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Mammalian Spermatogenesis

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ABSTRACT

The formation of mature spermatozoa is one of the most essential functions in life. A concerted sequence of events is needed to proliferate, maintain and mature germ cells starting with spermatogonial stem cells and culminating in mature gametes. Apart from the genetic background, this process requires highly organized tissue in which the complex process of spermatogenesis is strongly regulated by hormonal interplay, differential gene expression and cell-cell communication. Although similar overall principles of spermatogenesis are found in all mammalian testes in a much conserved pattern, numerous species-specific features such as efficiency and seasonality determine differences between the various mammals. In this article, we focus on morphological principles as well as on endocrine regulation and action of selected genes. Furthermore we report on recent experiments addressing the fate and physiology of spermatogonial stem cells, testis biology and development of the germ line and the somatic part of the testis by germ line transplantation and *in vitro* approaches.

Keywords: endocrine regulation, germ cell culture, germ line transplantation, testicular topography, testis

Abbreviations: **AMH**, anti-muellerian-hormone; **AR**, androgen receptor; **CDH-1**, formerly known as E-cadherin; **CG**, chorionic gonadotropin; **CREM**, cAMP response element modulator; **DAZ**, deleted in azoospermia; **DAZL**, deleted in azoospermia like; **ERM**, Ets related molecule; **ES cells**, embryonic stem cells; **FACS**, fluorescence activated cell sorting; **FSH**, follicle-stimulating hormone; **FSHR**, follicle-stimulating hormone receptor, **GDNF**, glia cell line-derived neurotrophic factor; **GFRα1**, GDNF family receptor alpha 1; **GnRH**, gonadotropin-releasing-hormone; **GPR54**, G-protein-coupled receptor 54; **JSD**, juvenile spermatogonial depletion; **LH**, luteinizing hormone; **LHR**, luteinizing hormone receptor; **LIF**, leukaemia inhibiting factor; **MACS**, magnetic activated cell sorting; **PGC**, primordial germ cells; **PR**, progesterone receptor; **SACS**, soft agar culture system; **SCF**, stem cell factor; **SCO**, Sertoli cell only syndrome; **SRY**, sex-determining region Y; **SSC**, spermatogonial stem cell; **TP**, transition proteins

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INTRODUCTORY REMARKS

Spermatogenesis is defined as the process of male gamete production. The site of spermatogenesis is the male gonad, the testis. Therefore, spermatogenesis summarizes all events that transform basic spermatogonia into highly specialized mature spermatozoa within the testis. Spermatogonia derive from primordial germ cells (PGCs) which, after entering the testis, develop into gonocytes. After spermatogenesis spermatozoa migrate from the testis into the epididymis where they are prepared to reach and fertilize eggs, and transfer the paternal genomic information to the next generation.

In the testis, the germ cells are located in tubules of which their inner side is covered by the seminiferous epithelium containing somatic Sertoli cells which provide nourishment and support cells of the germ line. Before a gamete can leave the testis, it has to pass through several stages of maturation. These processes include mitotic multiplication and propagation of the spermatogonial stem cells (SSCs), meiotic recombination of genetic material and testicular maturation of spermatozoa (Ehmcke *et al*. 2006).

Several developmental stages of germ cells are distinguished of which the haploidization of the genome is the major event, the meiotic division. In the fully developed mammalian testis, the majority of undifferentiated cells of the germ line are type A spermatogonia. This population of cells also includes the SSCs. These are the most important

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Invited Review

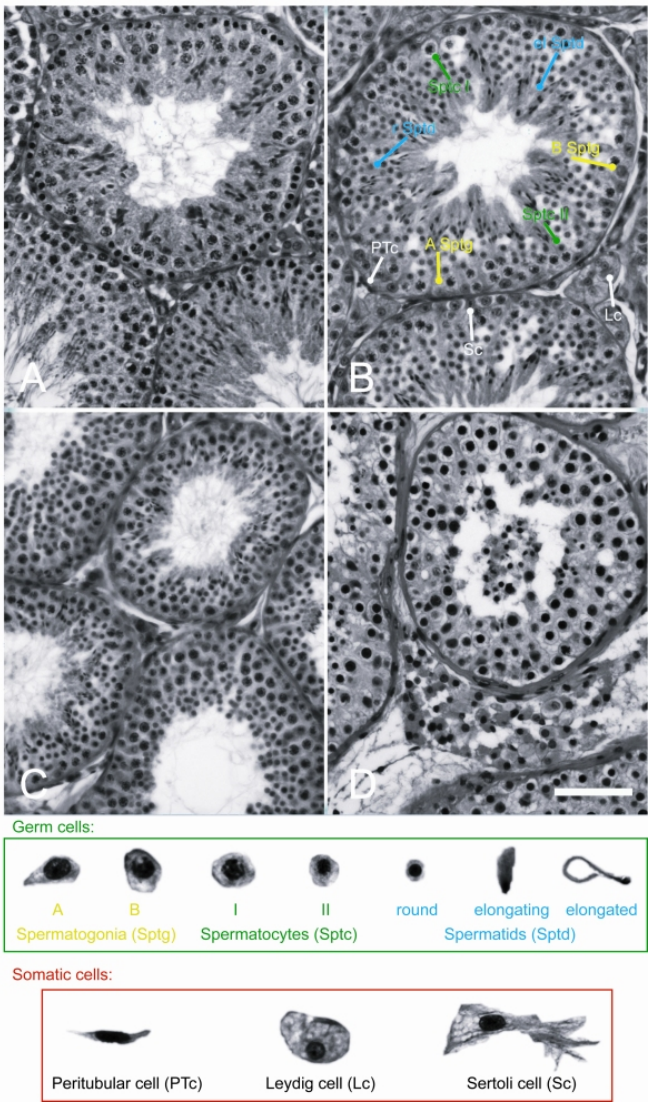

Fig. 1 Testicular histology of some mammalian testes. Different cross sections of the seminiferous tubules of (**A**) Djungarian hamster (*Phodopus sungorus*), (**B**) cat (*Felis catus*) (**C**) crab-eating macaque (*Macaca fascicularis*), (**D**) human. Among mammals, the basal organization of the germinal epithelium and the interstitium is similar. The germ cells and the somatic Sertoli cells of the various developmental stages are located in the seminiferous tubules, while the myoid peritubular cells cover the basal lamina and the Leydig cells are positioned in the interstitium. The various germ cell stages and the somatic testicular cell types are shown in detail in the lower row. Abbreviations: spermatogonia (Sptg); spermatocytes (Sptc), round spermatids (r Sptd), elongating and elongated spermatids (el Sptd), peritubular cells (PTc), Leydig cells (Lc), Sertoli cells (Sc). Scale bar = 200 µm.

