region of transition proteins and protamines are infertile due to spermatogenic arrest at the level of round spermatids. Male knockout mice for Ca^{2+}/calmodulin-dependent protein

kinase IV (Camk4), a serine/threonine protein kinase phosphorylating protamine-2, are infertile due to prolonged retention of transition protein-2 and complete absence of protamine-2 in elongated spermatids (Wu et al. 2000).

4.1 Male Infertility Caused by Incorrect Transcriptional Regulation

Transcriptional regulation in haploid spermatids involves methylation of cytosines and binding of transcription factors within the promoter region of genes (Steger 1999).

Methylation of specific cytosines following a guanine in the sequence CpG (p represents the phosphate group connecting C and G nucleotides) is known to be involved in transcriptional silencing and genetic imprinting, a gene regulation mechanism by which only one of the parental copies of a gene is expressed (Barlow 1995). Since imprints inherited from the previous generation have to be erased and re-established according to the sex, the germ line plays a key role in the imprinting process.

The promoter region of many genes expressed in haploid spermatids is known to contain a cAMP-responsive element (CRE) serving as binding site for the transcription factor cAMP-responsive element modulator (CREM). Due to alternative exon splicing, the CREM gene gives rise to functionally different proteins with either activating or repressing potential on target gene expression. During spermatogenesis, there is a switch in CREM gene expression resulting in CREM being converted from repressors to activators. Activation of CREM is thought to be mediated through phosphorylation of the serine residue at position 117 by protein kinase K (Walker and Habener 1996). However, recent data suggest a phosphorylation-independent mechanism of CREM activation in the testis by the specific transcription activator of CREM, referred to as activator of CREM in the testis (ACT) (Fimia et al. 1999). The essential role of CREM for spermatid differentiation has been reported simultaneously by Blendy et al. (1996) and Nantel et al. (1996), demonstrating that male mice lacking a functional CREM gene are sterile due to round spermatid maturation arrest. Infertile men exhibiting round spermatid maturation arrest reveal a lack or substantial reduction at the level of both CREM-mRNA (Steger et al. 1999) and CREM-protein (Weinbauer et al. 1998). Furthermore, the lack of a switch from repressors to activators as well as additional, unusual and inaccurately spliced transcripts have been reported in patients with impaired spermatogenesis (Behr and Weinbauer 2000).

4.2 Male Infertility Caused by Incorrect Translational Regulation

Translational regulation in haploid spermatids involves binding of mRNA-binding proteins within the 3′-untranslated region (UTR) and the poly-A tail of mRNAs (Steger 2001).

Transgenic mice revealing premature translation of protamine-1 have been demonstrated to be infertile due to precocious chromatin condensation (Lee et al. 1995). Male mice lacking the Tarbp2 gene encoding protamine-1 mRNA-binding protein (PRBP) are infertile (Zhong et al. 1999). Furthermore, deletion within AZF b encoding the mRNA-binding motif (RBM) protein is known to be followed by spermatogenic arrest at the level of primary spermatocytes resulting in male infertility (Elliott et al. 1997).

4.3 Male Infertility Caused by Incorrect Chromatin Condensation

Bedford et al. (1973) were the first to raise the question about a possible relationship between incomplete chromatin condensation in spermatozoa and male infertility. Subsequently, there have been many publications on this topic (Steger 2003). However, histone-to-protamine exchange in haploid spermatids seems to play a key role for correct spermatid differentiation. The integrity of the sperm chromatin, in addition, may also play the most important role, particularly in ICSI, where most of the natural selection mechanisms are bypassed. In in vitro fertilization (IVF), altered protamine-2 expression is already known to represent a common defect in infertility patients, but not in donors of known fertility. Although the mean sperm concentration was not significantly different, morphology, penetration rates and motility were all significantly decreased compared with patients exhibiting a measurable protamine-2 content (Carrell and Liu 2001).

The total protamine mass to DNA mass ratio has been demonstrated to be nearly identical in a variety of mammals (Bench et al. 1996). However, the relative proportion of protamine-1 and protamine-2 is tightly regulated within a genus, while the protamine-2 content of sperm chromatin is allowed to vary over a wide range between different species (Corzett et al. 2002). Although changes in the expression of the two protamine genes are observed in distantly related species and different genera, variation does not appear to be tolerated within a species. Normal levels of protamine-1 and protamine-2, therefore, seem to be indispensable for the production of structurally and functionally intact sperm.

Balhorn et al. (1987) reported that, in contrast to fertile men, sperm from infertile men displays an aberrant protamine-1 to protamine-2 protein ratio. In agreement with data from Balhorn et al. (1987), round spermatids in testicular biopsies representing the precursor cells of mature spermatozoa have been demonstrated to exhibit an aberrant protamine-1 to protamine-2 mRNA ratio (Steger et al. 2001, 2003). In addition, spermatozoa from testes in which less than 30% of round spermatids express the protamine genes were unable to fertilize an egg.

Interestingly, analysis of the human protamine-1 and protamine-2 sequences in 226 infertile patients and 270 proven-fertile volunteers revealed only four single nucleotide polymorphisms within the protamine-1 coding sequence, which did not cause any amino acid substitution, and solely one single nucleotide polymorphism within the protamine-2 coding sequence, which produced translational

termination (Tanaka et al. 2003). Although genes for protamine-1 and protamine-2 are clustered on chromosome 16p13.3 (Accession Z46940), data suggest that the expression of the protamine-1 and protamine-2 genes may actually be uncoupled in some developing spermatids of infertile men.

Furthermore, the focal reduction of protamine-1 and protamine-2 mRNA expression in seminiferous tubules with spermatogenic arrest at the level of round spermatids adjacent to tubules with at least qualitatively normal spermatogenesis (Steger et al. 2001) suggests local differences in the presence of regulating factors being responsible for the correct differentiation of round spermatids into mature spermatozoa.

5
Carcinoma In Situ of the Testis and Testicular Germ Cell Tumour: Impaired Intercellular Communication Due to Altered Sertoli Cell Differentiation?

5.1
Carcinoma In Situ of the Testis and Testicular Germ Cell Tumour

5.1.1
Incidence of Testicular Germ Cell Tumour

Testicular germ cell cancer is the most common cancer among men aged 17–45 years in Western industrialized countries and its incidence has increased steadily over the past 50 years (Adami et al. 1994; Moller 1998; Rorth et al. 2000). Known risk factors for developing testicular germ cell tumour are maldescensus of the testis (Giwercman et al. 1987), contralateral testicular tumour (Berthelsen et al. 1979; von der Maase et al. 1986) and gonadal dysgenesis (Muller et al. 1985). Furthermore, several clinical and epidemiological studies indicate a possible link between the rising incidence of reproductive abnormalities in men and the excessive exposure to environmental hormones or hormone disrupters (Rajpert-De Meyts and Skakkebaek 1993; Toppari et al. 1996; Skakkebaek et al. 1998, 2001; Sharpe et al. 2003). Genetic factors may, in addition, play a crucial role, as African Americans reveal significantly lower incidence than Caucasians living in the same areas of the United States. Furthermore, families with two or more cases of testicular cancer have been reported, indicating that testicular cancer susceptibility genes may exist (Rapley et al. 2003). In cases with some rare chromosome disorders associated with genital abnormalities including patients with so-called mixed gonadal dysgenesis and 45,X/46,XY karyotype or phenotypic 46,XY females with pure gonadal dysgenesis, there is an increased risk of developing germ cell cancer. The abnormal genetic constitution of these individuals may be associated with dysgenetic gonadal changes including microlithiasis, undifferentiated Sertoli cells/granulosa-like cells and persistence of undifferentiated gonocytes in the maldeveloped gonads, sometimes in the form of gonadoblastoma (Scully 1970; Muller et al. 1985; Skakkebaek 2004). Recent research demonstrated that similar dysgenetic changes can also

occur in testicular biopsies of men with normal genetic background associated with testicular dysgenesis syndrome (Skakkebaek 2001, 2004; Sharpe et al. 2003; Skakkebaek et al. 2003).

5.1.2 Origin of Testicular Germ Cell Tumour

Testicular germ cell tumours of young adults involve a wide variety of histological features, but can be divided into two major subtypes: seminoma resembling undifferentiated germ cells morphologically and immunohistochemically closely related to fetal germ cells, and non-seminoma including yolk sac tumour, choriocarcinoma, polyembryoma, embryonal carcinoma and teratoma representing embryonic or extraembryonic tissue as these tumour cells are able to differentiate to either direction (Mostofi and Sobin 1977) (Fig. 19).