cells for spermatogenesis because their task is to provide both self renewal of the SSCs and spermatogonia of type B that differentiate and divide mitotically into cell stages that are able to enter meiosis. Differentiated type B spermatogonia develop into primary spermatocytes undergoing meiosis, reducing the genomic content in the secondary spermatocytes. Thereafter another genomic reducing step leads to haploid spermatids, the beginning of spermiogenesis. During this period, the spermatids have to drastically alter their shape and content. In this process of spermiogenesis the cytoplasmic content decreases and the DNA condenses. The spermatids form the acrosom, an organelle which is essential for later interaction with the egg membrane during fertilization. Furthermore, the shape of the germ cells is transformed from relatively roundish into small spindle-shaped cells. After elongation of the spermatids, the typical form of the spermatozoa is achieved with a head bearing the nucleus, the acrosom, a midpiece that provides metabolic energy by mitochondria, and the flagellum the prerequisite for the ability of the sperm to progress through the female tract (see de Rooij and Grootegoed 1998; de Rooji and Russell 2000).

Among mammals, the basic processes of spermatogenesis resulting in mature male gametes are very similar. They are highly organized and require complex endocrine as well as genomic regulation supported and mediated by somatic cell types, the Sertoli cells in the tubules and the peritubular myoid cells and the Leydig cells in the testicular interstitium (**Fig. 1**).

The first part of this review presents the characteristic features of spermatogenesis, the genomic meaning and aspects of certain gene functions as well as the structural prerequisites and finally the underlying endocrine mechanisms. In the following three sections we summarize general principles of mammalian spermatogenesis and describe the main differences between spermatogenesis and oogenesis. We discuss some hypothetical aspects of the male-biased evolution with regard to spermatogenesis as a tool to protect genomic integrity. In detail, we focus on certain well examined genes essential for spermatogenic success.

Section five explains the structures of the testicular environment and summarizes observations on one of the most important features of the testis, the SSC populations, driving lifelong gamete production. Furthermore, we review recent findings on testicular organization and on the topography of efficient spermatogenesis (Wistuba *et al*. 2003; Luetjens *et al.* 2005).

The sixth section describes the endocrine axis in which hormones regulate spermatogenesis and male metabolism; in particular, we focus on the gonadotropins and their receptors since it was recently demonstrated that these hormone-receptor interplay differs among primates (Gromoll *et al*. 2003; Luetjens *et al.* 2005).

In the second part of this review, the two final sections elucidate novel experimental approaches addressing the manipulation of spermatogenesis by germline transplantation and finally by culture of germ cells to complete the highly complex spermatogenic processes *in vitro*.

SEXES ARE DIFFERENT: OOGENESIS VS. SPERMATOGENESIS

The formation of functional male gametes is one of the most essential functions in male life. This requirement underlies all of the metabolic actions that finally result in spermatogenesis, and which are unique and restricted to the male gonad. A gene localized on the Y chromosome, the Sry (sex-determining region Y) is responsible for the formation of a testis. Only if this gene is activated during early fetal development, the undifferentiated genital ridge, the gonadal *anlage*, will become a testis with the ability to support spermatogenesis (as reviewed by Wilhelm *et al*. 2007).

Interestingly, in mice it was demonstrated that embryonic germ cells as such can develop into oocytes as well as into spermatogonia. The molecular mechanisms underlying the individual decision on the fate of the germ cell are still poorly understood. During embryogenesis the potency of primordial germ cells (PGCs) allows both, either spermatogenesis or oogenesis. The environment provided by the genital ridge obviously influences the initiation of meiosis. If the cells enter a female genital ridge or are brought into a non-gonadal surrounding, the PGCs become meiotic oocytes. The somatic cells of a male genital ridge inhibit PGCs from entering meiosis and therefore direct them to a spermatogenic fate. The suggested underlying molecular mechanism is a feedback between PGCs and the environment of the gonadal *anlagen*. The response of PGCs to a masculinizing environment is the synthesis of a paracrine factor, prostaglandin D2. The effect of prostaglandin was proven by its ability to masculinize female embryonic gonads. This molecule induces a transformation in the embryonic female gonad of supporting cells into Sertoli cells (Adams and McLaren 2002). In the male, this signal estab-

lishes the interplay between the germ line and the developing testis, resulting in a suppression of meiosis and thereby the initiation of the spermatogenic pathway. A further candidate involved in the processes determining germ cell fate is retinoic acid. This metabolite is produced in the mesonephroi of both sexes but in an ovarian environment causes germ cells to enter meiosis and initiate oogenesis, while in the fetal testis meiosis is inhibited by enzymatic degradation of retinoic acid (Bowles *et al*. 2006). Moreover, the interplay between gonadal environment and retinoic acid levels appears to be an important mechanism for initiation of either oogenesis or spermatogenesis.

The spatial sequence of events required to generate mature gametes in female mammals is totally different from the male. The cause for the main differences between male and female gamete production and maturation is that in females no self-renewing stem cell population has to be maintained. In the male a highly organized sequence of differentiation processes results in a lifelong permanent production of billions of sperm. These are derived from a relatively low number of undifferentiated spermatogonial stem cells (SSCs). In the females, all germ cells are already generated at birth, differentiated and arrested at the prophase of meiosis I. This germ cell production and partial maturation takes place during fetal development, a phase in which the male gonad is far distant from meiosis or spermatogenic maturation.

In summary, oocyte numbers are limited and – once all are naturally depleted or lost by cytotoxic events – fertility and the chance to produce offspring is gone although the uterus maintains the capacity for a pregnancy as egg-donations to postmenopausal women have shown. In principle, a male can father offspring until his life ends; even disturbances can, at least in part, be compensated by the recovery of spermatogenesis as long as the stem cell population was not lost or destroyed (Kühnert and Nieschlag 2004). Furthermore, the goal of spermatogenesis is the production of compact, densely packed mobile cells, specialized to access and fertilize an oocyte. In contrast, oocytes are adapted to store essential components as ribosomes, cortical granules, mRNA and proteins to provide the early embryonic cleavage stages with metabolites and the genetic information for spatial events. The cytoplasm has the ability to reprogram the somatic cell nucleus and regulate embryonic development. This process is impossible in male germ cells because of the lack of cytoplasm. This difference results in an altered requirement fulfilled by the gonadal tissues and regulating signals. Thus it is not surprising that ovary and testis show a completely different organizational pattern. Although ovarian tissue has different cell types, they are not organized in a greater structural arrangement. After fetal development, the ovary is directed to maintain and nourish the pool of oocytes present until the onset of puberty and beyond. After onset of puberty, the ovary has the function to regularly produce female gametes through ovulation, thus maintaining the female reproductive cycling.