Interestingly, all these tumours originate from a common non-invasive precursor, the carcinoma in situ (CIS) cell (Skakkebaek 1972; Rorth et al. 2000). The morphological and/or immunohistochemical diagnosis of CIS, however, does not per se include a prediction of which histological type of germ cell tumour the cells might develop into. There is evidence for an antigenic heterogeneity of the phenotype of CIS cells despite their morphological uniformity. This was demonstrated by different expression patterns of immunohistochemical tumour cell markers, especially in the neighbourhood of mixed germ cell tumours (Rajpert-De Meyts et al. 1996, 2003). In addition, the partial phenotypic difference was confirmed by different cytogenetic studies (Looijenga et al. 1993, 2000; Ottesen et al. 2003),

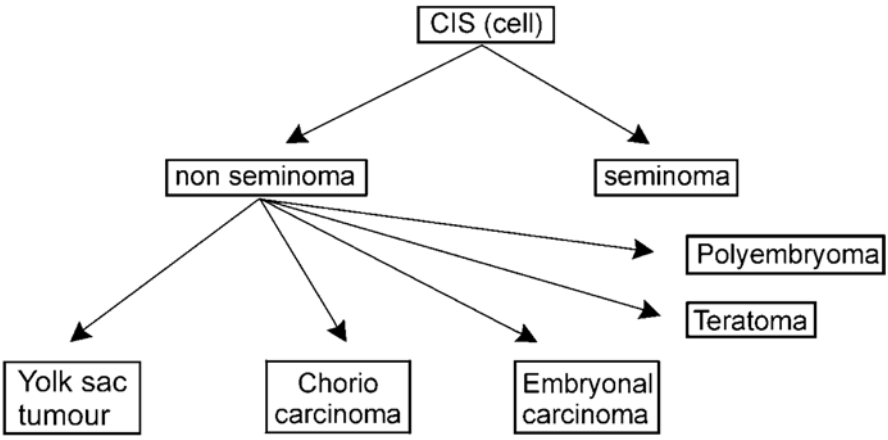


Fig. 19 Diagram showing a simplified classification of human testicular germ cell tumours. Testicular germ cell tumours of young adults can be divided into two major subtypes: seminoma and non-seminoma. Non-seminoma includes yolk sac tumour, choriocarcinoma, polyembryoma, embryonal carcinoma and teratoma. All these tumours originate from a common non-invasive precursor, the carcinoma in situ of the testis (*CIS*) cell

indicating that single CIS cells even within the same seminiferous tubule could represent different stages of differentiation making them predestined for further malignant progression into either seminoma or non-seminoma (Rajpert-De Meyts et al. 2003).

It has been suggested that the initiation of malignant transformation to atypical germ cells occurs during early development of fetal germ cells at weeks 7–10 of gestation (Skakkebaek et al. 1987; Jorgensen et al. 1995). At present, it seems to be confirmed that CIS cells derive from fetal germ cells that do not undergo normal development (Skakkebaek et al. 1987; Rorth et al. 2000). Inhibition of normal fetal germ cell differentiation into spermatogonia during pregnancy may be interpreted as a consequence of endocrinological or environmental imbalances (Skakkebaek et al. 1998; Dieckmann and Skakkebaek 1999). The intrauterine environment is thought to play an important role for initiating changes in primordial germ cells, possibly due to excessive levels of intrauterine oestrogen or ingestion of xeno-oestrogens or oestrogen-like substances or just altered relative levels of hormones during pregnancy (Rajpert-De Meyts et al. 1998; Skakkebaek et al. 1998; Dieckmann and Skakkebaek 1999; Sharpe 2003). Excess of oestrogens during early embryonic life might stimulate primordial germ cells to acquire the tumourigenic potential of CIS cells (Rajpert-De Meyts et al. 1998; Skakkebaek et al. 1998; Dieckmann and Skakkebaek 1999) making them less prone to responding to the normal regulatory influences of supporting Sertoli cells and directly or indirectly to the various hormonal, endocrine and paracrine stimuli regulating the differentiation of normal spermatogenic cells within the seminiferous tubules.

5.1.3 Diagnosis of Carcinoma In Situ of the Testis

After birth, CIS cells seem to remain in a dormant stage and divide only at a very slow pace during early childhood. Around puberty, concomitant with the terminal differentiation of Sertoli cells and in parallel with the initiation of spermatogenesis, CIS cells start to replicate strongly within the seminiferous tubules by replacing all normal spermatogenic cells leaving behind only CIS cells and Sertoli cells. This progressive development finally leads, via an intermediate stage of intratubular seminoma, to seminoma or non-seminoma. To date, the mechanisms underlying the phenomena of fetal initiation, long latency between malignant transformation and pubertal proliferation of CIS, as well as promotion and progression of intratubular CIS and intratubular seminoma to an invasive malignancy, are not known (Dieckmann and Skakkebaek 1999; Rorth et al. 2000). However, it is proposed that the changing endocrinological environment during puberty and other factors in addition to altered sex hormone levels may play a vital role (Rajpert-De Meyts et al. 1993; Skakkebaek et al. 1998; Dieckmann and Skakkebaek 1999) (Fig. 20).

CIS cells and fetal germ cells exhibit several morphological similarities, such as a large nucleus with one or more prominent nucleoli and large amounts of

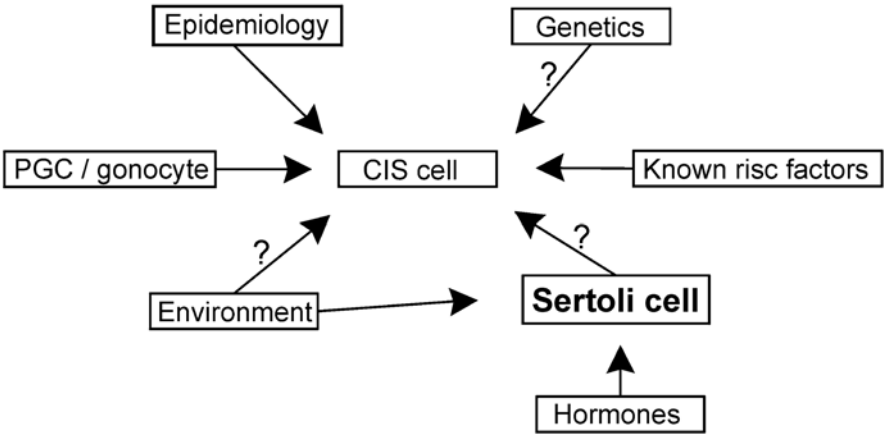


Fig. 20 Diagram showing multiple factors that are suggested to be involved in the pathogenesis of human testicular germ cell tumours. (Modified from De Kretser and Damjanov 1998)

glycogen granules within the cytoplasm (Sigg and Hedinger 1984; Holstein et al. 1987; Skakkebaek et al. 1987) (Fig. 21A, B).

The standard method for the identification of CIS is the immunohistochemical staining for placental alkaline phosphatase (PlAP). This enzyme is present in fetal germ cells, CIS cells, seminoma cells and several other types of testicular germ cell tumour cells, but cannot be detected in normal adult germ cells (Beckstaed 1983; Skakkebaek et al. 1987). The M2A antigen may be used as an additional marker, as it is expressed in fetal germ cells (Baumal et al. 1989; Jorgensen et al. 1995; Marks et al. 1999; Franke et al. 2004) and in abnormal germ cells in several testicular germ cell tumours and CIS (Bailey et al. 1986; Marks et al. 1999; Brehm et al. 2002) (Fig. 21C, D).

Recently, CIS cells have been demonstrated to exhibit features of stem cells, as they express a number of genes in common with gonocytes and embryonic stem cells, e.g. c-KIT and OCT-4 (Looijenga et al. 2003; Rajpert-De Meyts et al. 2003).

5.1.4
Models for Progression of Carcinoma In Situ to Testicular Germ Cell Tumour

When investigating development and differentiation of seminoma, it is important to be aware of different morphological stages of tumour development. Two developmental stages can be discriminated.

1. Seminiferous tubules with residual spermatogenesis containing single or a few CIS cells, Sertoli cells and normal germ cells (CIS tubules).
2. Seminiferous tubules that are devoid of any normal germ cells containing only CIS cells and Sertoli cells (CIS-only tubules).