Moreover, on the genomic level sex-specific features also reflect differences between male and female germ cells. The gametes, spermatozoa and oocytes, present a sex-specific methylation pattern that is established during oogenesis and spermatogenesis. These patterns are required for the allele-specific imprinted genes' expression in the somatic cells. To ensure the sex specificity of imprinting, the exons controlling the enzyme DNA methyltransferase responsible for methylation are differently regulated between the sexes, resulting in various expression and functions of this enzyme in spermatogenesis and oogenesis (Mertineit *et al*. 1998). The higher number of germ cell divisions taking place in sperm production gives rise to a higher number of genetic exchanges. Single point mutations in human sperm which are responsible for achondroplasia and Apert's syndrome, two autosomal dominant diseases, increase with the man's age (Kühnert and Nieschlag 2004). Wheras oocytes are at higher risk to undergo numerical or structural chromosomal changes which lead to, for example trisomy 21 due to the long period of meiotic arrest from birth to ovulation which, for example, may last between 10 and 50 years in humans.

THE MEANING OF SPERMATOGENESIS: PROTECTION OF GENOMIC INTEGRITY DURING GAMETE PRODUCTION

Sexual reproduction, a non-random process, is crucial for diversification and adaptation – key parameters of evolutionary progress. Apart from an optimized partner choice and quality control mechanisms during the fertilization processes (e.g. Fedina and Lewis 2004), a morphological and genetic selection of gametes occurs during the entire process of gametogenesis (e.g. Bernasconi *et al*. 2004).

In higher organisms, the mutation rate is used to describe the proportion of mutations per gamete per time unit. One explanation of varying mutation rates between the sexes and therefore different rates of genetic changes is the correlation between frequency and number of germ cell divisions (Haldane 1947; Ellegren 2007). Sex differences in replication and mutation are associated with the cell division number, which is extremely high in spermatogenesis and low in oogenesis. Errors during the replication processes are the main source of mutations. The more often DNA is replicated, the more possibilities occur for such errors. Therefore mutation rates rise when the number of cell divisions increases in the germ line. The mutation rate, being one main requirement for evolution, can be considered male biased (Li *et al*. 2002). When sequence divergences of chromosomes are compared, it becomes possible to calculate a male-to-female mutation ratio which differs among the taxonomic groups and is, for example, higher in primates than in rodents or carnivores (for review see Ellegren 2007).

Genetic diversity among individuals in both sexes rises during meiotic recombination of chromosomes. The meiotic process is restricted solely to male and female germ line cells. Recombination rates vary across species, sexes and chromosomes. For a long time it was thought that only the autosomal chromosomes play a role in building up newly grouped genetic combinations. In male mammals the sex chromosomes are heterogametic and cannot pair with its counterpart during the first meiotic division (male sex, e.g. XY karyotype in placentaria) leading to no new combination. The Y-chromosome has adopted a special recombination mode resulting in recombination within the chromosome (Rozen *et al*. 2003; Skaletsky *et al*. 2003; Willard 2003). The chromosome containing many highly repetitive sequences pairs with itself to gain recombination of the gene sequences. Although only one allele per individual exists, several gene copies may be present. As a possible reason for this difference it was assumed that too much recombination could impair the stability of a successful genome. The heterogametic male selection may negatively influence the reproductive success. Thus a balanced stable genetic combination can be of advantage to gain access to the oocytes and achieve fertility (Lenormand and Dutheil 2005). A suggestion explaining this phenomenon may be that selection is not limited to a taxonomic group or to an individual but affects the gametes as such ("haploid selection"; Lenormand and Dutheil 2005). In most species male gametes would undergo a stronger selection process than female gametes because sperm production offers more mutational possibilities and therefore more "quality control" mechanisms are needed. In summary, male germ cells may change their genetic contents more rapidly than female gametes (number of germ cell divisions). For the same reason they have to be controlled more strongly on the chromosomal level to provide genomic integrity for reproductive success , i.e. by maximized offspring.

The characteristics of spermatogonial stem cells (SSCs), which represent the cell population in the testis, driving spermatogenesis by both, self-renewal and mitotic propagation (see below), seem to vary between different species but definitely differ among mammalian taxa. This ambivalent

cell population mainly fulfils two tasks in parallel: firstly the maintenance of the individual male germ line (self renewal) being adult stem cells and secondly to generate daughter cells differentiating into gametes transferring the genome into the next generation. Accepting that testicular stem cells work as a mitotically minor active germ line reserve, their most important function is to protect the integrity of the prospective gene pool. Only if this task can be fulfilled, a permanent replacement of healthy germ cells maintaining normal spermatogenesis is assured. When comparing SSCs and early differential steps of spermatogenesis among rodents and primates, primates exhibit a further population of progenitor germ cells, whilst rodents obviously do not need such precursors. They have a single-type spermatogonia (type A) covering two tasks – building up a regenerative selfrenewing stem cell (i.e. SSCs) reservoir and providing cells for reproductive differentiation. These differences must be linked to differences among primates' and rodents' life expectancy and number of potential offspring, i.e. different reproductive strategies. Hence, long-living species with a relatively small number of offspring are at higher risk of being exposed to adverse environmental events affecting the germ line integrity than species with a shorter life expectancy with numerous offspring (Ehmcke *et al*. 2006). Concluding, both evolutionary strategies are differentially affected by their ability to protect the genome of their gametes.

Therefore in primates the testicular progenitor cells of the germ line are implemented as an additional tool for germ line protection, a mechanism obviously not necessary in rodents, probably due to a reproductive strategy adapted to shorter life expectancy. The different strategies of reproduction are reflected by the differing organization of the SSC population in the testis – giving evidence that phylogeny has influenced the physiological features of germ line stem cells (Ehmcke *et al*. 2006).

In conclusion, production of male gametes appears on the one hand to offer a playground for evolutionary processes due to the mode of spermatogenesis; on the other hand, gamete quality is supported by a pool of germ line spermatogonial stem cells subjected to a restrictive control.

GENE EXPRESSION AND SPERMATOGENESIS

Deciphering genomes is a milestone of modern biology. A major result of the sequenced DNA bases is that we understand only a minor portion of how the entire genome actually works. Comparative genomic analysis between rodents, primates and the human showed the surprising fact that the number of genes does not seem to be the main cause of the huge phenotypyical diversity among species. Alternative splicing of genes and the resulting variety of protein isoforms may make the difference and drive the functional complexity (Modrek and Lee 2002).

Interestingly, even if species are as closely related as humans to chimpanzees, spermatogenesis-related gene expression patterns in the testis can differ much more than in other organs (Khaitovich *et al*. 2005). In contrast to other metabolic processes, most spermatogenic genes seem to lack backup mechanisms. If the expression of only one gene involved in a certain sequence of spermatogenic events is disturbed, spermatogenesis will fail, although numerous genes are involved in the completion of gamete maturation. The effects observed in such cases – mostly spermatogenic arrest at different stages of spermatogenic development, as Sertoli-cell-only (SCO, germ cells are completely lost in the seminiferous tubules), spermatogonial, pre-meiotic or post-meiotic arrest – are morphologically comparable but are caused by the failure of various different genes (**Fig. 2**). The majority of relevant spermatogenic genes analyzed in more detail so far have come into focus due to experiments with knockout mice or because of genetically caused infertility problems of men.

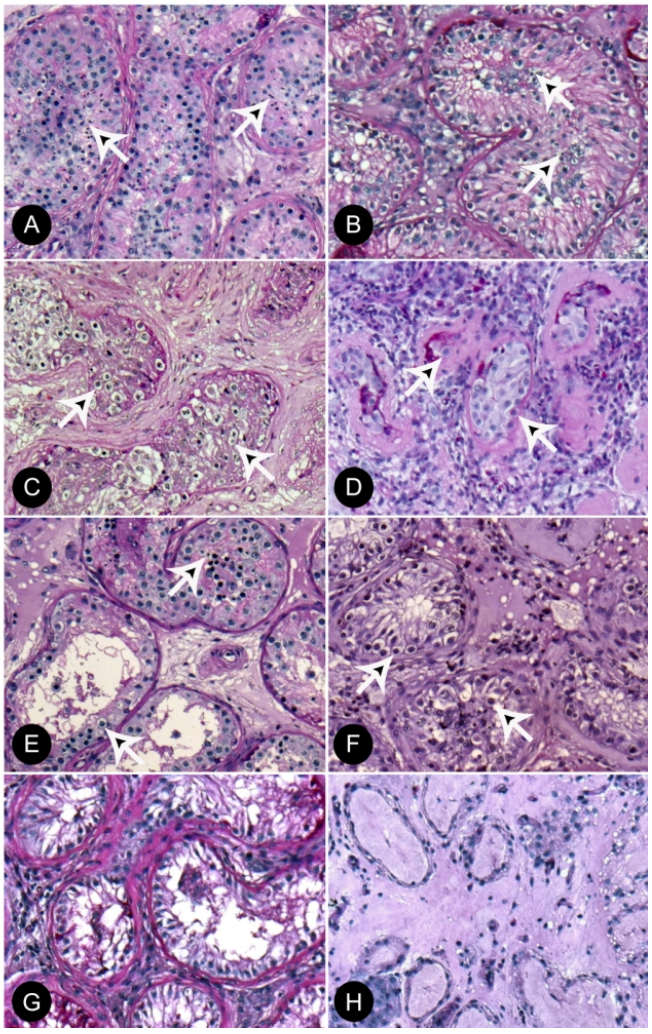

Fig. 2 Testicular histology of samples from infertile patients. All stages of spermatogenic development and types of possible spermatogenic arrests are representatively shown. (**A**) Full spermatogenesis with elongated spermatids; (**B**) spermatogenesis up to the round spermatid stage; (**C**) meiotic arrest with spermatocytes as last developmental stage; (**D**) spermatogonial arrest, (**E, F**) Mixed atrophy with tubules all containing different developmental arrests; (**G, H**) no germ cells in the tubules: (**G**) Sertoli cell only; (**H**) tubules contain no cells anymore and are filled with extracellular matrix. Arrows indicate the most advanced germ cell stage per tubule. Scale bar = 200 μm.

Arrested spermatogenesis is correlated to failure of gene expression

Due to the massive number of possibly relevant genes, this review can only concentrate on selected genes related to spermatogenesis. These few examples demonstrate how the failure of gene expression at certain crucial points of spermatogenesis results in the typical morphological paradigm of spermatogenic arrest and failure to generate intact male gametes.

The spermatogonial stem cells (SSCs), exhibiting the most fascinating feature to be able to maintain the fundamental germ line potential of an individual by their ability to self renew as adult stem cells and to give simultaneously rise to daughter cells that enter the route of differentiation, inhabit a certain niche within the testicular tubules before they enter differentiating mitotic cycles regulated by the Sertoli cells (Hess *et al*. 2006). Even if the SSCs manage to colonize a niche within the seminiferous epithelium, this stem cell niche has to communicate with other spermatogonial cells to initiate the differentiation processes. Spermatogenesis only starts when SSCs begin the process of differentiation and undergo mitotic division to form germ cell clones.

As we learned from studies performed in mutant mice, during this key event, the spermatogonial membrane receptor c-kit and its ligand stem cell factor (SCF) (Yoshinaga *et al*. 1991) as well as Ets-related molecule (ERM) (Hess *et al*. 2006) play a crucial role in regulating stem cell colonization, expansion and differentiation (Ohta *et al*. 2000). If one of the molecules is missing due to mutation, c-kit (encoded at the *dominant white spotting*, *W locus*) or SCF (encoded at the *Steel* locus), spermatogenesis is interrupted at a very early stage. Morphologically it arrests at the level of spermatogonial cells if they manage to remain intact in the seminiferous epithelium or only SCO tubules remain. While c-kit is a molecule expressed in early differentiating spermatogonia, SCF and ERM are products of the Sertoli cells. Both are involved in the formation of the stem cell niche. In mutant mice lacking c-kit endogenous spermatogenesis is completely lost. Therefore, this mouse has become a worthy tool in transplantation assays, being a naturally aspermatogenic recipient. It offers the advantage of still being able to support donor-derived spermatogenesis by offering an intact somatic testicular environment (Wistuba and Schlatt 2002). In contrast, mice that do not express SCF in their Sertoli cells show arrested spermatogenesis because of the impossibility to support germ cell differentiation.