Intratubular seminoma is most probably the morphological substrate of CIS cells becoming independent from the feeder support of Sertoli cells and is defined

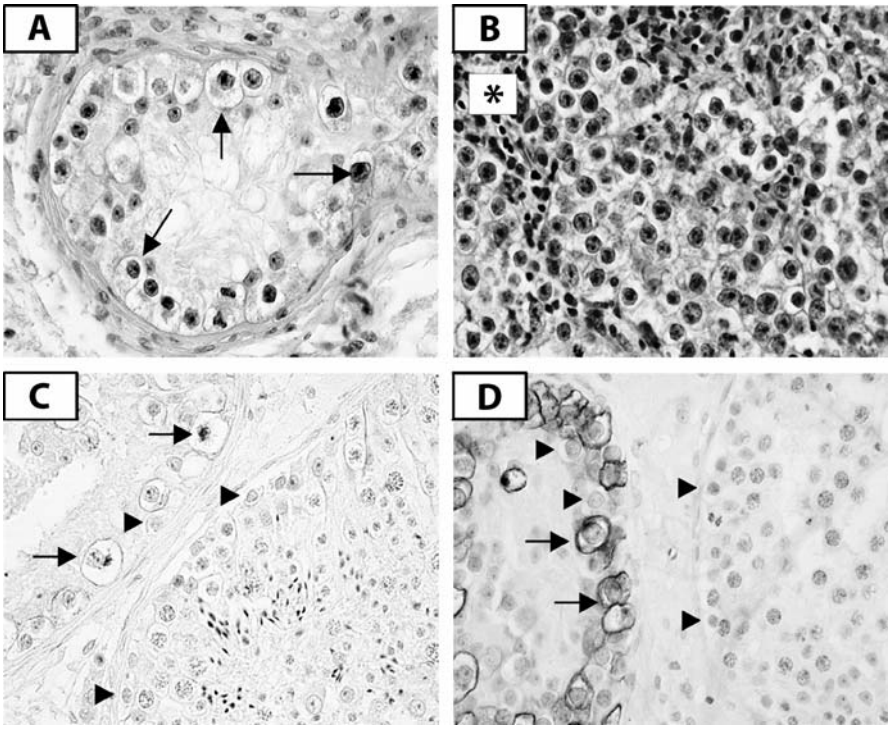


Fig. 21A–D Paraffin sections showing histology of carcinoma in situ of the testis (**A**) and classical seminoma (**B**), as well as immunohistochemical staining for placental alkaline phosphatase (*PlAP*, **C**) and M2A antigen (**D**). **A** Seminiferous tubule showing CIS cells (*arrows*) and Sertoli cells. **B** Classical seminoma composed of uniform tumour cells surrounded by fine fibrovascular septa containing lymphocytes (*). Note distinct cellular borders and sharp nuclear membranes. **C** Within the CIS tubule (*left*), CIS cells (*arrows*) are immunopositive for PlAP, while normal spermatogonia (*arrowheads*) remain unstained, as can also be demonstrated in the tubule revealing normal spermatogenesis (*right*). **D** Within the CIS tubule (*left*), CIS cells (*arrows*) are immunopositive for M2A antigen, while normal spermatogonia (*arrowheads*) remain unstained, as can also be demonstrated in the tubule revealing normal spermatogenesis (*right*). In both cases, Sertoli cells are immunonegative. H&E (**A**, **B**), ABC method, positive signal brown (**C**, **D**), ×40

by an often extended seminiferous tubule whose lumen is filled by seminomatous cells and its Sertoli cell lining is reduced or often gone (Oosterhuis et al. 2003).

As parenchyma surrounding seminoma usually exhibits a heterogeneous pattern with varying amounts of CIS, CIS-only, intratubular seminoma, as well as completely fibrotic or atrophic tubules and intertubular tissue (Schulze and Holstein 1977; Oosterhuis et al. 2003; Donner et al. 2004), and CIS is thought to represent the non-invasive precursor of testicular germ cell tumours (Skakkebaek 1972), great efforts have been made to understand the mechanisms of the development from preinvasive CIS to invasive neoplasia resulting in two different theories.

5.1.4.1
Progression of Tumour Development Through Migration

According to the first theory, neoplastic CIS or intratubular seminoma cells migrate through the tubular wall of a seminiferous tubule (Schulze and Holstein 1977). This is in accordance with several other neoplasms, especially with CIS of breast and skin and their invasive successors (Liotta et al. 1983). Here, it has been shown that the basement membrane gets lost with invasiveness. However, in testicular tumours, there exist no histological studies on CIS cells passing the tubular walls, except that of Jacobsen and Talerman (1989) reporting an invasion of the tubular wall by malignant germ cells (Fig. 22).

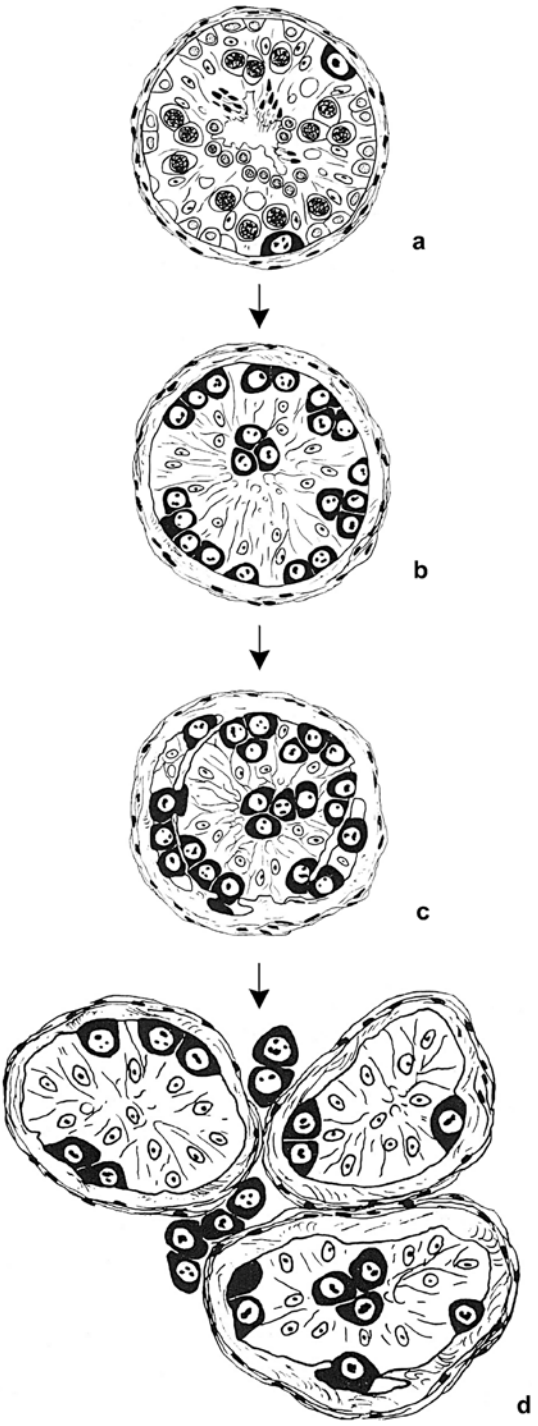
5.1.4.2
Progression of Tumour Development Through Tubular Enlargement

According to the second theory, the switch from preinvasive CIS to invasive germ cell tumour takes place in situ by intratubular or tubular enlargement due to tumour cell proliferation. This process is accompanied by Sertoli cell degeneration and conversion of the tubular wall into connective tissue, as is detectable in testicular specimens adjacent to germ cell tumours and in patients with CIS, where areas can be identified with tumour cells surrounded by fibrous sheets resembling enlarged seminiferous tubules in an intermediate stage (Donner et al. 2004). The enlarged tubules, as well as tumour cell clusters, have been demonstrated to contain not only numerous tumour cells, but also residual Sertoli cells in an altered differentiation state. The walls of affected seminiferous tubules were demonstrated to have turned into connective tissue exhibiting an intact basement membrane with a functional loss of contractility (Donner et al. 2004) (Fig. 23).

5.2
Sertoli Cell Differentiation in Seminiferous Tubules Infiltrated with Carcinoma In Situ of the Testis

Infertility and testicular dysgenesis syndrome including testicular cancer have been suggested to be correlated with an impaired function and/or differentiation of Sertoli cells (Kliesch et al. 1998; Skakkebaek et al. 1998, 2001; Brehm et al.