Compared to mice models, some azoospermic men show similar phenotypes in testis biopsies. In these biopsies various types of spermatogenic arrest have been found. One prominent group of these arrested phenotypes is the so-called spermatocyte arrest which mainly comprises all sorts of arrested germ cells in the first meiotic cleavage. In an attempt to determine what mechanism halts these cells in their further development, cell cycle participating proteins came into focus. Because the cell cycle has been extremely conservative during evolution, the knowledge gained in fruit flies helped to unveil such mechanisms. A mutation in the *boule* gene of the fruit fly (*Drosophila melanogaster*) leads to a phenotype with germ cells halter at the spermatocyte stage. This phenotype can be rescued by inserting the human gene *BOULE* into the genome of the fly (Xu *et al*. 2003). Interestingly, azoospermic men with a similar phenotype have no BOULE expression in their testes (Luetjens *et al*. 2004). This leads to the halt, due to the effect that BOULE has to regulate the translation of *CDC25* mRNA into its protein. Subsequently, CDC25 forms with cyclin B the maturation promoting factor, driving meiosis. Interestingly, the above mentioned patients have no CDC25 expression and therefore show no later germ cell stages (Luetjens *et al*. 2004). The *BOULE* gene belongs to the DAZ (*Deleted in AZoospermia*) gene family which was first identified in azoospermic men with Y-chromosomal deletions (Reijo *et al*. 1995) including the genes *BOULE*, *DAZ-like* (*DAZL*) and *DAZ* (Xu *et al*. 2003). Following a gene duplication of *boule*, the new gene *Dazl* originated in early ancestors of the vertebrates and during evolution of primates the *DAZ* gene emerged from duplication of the *DAZL* gene (Tung *et al*. 2006). Both evolutionary early genes *BOULE* and *DAZL* remain within the autosomes but the *DAZ* gene was translocated to the Y-chromosome. Therefore only spermatogenesis is affected and only males can have this gene (Xu *et al*. 2003). In men, where *DAZ* and *DAZL* co-exist, both genes share a high homology of up to 90% (Saxena *et al*. 1996). A striking characteristic of the Y-chromosomal *DAZ* gene is the intragenic amplification giving rise to a protein domain referred to as the DAZ repeat region (Saxena *et al*. 1996). Studies in infertile men indicate that deletions of the *DAZ* genes may cause defects in spermatogenesis beginning in the stem cell line.

Targeted disruption of *Dazl* expression in the mouse results in complete sterility of both sexes caused by germ cell maintenance and maturation failures (Ruggiu *et al*. 1997). *Dazl* was detectable in type-B spermatogonia, preleptotene and zygotene spermatocytes. However, histological staining of testis from 9-week-old down regulated rats revealed that mature sperm were not produced, but germ cells were present and developed beyond meiosis. The testes contained only abnormal cells with rounded nuclear morphology in the lumen of the seminiferous tubules (Dann *et al*. 2006). These data show that DAZ is required in spermatogonia during the early differentiation of germ cells and DAZL mainly in early spermatogenesis but also for the maturation process of elongating spermatids, i.e. spermiogenesis.

Obviously, the cAMP response element modulator (CREM), a transcription factor of which several isoforms have been identified in the mouse (Foulkes *et al*. 1992) and in the primate testis (Behr and Weinbauer 2001), is also involved in the regulation of the expression of genes that are necessary for spermiogenesis (Sassone-Corsi 1995). CREM expression is modulated by FSH. Male CREM knockout mice exhibited a round spermatid maturation arrest and patients lacking the CREM expression were also found to be arrested at this level (Blendy *et al*. 1996; Weinbauer *et al*. 1998). CREM proteins are expressed in both germ cells and Sertoli cells. Therefore it was not clear whether the CREM expression in the germ line or in the somatic Sertoli cells or in both is responsible for correct spermiogenic development. To answer this question a cross-over germ cell transplantation experiment was conducted in CREM-deficient mice. Transplanted wild-type spermatogonia colonized the testes of the CREM-deficient recipients successfully and produced mature sperm, indicating that the germ cell but not the somatic testicular environment is the trigger for spermatogenic arrest under the CREM-deficient condition. Obviously, CREM deficiency does not disturb Sertoli cell function because the nourishing support of wild-type germ cells during maturation is qualitatively maintained in the absence of CREM and donor derived gametes were obtained from transplanted CREM-deficient hosts (Wistuba *et al*. 2002).

During spermiogenic maturation, histones of the DNA are replaced and protamines assemble in a morphologically newly formed male germ cell nucleus. This process requires a well orchestrated setting of transitionally expressed proteins, transition proteins (TP), which help disintegrate the histones and integrate protamines. In knockout mice lacking TP, DNA transcription was repressed; nuclear formation, histone displacement, and protamine deposition advanced normally, but chromatin condensation was irregular and many late spermatids showed DNA breaks. However, many mature spermatids remained in the testis, the number of epididymal spermatozoa was drastically reduced and the cells were abnormal. All male mice were sterile. Thus, in male animals TPs are required for normal sperm chromatin condensation, to reduce the number of DNA breaks, and to prevent formation of secondary defect spermatozoa, eventual loss of genomic integrity, and sterility (Zhao *et al*. 2004).

Even if spermatogenesis has been completed once, it may stop and fail later. This phenomenon was observed in a mutated mouse line developing an interesting testicular phenotype. In the strain of ''*juvenile spermatogonial depletion* (*jsd*)'' – mice, male animals homozygous for the mutation have only a single wave of spermatogenesis occurring at puberty, followed by its complete failure and ending in a spermatogonial arrest at the level of type A spermatogonia (Beamer *et al*. 1988). The mutant males are obviously endocrinologically normal, with the exception that FSH serum levels are increased between week 4 and week 20, but become normal once again at one year of age. Germ cell transplantation experiments showed that the mutant testicular environment is able to support the differentiation of transplanted normal spermatogonia and that an intrinsic defect of the germ cells is the cause of male infertility in the *jsd/jsd* mice (for review see Wistuba and Schlatt 2002).

A recent study (Rohozinski and Bishop 2004) reported the loss of an X-linked gene, mUtp14b, to be responsible for the spermatogenic failure. In the testis, this gene is mainly expressed from the zygotene stage up to round sper-

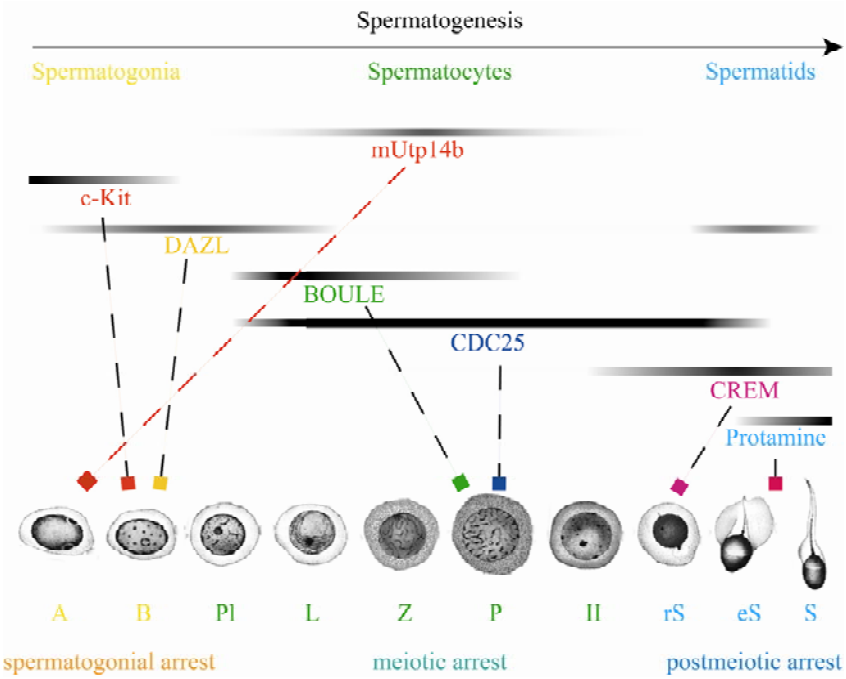

Fig. 3 Expression pattern of seven genes related to spermatogenesis. Disruption of the gene function results in spermatogenic arrest at different levels of spermatogenesis. This model depicts the 10 different steps of spermatogenesis and associates them with the protein expression of jsd, c-kit, Dazl, boule, cdc25, CREM and protamine. Lack of jsd, c-kit and Dazl result in a spermatogonial arrest, Boule and cdc25-deficiency leads to a meiotic halt while missing CREM and protamine expression arrests spermatogenesis postmeiotically. Abbreviations: Spermatogonia A (A), spermatogonia B (B), preleptotene (Pl), leptotene (L), zygotene (Z), pachytene (P), secondary spermatocyte (II), round spermatids (rS), elongating spermatids (eS), elongated spermatids (S).

matids. In spermatogonia there is very likely already weak expression. Failure of this expression is obviously sufficient to destroy spermatogenesis by loss of mitotic germ cell accumulation albeit in the presence of normal apoptosis. As a consequence, cell death results in spermatogonial arrest of spermatogenesis, once the first spermatogonial wave has passed and gone. Why this first wave can be produced is still unclear, but there is evidence from other experiments (Yoshida *et al*. 2006) that the first spermatogonial wave in mice starts directly from the gonocytes before the spermatogonial cell population is established. The findings in the *jsd/jsd* model would support this concept of a differently regulated first spermatogenic wave.

Considering these few selected examples of genetic disturbances, it would seem that even minor influences on the genomic information needed to maintain spermatogenesis correctly are sufficient to interrupt the complete processes (**Fig. 3**). These observations indicate that the genomic background of spermatogenesis is extremely complex and is to date only poorly analysed and understood.

TESTICULAR PRINCIPLES: TOPOGRAPHY OF THE SEMINIFEROUS EPITHELIUM AND SPERMATOGENIC EFFICIENCY

Testicular topography

In general, the mammalian testis consists of two compartments, the seminiferous tubules and the interstitium. The latter is responsible for blood supply, immunological responses and contains Leydig cells mediating endocrine signals of the pituitary to the testis and back to other body functions.

The tubules contain androgen-sensitive Sertoli cells and the entire germ line. They are shaped by a closed basal lamina produced by covering peritubular epithelial cells on the outside dividing the testes into two intratesticular compartments of which the inner tubular compartment becomes immunologically privileged. The peritubular cells are myoid and drive the peristalsis necessary to move the non-motile elongated testicular spermatozoa released from the nourishing Sertoli cells in the direction of the efferent ducts. From there they are forced into the epididymis where they mature until capable of fertilizing an oocyte. The polarized Sertoli cells attach to this inner extracellular matrix of the basal lamina, forming the blood-testis barrier. In addition to the structural and supportive function for the germ line, the Sertoli cells´ main importance lies in transducing androgenic signals from the outside into the propagating germ line (Sharpe *et al*. 2003). The key hormone players for the Sertoli cells are testosterone, FSH, anti-muellerian hormone (AMH), and inhibin.

In neonatal mammals, FSH induces Sertoli cell proliferation, producing a final number of cells that differentiate terminally during puberty; afterwards the Sertoli cell population loses its proliferation ability and, apart from one known exception (see below), remains stable lifelong. Typically, the terminal differentiation is marked by down-regulated AMH expression and by up-regulated androgen receptor (AR) expression (Tan *et al*. 2005).

The exception to this principle is the Djungarian hamster, a highly seasonal rodent model (Meachem *et al*. 2005; Tarulli *et al*. 2006). This hamster species is adapted in Siberia to an environment that imposes an extreme breeding seasonality during which testicular regression effectively stops spermatogenic activity completely during hibernation. This process is dependent on total light exposure i.e. day length (Schlatt *et al*. 1995). Before reinitiation of the breeding season, the testis is functionally rebuilt. Interestingly, this plasticity involves repeated formation of junction proteins a feature of terminal Sertoli cell differentiation. The expression of junction proteins indicates that the Sertoli cells are not terminally differentiated in these seasonally extreme animals (Tarulli *et al*. 2006).

Spermatogenic stages, spermatogonia and clonal size

A single terminally differentiated Sertoli cell supports only a limited number of germ cells; the number of Sertoli cells determines the final testis size in mammals. This ratio between Sertoli cell and germ cells defines the Sertoli cell workload which obviously does not influence the spermatogenic efficiency, but is very likely to be a species-specific feature of the seminiferous epithelium (Luetjens *et al*. 2005). However, this has no impact on the number of the gametes finally produced. The total number of spermatozoa produced is positively correlated only with the testis size, which is determined by the Sertoli cell number (Luetjens *et al*. 2005).

Recent findings in primates provide evidence that the Sertoli cell workload is linked to the size of germ cell clones. This parameter is the most important characteristic of a Sertoli cell for the organization of the intratubular architecture of the germinal epithelium (Ehmcke *et al*. 2005; Luetjens *et al*. 2005; Wistuba *et al*. 2005; Ehmcke *et al*.

2006).