Fig. 22 Diagram showing progression of tumour development via migration of CIS or intratubular seminoma cells through the tubular wall. At the beginning, single tumour cells can be observed in the basal compartment of the seminiferous tubule (*a*). At that time, the seminiferous tubule still displays spermatogenic activity. Subsequently, the seminiferous tubule resembles CIS-only or intratubular seminoma with tumour cells detectable in the basal and adluminal compartments (*b*). Then, intratubular tumour cells penetrate or migrate through the tubular wall (*c*). Finally, tumour cells occur within atrophic tubules and in the interstitium (*d*). (Modified from Holstein et al. 1988)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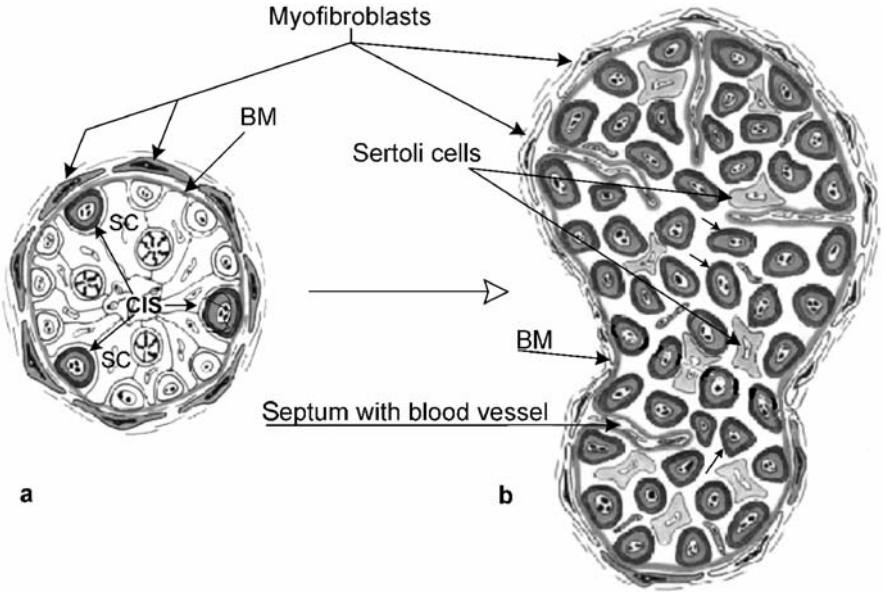


Fig. 23 Diagram showing progression of tumour development via tubular enlargement. In a first step (*a*), single tumour cells (*arrows*) are lying in the basal compartment of the seminiferous tubule displaying residual spermatogenesis. Note presence of myofibroblasts which are immunopositive for both actin and myosin (Donner et al. 2004). In a second step (*b*), preinvasive CIS switch to germ cell tumour. This takes place by tubular enlargement due to tumour cell (*arrows*) proliferation followed by degeneration of Sertoli cells and conversion of the tubular wall into connective tissue. Note presence of myofibroblasts that are now immunonegative for both actin and myosin and Sertoli cells which are immunopositive for cytokeratin 18 and connexin 26 (Donner et al. 2004). *BM*, basement membrane; *Sc*, Sertoli cells

2002; Sharpe et al. 2003). It was postulated that Sertoli cells play a crucial role for the fetal development of abnormal germ cells (Rajpert-De Meyts and Skakkebaek 1993; Skakkebaek et al. 1998; Sharpe et al. 2003) and for pubertal progression of the neoplasm to invasiveness (Brehm et al. 2002), but information on this issue is still scarce. To date, two main hypotheses are discussed concerning the status of Sertoli cell differentiation in seminiferous tubules infiltrated with CIS cells (Kliesch et al. 1998; Skakkebaek et al. 2001; Brehm et al. 2002; Sharpe et al. 2003).

5.2.1 Hypothesis of Primary Undifferentiated Sertoli Cells

Although it is difficult to distinguish failure of maturation, which may occur at a certain step, from dedifferentiation unless there are obvious differences in Sertoli cell nuclear morphology, the occurrence of immature features of Sertoli cells, like cytokeratin 18 expression in the testes of adult men with various abnormalities including CIS, is interpreted as a reflection of failure of maturation (Sharpe et al.

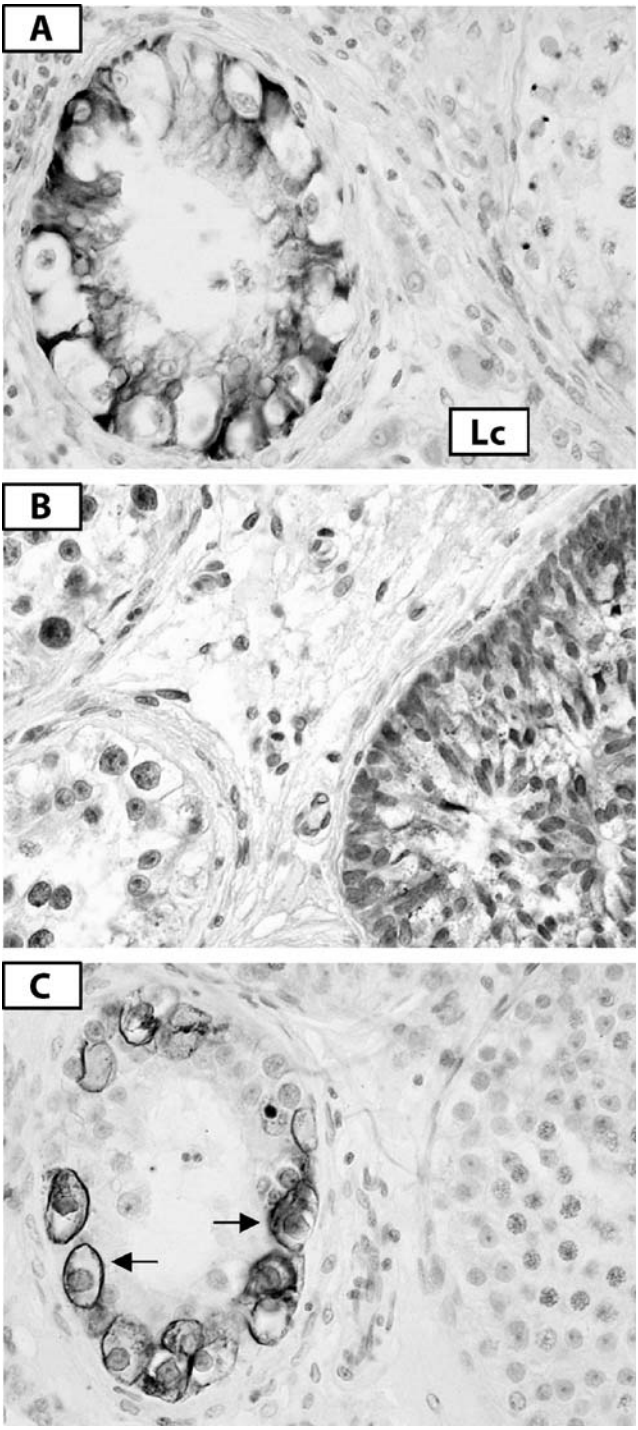
2003). Within the testicular dysgenesis syndrome, testicular germ cell cancer, cryptorchidism, hypospadias and some cases of low sperm counts have been proposed to have a common origin in fetal life (Skakkebaek et al. 2001; Sharpe et al. 2003). Single expression of cytokeratin 18 is a sign of Sertoli cell immaturity. Interestingly, it is expressed at a time (until the 20th week of gestation) when abnormal germ cells are derived due to impaired testicular differentiation (Franke et al. 2004). Thus, it is possible that both Sertoli cells and germ cells are fetally targeted by so far unknown factors and are altered in their normal differentiation programme.

5.2.2 Hypothesis of Secondary Dedifferentiated Sertoli Cells

It has been demonstrated that seminiferous tubules bearing CIS show positive cytokeratin 18 expression in Sertoli cells, only if tubules were devoid of normal germ cells. However, in the presence of CIS cells together with round or elongated spermatids, adjacent Sertoli cells did not express cytokeratin 18. In this context, cytokeratin 18 expression may be interpreted as a sign for fetal dedifferentiation or as a re-emergence of an immature phenotype of previously mature Sertoli cells associated with tumour progression (Kliesch et al. 1998; Brehm et al, 2002). It was further suggested that this dedifferentiation may result in a loss of Sertoli cell function and cessation of spermatogenic activity (Kliesch et al. 1998). As it is obvious that the expression of a single immunohistochemical marker should always be interpreted carefully in the context with the expression patterns of additional markers and with morphological appearance of Sertoli cells and/or Sertoli cell nuclei, it has further been shown that Sertoli cells in tubules infiltrated with CIS did not express the later maturation markers M2A antigen or AMH (Brehm et al. 2002), whose loss is known to follow temporally the loss of cytokeratin 18 (Steger et al. 1996, 1999) (Fig. 24). These data are further supported by results of ultrastructural studies demonstrating that Sertoli cell nuclei in seminiferous tubules infiltrated with CIS exhibit an adult phenotype (Nielsen et al. 1974; Jorgensen et al. 1990).