The spermatogenic process leading to the production of fertile male gametes comprises the proliferation of spermatogonial stem cells (SSCs), the meiotic division, the differentiation and maturation of spermatids (for review see Sharpe 1994). All these tasks require a germinal epithelium to be highly organized. The complex pattern of germ cell differentiation results in specific arrangements of associated cell types designated as stages of spermatogenesis (Wistuba *et al*. 2003; Luetjens *et al*. 2005). The sequence and number of these stages have been (often arbitrarily) described in various mammals and have been assumed to be species-specific.

Many publications demonstrate distinct organizational differences of the germinal epithelium between primates and non-primates such as rodents (Clermont 1963; Alastalo *et al*. 1998) but also among the primate order itself (Weinbauer *et al*. 2001; Wistuba *et al*. 2003; Luetjens *et al*. 2005). Rodents reveal several generations of A-type spermatogonia and also several generations of proliferating A-type spermatogonia leading to large clonal cell sizes (de Rooij and Grootegoed 1998). In the murine testis, the population of A spermatogonia can be divided into A_{single}, A_{pair}, $A_{aligned}$, A1, A2, A3 and A4 (de Rooij and Grootegoed 1998; de Rooij and Russell 2000; Dettin *et al*. 2003). Of those, only the A_{single} spermatogonia are considered to be the SSCs. All other spermatogonial cell types are already in the process of early differentiation and have lost the potential to self-renew. The first spermatogonial cell types, A_{pair} and $A_{aligned}$, form unsynchronized clones with the rest of the seminiferous epithelium. The stages derived from these later, A1–A4 spermatogonia, synchronize their expansion with the seminiferous epithelial cycle (Ehmcke *et al*. 2006). However, although the mitotic turnover rate in the rodent testis appears to be low, a large number of sperm is produced from the SSCs through many mitotic divisions.

In contrast, in the order of primates only four different types of spermatogonia have been identified: the A_{dark}, the A_{pale}, the $A_{transition}$, and several B-spermatogonia types (Clermont 1969; Meistrich and van Beek 1993; Zhengwei *et al*. 1997; Ehmke *et al*. 2005). The A_{dark} spermatogonia are considered as reserve stem cells that do not divide when spermatogenesis is intact, but start proliferating upon severe testicular damage (van Alphen *et al*. 1988; Ehmke *et al*. 2005). The A_{pale} spermatogonia divide during every spermatogenic cycle and provide two types of daughter cells. These cells are either again self-renewal A_{pale} spermatogonia or $A_{transition}$, the last cell stage prior to B-spermatogonia, which means that to some extent A_{pale} spermatogonia have stem cell properties. The two different modes of germ line development have to be kept in mind to understand the organizational consequences in the seminiferous epithelium.

The seminiferous epithelium is characterized by specific germ cell associations derived from topographic relationships of the developing and proliferating germ cells. These associations lead to different and species-specific stages of spermatogenesis which can be observed histologically in tubule cross sections (**Fig. 4**).

A single, specific germ cell association can occur in a tubular cross section, filling the complete circular epithelial space. This is designated as a single-stage organization and is observed in most mammals analyzed so far (always one spermatogenic stage per tubular cross-section: shrew

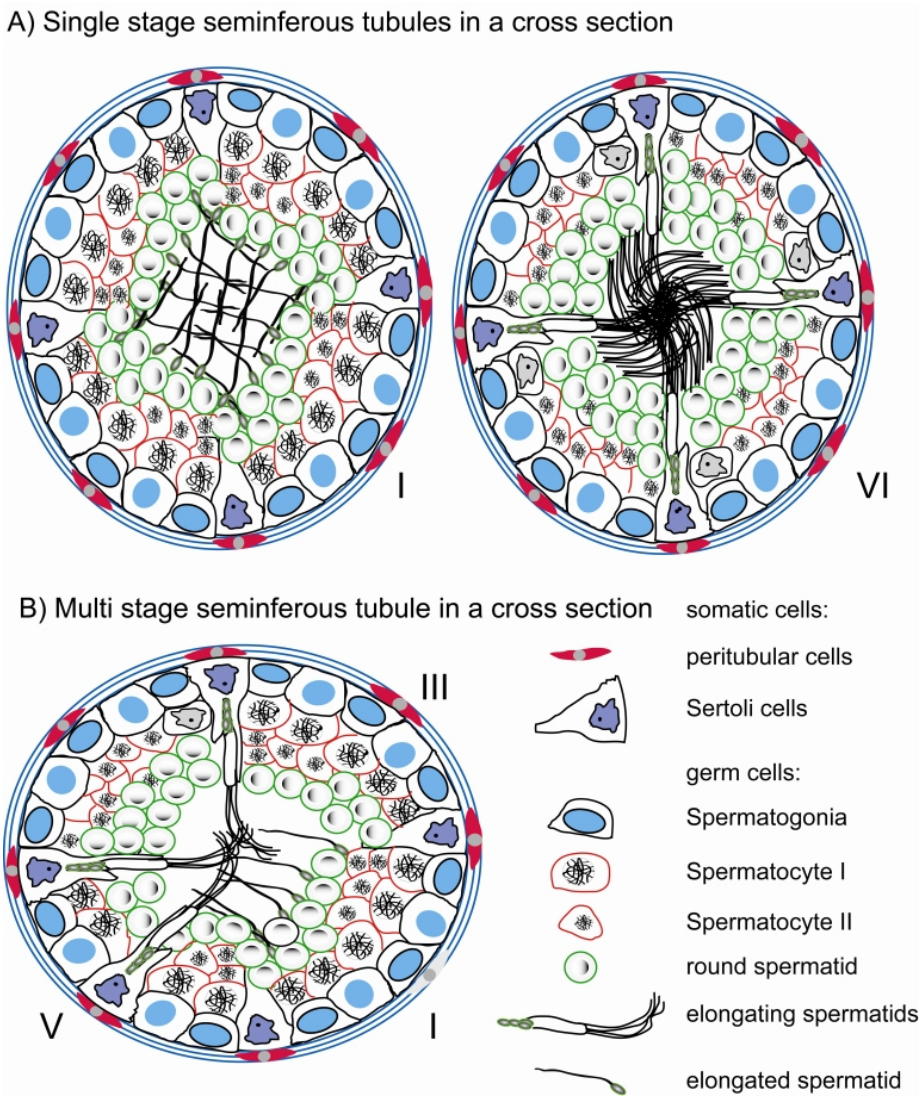

Fig. 4 Model of the possible cross-sectional arrangements of spermatogenic stages (according to the simplified six-stage-scheme developed for the assessment of human spermatogenesis, Clermont 1963; McLachlan *et al*. 2002) in mammalian seminiferous tubules. (**A**) Two single-stage tubules based upon segmental development (stage: I and VI). (**B**) Multi-stage tubules comprise between one and four different stages per cross section (here: stages I, III and V).