In addition, an inverse correlation between intratubular connexin 43 expression and progression of tumour development was detected. For the pubertal differen-

Fig. 24A–C Immunohistochemistry showing expression of cytokeratin 18 (**A**), AMH (**B**) and M2A (**C**) in seminiferous tubules infiltrated with carcinoma in situ (CIS) of adult men. **A** Sertoli cells associated with CIS are immunopositive for cytokeratin 18 (*left tubule*), while Sertoli cells associated with normal spermatogenesis are immunonegative (*right tubule*). **B** A strong immunoreaction for AMH can be observed in pre-Sertoli cells of a prepubertal seminiferous cord (*right*), while Sertoli cells in CIS-only tubules (*left upper* and *lower*) are completely immunonegative. **C** While the seminiferous tubule with normal spermatogenesis (*right*) is completely immunonegative, within the CIS-only tubule (*left*), CIS cells (*arrows*) reveal a strong signal for M2A. *Lc*, interstitial Leydig cells. ABC method, positive signal brown. ×40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Lc
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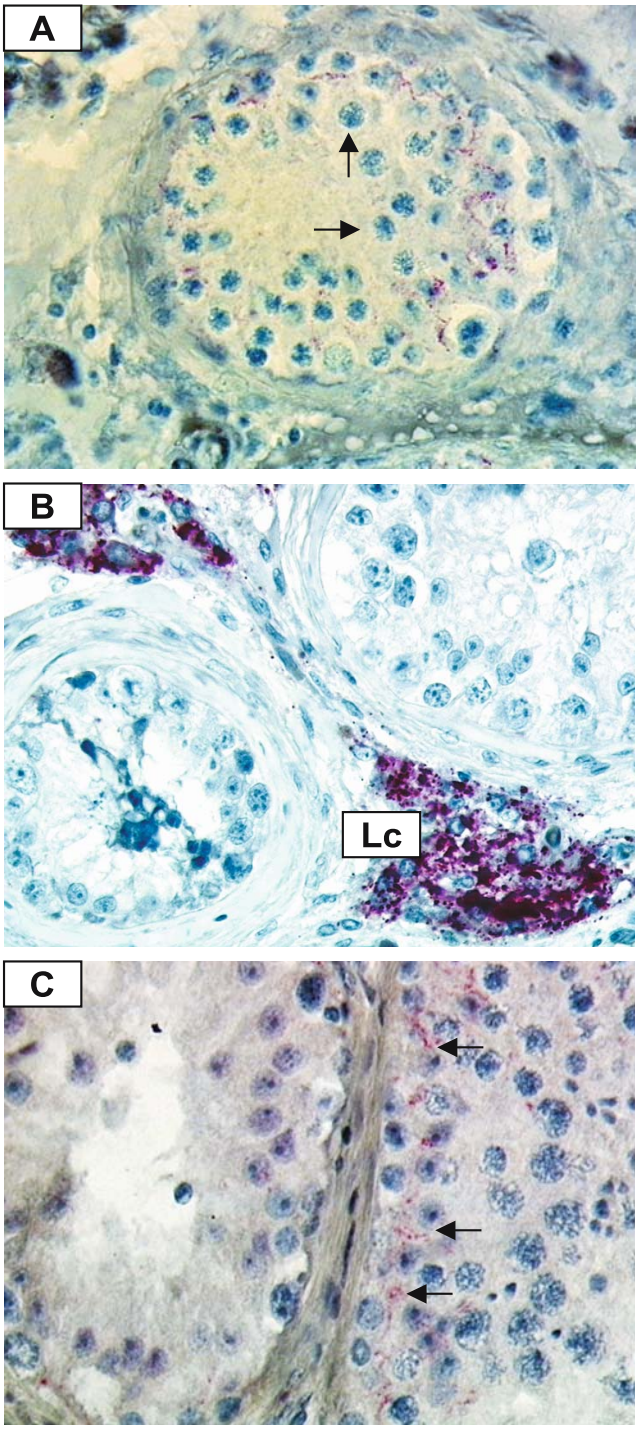
tiation marker connexin 43, a reduction of the protein from its normal intercellular location between Sertoli cells and between Sertoli cells and germ cells in tubules infiltrated with CIS and residual spermatogenesis was observed, whereas in seminiferous tubules containing only Sertoli cells and CIS cells, no intratubular connexin 43 immunoreactivity was observed (Brehm et al. 2002) (Fig. 25). This corroborates findings in seminiferous tubules with spermatogenic arrest at the level of spermatogonia or SCO associated with infertility (Steger et al. 1999) and is also likely to be a manifestation of a phenotypic reversion of Sertoli cells to a less differentiated maturation state associated with neoplasia (Brehm et al. 2002). This suggests that normal intercellular communication through gap junctions between Sertoli cells, on the one hand, and between Sertoli cells and germ cells, on the other hand, is disrupted in these tubules. However, it remains to be elucidated whether this disruption of intercellular communication involving Sertoli cells is a consequence of CIS proliferation or whether both the Sertoli cell derangement and CIS proliferation originate from a common antecedent cause. It is also possible to envisage the existence of intercellular communication through gap junctions between Sertoli cells and isolated CIS cells. This intriguing conjecture raises the possibility that adult or differentiated Sertoli cells play a role in maintaining the long latency of CIS cells. Thus, a phenotypic reversion of Sertoli cells resulting in the loss of connexin 43 expression and disruption of intercellular communication might actually proceed and possibly allow the uninhibited pubertal proliferation of CIS cells.

The gap junctional protein connexin 26 was demonstrated to be expressed in the cytoplasm of Sertoli cells, weak in normal tubules and strong in CIS-infiltrated tubules. This indicates an aberrant accumulation of this protein in the cytoplasm of Sertoli cells associated with neoplastic testicular pathology (Fig. 26).

Fig. 25A–C Immunohistochemical expression of connexin 43 in seminiferous tubules infiltrated with carcinoma in situ. **A** Reduced intratubular expression of connexin 43 in an adult seminiferous tubule infiltrated with CIS but displaying residual spermatogenesis. Note single primary spermatocytes (*arrows*). **B** Connexin 43 is completely absent in seminiferous tubules showing CIS only. Note that interstitial Leydig cells (*Lc*) still express connexin 43 and, therefore, can be used as internal positive control. **C** While no expression of connexin 43 can be detected in the seminiferous tubule exhibiting CIS-only (*left*), typical immunoreactive signals (*arrows*) can be appreciated in the adjacent tubule showing normal spermatogenesis (*right*). APAAP method, positive signal red. ×40

Fig. 26A–C Immunohistochemical expression of connexin 26 in seminiferous tubules infiltrated with carcinoma in situ. **A** Aberrant intracytoplasmic expression of connexin 26 in adult Sertoli cells associated with CIS but displaying residual spermatogenesis. Note single primary spermatocytes (*arrows*). **B**, **C** Aberrant intracytoplasmic expression of connexin 26 in Sertoli cells associated with CIS only. In contrast, weak or no expression of connexin 26 can be detected in adjacent seminiferous tubules showing normal spermatogenesis. Interstitial Leydig cells (*Lc*) are immunonegative. APAAP method, positive signal red. ×40 (**A**, **C**), ×20 (**B**)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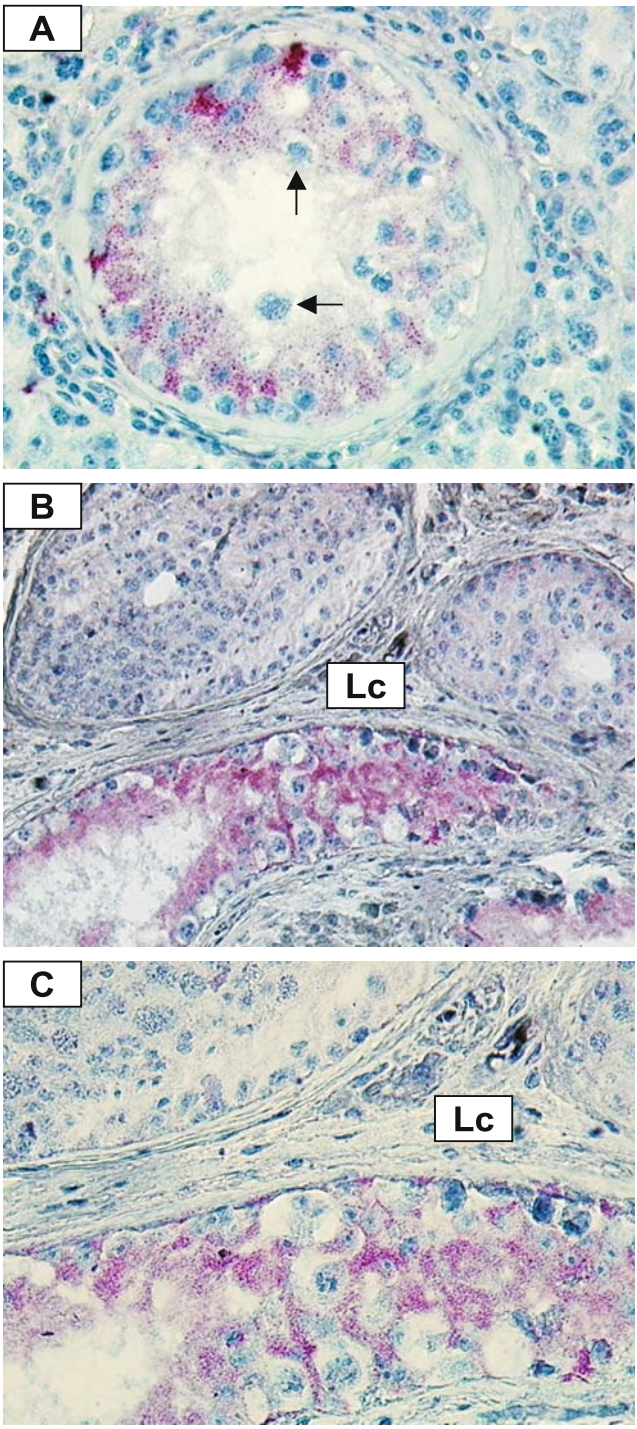
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The expression of individual connexins is responsive to various physiological states and pathological processes (Bruzzone et al. 1996). Increased levels of connexin 26 expression have been reported in preneoplastic foci in experimental carcinogenesis in rat liver (Neveu et al. 1994). Therefore, it is possible that the expression of connexin 26 is upregulated in human Sertoli cells as an adaptive response to the loss of expression of predominant connexin 43 due to phenotypic reversion associated with testicular neoplasia. However, connexin 26 fails to assemble properly for membrane insertion in this situation and accumulates in the cytoplasm.

Although it can not be totally excluded that there are also different populations of Sertoli cells in tubules infiltrated with CIS as detected in seminiferous tubules with SCO syndrome (Nistal et al. 1990), observations concerning differentiation markers connexin 43 and connexin 26 (Brehm et al. 2002) seem to confirm and favour the hypothesis of phenotypic reversion of Sertoli cells in the neoplastic tubule from their previous adult differentiated state (Kliesch et al. 1998).

Summarized, human Sertoli cells in seminiferous tubules infiltrated with CIS seem to develop and differentiate normally until puberty, showing different immunohistochemical and morphological signs of adult Sertoli cells. They do not express the prepubertal differentiation markers M2A and AMH, but the pubertal differentiation marker connexin 43. With the initiation of spermatogenesis at puberty and with the beginning of increased proliferative activity of CIS cells, a process of dedifferentiation of associated Sertoli cells starts, as has been demonstrated at the protein level by:

- Reduced or absent intratubular expression of the pubertal differentiation marker connexin 43
- Increase of connexin 26 expression with aberrant intracytoplasmic localization
- Re-expression of the prepubertal differentiation marker cytokeratin 18

The change of connexin 43 and connexin 26 expression associated with CIS indicates a process of epithelial dedifferentiation associated with a functional alteration or functional loss of Sertoli cells. The altered connexin expression and distribution in Sertoli cells further suggests a derangement in intercellular communication between Sertoli cells and between Sertoli cells and CIS cells through gap junctions. Thus, altered gap junctional intercellular communication and the dedifferentiation of Sertoli cells may play a role in the resulting spermatogenic impairment, the proliferation and neoplastic progression of CIS cells at puberty and the manifestation of testicular cancer.

5.3
Role of Gap Junctional Intercellular Communication in Carcinogenesis

Gap junctions are thought to be involved in the regulation of development, cell differentiation, cell proliferation, maintenance of tissue homeostasis, regulation of cellular growth, expression of the neoplastic phenotype and seem to participate in oncogenic transformation processes (Yamasaki 1990a, b; Bennett et al.

1991; Yamasaki et al. 1999). Reflecting the importance of gap junctional intercellular communication in various physiological functions, deletion of different connexin genes in mice resulted in various disorders including heart malformation, cataract or cancer (Yamasaki 1990a, b; Donaldson et al. 1997; Krutovskikh and Yamasaki 1997; Yamasaki et al. 1999). Cancer is supposed to be a disease of abnormal homeostasis mediated by defects in intra-, extra- and intercellular forms of communication that disrupt the balances between cellular proliferation, differentiation, apoptosis and adaptation (Trosko and Ruch 1998).

The first evidence for the involvement of aberrant gap junctional intercellular communication in carcinogenesis was the observation that human cancer cells exhibit a reduced level of gap junctional intercellular communication (Loewenstein and Kanno 1966). It was observed that tumour cells usually have a low capacity to communicate between each other (homotypic gap junctional intercellular communication). Later, the lack of gap junctional intercellular communication in cancer cells was extended to cancer cell–normal cell communication (heterotypic gap junctional intercellular communication) (Yamasaki 1990a, b; Krutovskikh and Yamasaki 1997). Therefore, it has been suggested that selective communicational isolation of tumour cells from surrounding normal tissue helps them to escape from signals keeping proliferation in normal tissues under negative control (Krutovskikh and Yamasaki 1997). Since then, there have been numerous studies on gap junctional intercellular communication in various tumours (Trosko and Ruch 1998). In almost all tumours, some abnormality in gap junction has been observed. Known mechanisms for aberrant gap junctional intercellular communication behaviour include reduction or loss of the transcription level of connexin genes, abnormal phosphorylation and/or localization pattern (intracytoplasmic or perinuclear localization) of connexin or gap junction and failure of gap junction assembly suggesting their functional impairment (Oyamada et al. 1990; Yamasaki 1990; Krutovskikh et al. 1991, 1994; Lee et al. 1991; Mesnil et al. 1994; Neveu et al. 1994; Omori et al. 1996; Yamasaki and Naus 1996; Omori et al. 1998). Additionally, it was demonstrated that impaired gap junctional intercellular communication and/or loss of connexin expression have been implicated to correlate with neoplastic transformation and increased invasiveness of several human tissues, such as prostate (Hossain et al. 1999), breast (Laird et al. 1999), lung (Ruch et al. 2001), brain (Soroceanu et al. 2001) and testis (Brehm et al. 2002; Segretain et al. 2003; Roger et al. 2004). Furthermore, connexin expression was found to be severely reduced in different preneoplastic lesions in several organs (Kamibayashi et al. 1995; Tsai et al. 1996; King et al. 2000; Saito et al. 2001; Habermann et al. 2002).

Besides environmental and endocrinological substances, gap junctional intercellular communication can be blocked or altered by various agents or genes which are involved in carcinogenesis (Swierenga and Yamasaki 1992; Budunova and Williams 1994). Various oncogenes inhibit gap junctional intercellular communication including Ras, src, SV40 t-antigen, neu and fps oncogenes (Yamasaki 1990a, b). Cell proliferation and tumourigenicity are reduced when poorly communicating neoplastic cells are transfected with connexin genes (Mesnil et al. 1995).

Conversely, inhibition of gap junctions by dominant negative connexin genes or connexin gene deletion increases cell proliferation and neoplastic transformation (Temme et al. 1997; Omori and Yamasaki 1998) which implies that downregulation of gap junctions may also be involved in cell cycle progression. It remains to be elucidated whether oncogenes, tumour suppressor genes or cell cycle-dependent kinase inhibitors, like p21 or members of the Ras family that are known to play a role in human testicular germ cell tumours, have influence on intratubular connexin 43 expression (Shuin et al. 1994; Datta et al. 2001; Skotheim and Lothe 2003).

Activation of the Ras/Raf-mitogen activated protein kinase (MAPK) signalling cascade is initiated by activation of growth factor receptors regulating many cellular events including cell cycle control. It was shown that the phosphoprotein connexin 43 is a MAPK substrate in vivo and that connexin 43 phosphorylation at different phosphorylation sites of connexin 43 initiates a downregulation of gap junctional communication being sufficient to disrupt gap junctional intercellular communication (Warn-Cramer et al. 1998; Mograbi et al. 2003). Since the control of the cell cycle plays an essential role in cell growth and its dysregulation is considered a major aetiologic factor in carcinogenesis, genetic alterations of cell cycle regulatory genes are studied intensively. It is getting more and more obvious that also cell-to-cell interaction via gap junctions plays an important role in growth control and thus in tumourigenesis as well (Krutovskikh and Yamasaki 1997).

Connexin 43 is known to have a tumour-suppressing effect. The principal role of connexin proteins has been thought to be the formation of the gap junction channels that mediate the tissue homeostasis. Therefore, the mechanisms by which connexins inhibit tumour growth were originally proposed to act through the diffusion of putative growth inhibitory factors via the connexin-modulated gap junctions. However, increasing evidence suggests that gap junction independent roles, for example interaction with other proteins, influence of connexin cDNA and/or mRNA expression, genetic and epigenetic changes, may also be involved in the connexin-induced growth inhibition (Mesnil et al. 1995; Yamasaki et al. 1999; Zhang et al. 2003).

Results of different studies led to the assumption that connexin genes form a family of tumour-suppressing genes, but so far, only two connexin gene mutations have been found in chemically induced rat tumours (Omori et al. 1996; Saito et al. 1997). However, recent studies suggest that connexin genes (connexin 32 and connexin 43) may be inactivated by hypermethylation of their promoter regions, suggesting that epigenetic inactivation of connexin genes may be a mechanism of gap junctional intercellular communication disturbance in certain tumours and neoplastic cells (Piechocki et al. 1999; Yamasaki et al. 1999). Additionally, the connexin 43 gene is frequently silenced or poorly expressed in many types of neoplastic cells and this does not appear to be due to connexin 43 mutation, but might be due to DNA methylation (Trosko and Ruch 1998). DNA methylation is one mechanism regulating the transcription of connexin 43, as has been reported in rat liver cells. Other mechanisms including transcript stability may also be important

in the intratubular specific expression of connexin 43. The half-life of connexin 32 mRNA for instance is reduced in regenerating liver (Kren et al. 1993) and domains within the 3′-UTR of the connexin 43 transcript have been identified that control its stability in a hormone-specific manner. Another mechanism for the reduced connexin expression frequently observed in neoplastic tissues could be *cis*/*trans* regulation of connexin genes (Piechocki et al. 1999). Further investigations at the DNA level using Southern blot analysis, RT-PCR with single microdissected CIS-, intratubular seminoma and seminoma cells and/or FISH have to reveal whether there are possible underlying mutational changes within the connexin 43 gene on chromosome 6 or non-mutational changes, for example in the promoter region of connexin 43 (e.g. hypermethylation).

5.4 Role of Gap Junctional Intercellular Communication in the Development of Carcinoma In Situ of the Testis to Testicular Germ Cell Tumour

At present, it is not known whether CIS cells have the potential to grow invasively or intratubularily from the beginning or whether the acquisition of invasiveness is the result of a second (pubertal) event, which would correspond to the co-carcinogenic effects of promoters in other tumour systems of multistep carcinogenesis (Pitot et al. 1988; Damjanov 1991). Ploidy studies of testicular tumours (Oosterhuis et al. 1989) are consistent with an at least two-step event leading to the transition of initially polyploidized non-invasive CIS cells into invasive cancer. In addition, it was demonstrated that intratubular seminoma cells and seminoma cells, but not CIS cells, show an overrepresentation of the 12p chromosome (Looijenga et al. 2000; Rosenberg et al. 2000; Oosterhuis et al. 2003). It may be speculated that altered gap junctional intercellular communication may play a role in this multistage carcinogenic process, as it was shown in normal testis that several cell types have to interact with each other and with other components of the tissue microenvironment properly and coordinated, including neighbouring mesenchymal cells and extracellular matrix (Jegou 1993). It is obvious that disturbances in these complex cell-to-cell interactions may lead to progression of testicular germ cell tumour development.

As the mechanisms underlying the phenomenon of latency between fetal malignant transformation, pubertal proliferation of CIS and progression of CIS to an invasive malignancy are still not known, it was proposed that altered cell–cell communication via gap junctions may play a role in pubertal testicular cancer progression (Brehm et al. 2002). Different scenarios underlying reduction and/or loss of intratubular connexin 43 expression can be considered.

5.4.1 Loss of Contact Inhibition

During testicular tumour development, loss of gap junctional intercellular communication may occur due to suppressed expression of connexin 43 at early stages

(Brehm et al. 2002). Several lines of evidence suggest that a disturbance of gap junctional intercellular communication facilitates the clonal growth of potential cancer cells and connexin genes may act as tumour suppressors (Lee et al. 1991; Mesnil et al. 1995; Saito et al. 2001), and several reports have demonstrated that connexin expression is decreased in precancerous lesions (Krutovskikh et al. 1991; Kamibayashi et al. 1995; Temme et al. 1997). Thus, it may be speculated that loss of gap junctional intercellular communication enhances clonal dispersion of CIS cells, causing loss of growth suppressing signals from the surrounding Sertoli cells accreting cell proliferation. It was hypothesized a long time ago that gap junctional intercellular communication controls cell growth by transmission of growth regulatory signals and that loss of growth control in cancer cells may be due to decreased communication capacity (Yamasaki 1996). Most cancer cells either lack or display decreased gap junctional structure and function, and aberrant expression and function of connexins are associated with tumour progression (Krutovskikh et al. 1991; Lee et al. 1991). Consistent with results from Brehm et al. (2002), Wilgenbus et al. (1992) failed to identify connexin 43 in a small number of human testicular tumours by immunofluorescence, and Roger et al. (2004) found an aberrant intracytoplasmic connexin 43 immunostaining in seminoma cells of a small number of human seminoma patients and in the seminoma cell line JKT-1, but no typical bands in Western blots. Taken together, all studies in neoplastic human testes suggest a role of connexin 43 in human seminoma development from CIS and show that human seminoma cells are not able to communicate normal via functional connexin 43 gap junctions.

5.4.2
Loss of Gap Junctional Intercellular Communication

One conceivable way by which CIS cells become invasive and penetrate the tubular wall (Schulze and Holstein 1977) or start to proliferate in situ (Donner et al. 2004) may be a loss in homotypic or heterotypic gap junctional intercellular communication via connexin 43 with adjacent Sertoli cells. In both cases, tumour cells become isolated from adjacent Sertoli cells, which may lead to an increase of invasive potential and proliferation rate of CIS or intratubular seminoma cells. In neoplastic uterine cervix, a drop in gap junctions has been demonstrated to represent one step towards invasiveness and malignant transformation (Kocher et al. 1981; Schindler et al. 1981). Here, the concomitant and gradual decrease of gap junctions paralleled the histologic progression towards malignancy in testicular premalignant and malignant lesions, as junctions became rarer with each increasing degree of malignancy. The loss of intercellular junctions of Sertoli cells and CIS cells might, therefore, express failure of their differentiation and could be associated with a certain cellular autonomy, increased invasive potential and abnormal behaviour of preinvasive and invasive cancer cells.

Data from the connexin 43 protein level (Brehm et al. 2002; Roger et al. 2004) are supported by ultrastructural studies (Gondos et al. 1983; Gondos 1993) sug-

gesting that pathogenesis of germ cell neoplasia involves excessive proliferation of precursor germ cells associated with a loss of intercellular communication. Cell borders of adjacent CIS cells were found to be closely approximated, but junctional attachments or other surface modifications could not be appreciated. Cell membranes were closely apposed without recognizable junctional attachments and intercellular bridges were not seen. Their absence might indicate a lack of a normal constituent of germ cell differentiation (Holstein and Korner 1974). Germ cell interconnections are normally present in large numbers in the testis and are considered to be responsible for the synchronization of spermatogenesis (Gondos 1993), and cytoplasmic bridges between normal germ cells of the same stage may permit rapid and constant communication from Sertoli cells throughout the germ cell clone (Risley et al. 2000).

Although the original descriptions of such bridges were in groups of spermatocytes and spermatids, some studies have demonstrated their presence at spermatogonial and prespermatogonial stages of differentiation (Gondos and Hobel 1971), indicating that their absence in CIS cells represents a significant departure from the normal appearance. In addition, seminoma cell surfaces exhibit only scattered short projections and simple infrequent contact with adjacent cells, and intercellular bridges or gap junctions were not detected. Thus, it is well possible that a factor which normally limits germ cell mitosis in seminiferous tubules is lacking or defective in cases of neoplasia or neoplastic CIS cells. One such mechanism could involve the contact inhibition of CIS cells by gap junctional intercellular communication or controlled cell proliferation provided by normal gap junctional intercellular communication. It seems that failure of gap junctional intercellular communication or intercellular bridge formation leads to continued uncontrolled mitotic division or neoplasia. Lack of intercellular bridges could also affect intercellular communication as different pathways of testicular communication are closely combined. The excessive pubertal proliferation of CIS cells could thus be related to a lack of intercellular communication. Some other ultrastructural studies of CIS and seminoma cells showed similar results, demonstrating that no desmosomal attachments between neoplastic cells and remaining Sertoli cells have been observed, intercellular bridges of the type connecting normal developing germ cells were absent and gap junctions not explicitly mentioned (Holstein and Korner 1974; Gondos et al. 1983; Gondos and Migliozzi 1987; Gondos 1993), whereas electron microscopy has produced morphological evidence for the existence of gap junctions between Sertoli cells and between Sertoli cells and normal germ cells (Russell 1977b, 1993b; McGinley et al. 1979; Russell and Peterson 1985; Enders 1993). It is proposed that failure, reduction or loss of gap junctional intercellular communication via connexin 43 gap junctions in addition to detected failure of intercellular bridge formation might lead to continued uncontrolled mitotic division of CIS cells and finally to solid invasive neoplasia. Additional support for this hypothesis comes from a mutant species of *Drosophila* in which absence of germ cell connections was found to be associated with spontaneous germ cell tumours (Gollin and King 1981).

5.4.3
Role of Connexin 43 in Disintegration of the Blood–Testis Barrier

Connexin 43 is known to play a role in the regulated and coordinated formation of the blood–testis barrier at puberty (Pelletier 1995). Increasing numbers of intratubular CIS cells concomitant with increasing severity of disease and reduction of direct cell-to-cell communication between CIS cells and Sertoli cells contribute (a) to the loss of Sertoli cell functional and morphological integrity, (b) to an advanced degeneration and dedifferentiation of Sertoli cells leading to a cessation of spermatogenic activity, (c) to a reduction of inhibitory and controlling effects on growth and differentiation of precancerous CIS cells exerted by adjacent Sertoli and (d) to a change or damage of the Sertoli cell junctional complexes. It is well possible that a loss of regulator connexin 43 leads to damage and disruption of the barrier. These findings could be interpreted as additional signs of disintegration of normal structures within the seminiferous epithelium going along with tumour progression or invasiveness of CIS cells.

5.4.4
Effects of Environmental and Endocrinological Substances on Gap Junctional Intercellular Communication and Connexin 43 Expression

An interesting and intriguing question remains: Which are the target cells during fetal initiation and/or pubertal progression of human testicular cancer for proposed environmental and endocrinological substances (Skakkebaek et al. 1998; Rorth et al. 2000; Sharpe et al. 2003)?

Some pesticides are known to possess, in vitro, weak oestrogenic activities, as they are able to bind to oestrogen nuclear receptors and to induce proliferation of MCF-7 breast cancer cells (Cooper and Kavlock 1997). Based on these features, it could be possible that these chemicals mimic or interfere with the action of normal sexual steroid hormones (McLachlan 2001; Skakkebaek 2002). As connexins or gap junction channels are known to be direct targets for pesticides (Trosko and Ruch 1998), there is strong evidence that environmental chemicals are able to target gap junctional intercellular communication (Yamasaki 1996; Trosko and Ruch 2002).

This assumption was confirmed using the Sertoli cell line 42GPA9. It was reported that Lindane, a member of the lipid-soluble pesticide family, is able to impair and modulate testicular gap junctional intercellular communication via connexin 43-containing channels between Sertoli cells using the mitogenic MAPK pathway by promoting the intracellular localization of this tumour suppressor within endosomes. These results give rise to the theory that such inappropriate activation of the mitogenic MAPK pathway and consequently inactivation of the tumour suppressor connexin 43 by Lindane may indirectly participate via Sertoli cells in the promotion of neoplastic cell growth (Mograbi et al. 2003). Investigation of connexin 43 expression in human seminoma cells showed a disrupted traffic of this gap junctional protein to the plasma membranes (Roger et al. 2004). Unfortunately, this study only investigated a very small number of seminoma patients and

the seminoma cell line JKT-1. Here, connexin 43 was found to be immunolocalized aberrantly in the Golgi apparatus without membrane expression indicating an impaired transport or assembly of functional connexin 43 gap junctions to the plasma membrane in seminoma cells. Applying Western blotting in the seminoma cell line JKT-1 and in patients with normal spermatogenesis, an abnormal 70-kDa band, but no classical 43-kDa isoforms, was detected, leading to the conclusion that prevention of connexin 43 membrane expression may contribute to tumoural proliferation (Roger et al. 2004).

5.4.5 Role of Connexin 43 in Cell Cycle Control

It is known that connexin 43 plays a role in cell cycle control (Chen et al. 1995; Zhang et al. 2003b). Loss of cell cycle-dependent kinase inhibitor p21 expression was shown to be associated with the development of invasive germ cell tumours from CIS (Datta et al. 2001), as p21 expression was noted in most cases of CIS, but the corresponding invasive tumour had lost p21 expression. Both N-cadherin and connexin 43 were demonstrated to induce p21 expression leading to a decreased cyclinB-Cdc2 kinase activity and to reduced cell proliferation (Kamei et al. 2003). Upregulation of p21 in cells expressing N-cadherin and connexin 43 was greater than in cells expressing N-cadherin alone. The synergistic effect of connexin 43 is thus dependent on its ability to mediate intercellular communication via which channels from G_2 arrested cells, like Sertoli cells, may directly activate a signal transduction pathway leading to p21 induction in neighbouring germ cells. It may be suggested that, if there is no connexin 43 expression, as is the case for seminiferous tubules containing only CIS cells and Sertoli cells, no possible induction of p21 in abnormal CIS cells can occur leading to an increased cell proliferation rate.

5.4.6 Genetic Background for Downregulation of Connexin 43

Chromosomal constitution of CIS, intratubular seminoma and adjacent invasive seminoma was found to be very similar using comparative genomic hybridization (CGH). Most of the imbalances present in the intratubular seminoma and invasive seminoma were already observed in CIS and seem to be involved in the progression from CIS to invasiveness. Data from a CGH study of microdissected samples from different stages in the development of seminoma revealed numerous changes in parts of chromosomes being involved in the early development and progression of this cancer (Looijenga et al. 2000). Interestingly, in microdissected CIS tubules significant alterations of parts of chromosome 6q (gains of 6q in CIS tubules preceding seminoma and losses of parts of 6q in CIS tubules preceding non-seminoma) were detected in CIS, indicating that changes of this chromosome could play a role in early development and/or promotion or progression phase of germ cell cancer. Since Sertoli cell nuclei were isolated, it cannot be excluded that

also these somatic cells show chromosomal changes. Furthermore, other groups have detected changes at chromosome 6 in seminoma or human germ cell tumour cell lines including teratoma and yolk sac tumour, indicating that this region may contain genes involved in the divergent development of testicular germ cell tumours (Oosterhuis et al. 1997; Summersgill et al. 1998, 2001). Based on these data, it can be speculated that changes on chromosome 6q harbouring the connexin 43 gene could be a reason for changes in connexin 43 expression in CIS.

Finally, the lack of connexin 43 gap junctions in CIS and seminoma may act as a new potential therapeutic target. Earlier studies indicated that restoration of connexin 43 in both preinvasive and invasive tumour cells lacking connexin 43 can reverse the transformed phenotype (Hirschi et al. 1996), suggesting that a strategy to express functional connexin 43 may be of therapeutic value. Furthermore, restoration of connexin 43 expression or a chemical induction of connexin 43 in tumour cells can enhance the bystander effect in gene therapy approaches using suicide gene pro-drug targeting (Dilber et al. 1997; Trosko and Ruch 1998; Carystinos et al. 1999; Laird et al. 1999). These observations indicate that specific modulators of testicular connexin 43 should have therapeutic implications in cancer making preinvasive CIS cells prone to normal regulatory effects from normal adjacent Sertoli cells. One possible mechanism to increase drug penetration and dispersal in testicular preinvasive CIS would be to reinstall gap junctional intercellular communication. This increase in gap junctional intercellular communication might be achieved by increasing tumour cell connexin expression pharmacologically (e.g. with forskolin, steroids, and retinoids) or by introducing active connexin genes (gene therapy approach).

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