moles: Mizukam *et al*. 2001; mice: Oakberg 1956; rats: Leblond and Clermont 1952; gerbils: Segatelli *et al*. 2004; dogs: Ibach *et al*. 1976; wolves: Bitencourt *et al*. 2007; bats: Morigaki *et al*. 2001; cats: Franca *et al*. 2003; pumas: Leite *et al*. 2006; goats: Franca *et al*. 1999; marsupials: possum: Lin *et al*. 2004; plain rats: Peirce and Breed 1987, 2001; donkeys: Neves *et al*. 2002; primates: strepsirhini and some catarrhini: Wistuba *et al*. 2003; Luetjens *et al*. 2005). In contrast, when different germ cell associations are present simultaneously in a tubular cross section, this arrangement is characterized as multi-stage organization: more than one spermatogenic stage per tubular cross-section (marsupials: hopping mice: Peirce and Breed 1987, 2001; bears: Komatsu *et al*. 1996, primates: some catarrhini, platyrhini, great apes and men: Wistuba *et al*. 2003; Enomoto *et al*. 2004; Luetjens *et al*. 2005; Ehmke *et al*. 2005, 2006). While in non-primates this multi-stage pattern is an exception, it appears to occur more regularly in primate testes.

After the staged morphological organization of the seminiferous epithelium was analyzed in a more simplified way using a six-stage scheme (Clermont 1963; McLachlan *et al*. 2002), comparative analysis of spermatogenic organization became possible. As a major achievement, this simplification showed new phylogenetic aspects of testis development (Wistuba *et al*. 2003; Luetjens *et al*. 2005). In the primates, one recently evolved feature of testicular organization is the alteration of the single-stage morphology in the testis. During the evolution of primates the development of a multi-stage organization occurred convergently and independently in both taxonomic groups, the New World monkey and in the Great Apes.

The single-stage or multi-stage organization is very likely to be related to the clonal size of the spermatogonia. In a system with many divisions before the onset of meiosis, the entire circumference of the tubule becomes filled with one cell type at a time which develops in a longitudinal fashion. If many different cell types have to be generated, as in higher primates, the clonal sizes are small and cannot fill up the entire tubule circumference leaving space for other spermatogonial clones at different developmental stages (Ehmke *et al*. 2005, 2006). Although it was originally thought that a multi-stage organization might influence the spermatogenic efficiency of the testis negatively, comparative analysis showed no differences among the primate order nor between primates and rodents which were assumed to have the most efficient testes of all mammals analyzed (Wistuba *et al*. 2003; Luetjens *et al*. 2005).

ENDOCRINE REGULATION -A COMPLEX CONCERT OF HORMONE ACTION

After the onset of puberty, male mammalians depend strongly on the production of testosterone. Although testosterone is produced upon LH stimulation in Leydig cells, many male functions are targeted by the androgen or its metabolites. Target organs are skin and hair follicles, muscles, bones, brain, voice, blood, penis, epididymis, prostate and the testes (Nieschlag and Behre 2004). Moreover, building up and maintaining a normally working testis that produces mature spermatozoa requires a highly complex endocrine regulation. Reproductive function is controlled along an endocrine axis linking the brain, the hypothalamus, the pituitary and the gonads. Each of these organs contributes and reacts to hormones, being both target and source of signals (**Fig. 5**; for review see Nieschlag and Behre 2004).

Kisspeptin, GnRH, Inhibin, FSH and LH

Prior to the onset of puberty the hypothalamus pituitary gonadal axis has to be stimulated to initiate the sexual life of a mammal. In the arcuate nucleus of the brain specialized neurons (Kiss-1 neurons) release kisspeptin to induce gonadotropin-releasing-hormone (GnRH) secretion. After being contacted by the Kiss-1 neurons, GnRH neurons in the hypothalamus express a GPR54 receptor which binds kisspeptin, via a G protein cascade, and leads to the release of GnRH (for review see Smith *et al*. 2006). The initial release of kisspeptin, starting pubertal events in both sexes, seems to occur in all mammals analyzed so far. This mechanism has been shown for mice (Gottsch *et al*. 2004), rats (Matsui *et al*. 2004; Navarro *et al*. 2004), sheep (Messager *et al*. 2005), monkeys (Shahab *et al*. 2005; Plant *et al*. 2006) and humans (Dhillo *et al*. 2005).

Pulsatile GnRH secretion from the hypothalamus drives the pituitary to release both follicle-stimulating hormone (FSH) as well as luteinizing hormone (LH). Although both hormones are essential in males and females their appellation refers only to the female situation. These gonadotropins are also essential for normal spermatogenesis. FSH binds to its receptors expressed by the Sertoli cells, acting to stimulate spermatogenesis, whilst LH induces the Leydig cells to produce testosterone (e.g. Nieschlag *et al*. 1999; Luetjens *et al*. 2007).

In adult primates as well as in adult rodents, gonadotropin secretion was reported to be regulated by hypothalamic GnRH secretion, the intratesticular hormone inhibin and testosterone in a feedback loop (Winters *et al*. 1996; Fingscheidt *et al*. 1998; Winters and Moore 2004). Inhibin, a testicular glycoprotein hormone negatively affecting FSH levels and consisting of two covalently linked subunits, is biologically active only in two dimeric forms inhibin A and B. Sources of the relevant isoform, inhibin B, are mainly the Sertoli cells, but also the germ cells and even the Leydig cells may contribute to the production of inhibin (for review see Meachem *et al*. 2001). *In vitro*, LH and FSH stimulate inhibin B secretion of isolated and cultured testicular cell suspensions (Berensztein *et al*. 2000). Whilst it remains unresolved whether Leydig cells are really capable of inhibin production, the role of germ cells in the regulation of inhibin secretion has been demonstrated. Changes of inhibin B levels are thought to serve as an early signal for testicular damage.

In terms of the regulative processes, inhibin B is strongly interlinked to the gonadotropin FSH and in the male sex this endocrine interplay naturally undergoes changes in the course of life (Meachem *et al*. 2001).

Production of inhibin B switches around puberty onset. An early postnatal increase of inhibin B levels is observed that correlates with activation of the hypothalamic-pituitary-gonadal axis and as such seems to parallel the changes in testosterone and LH levels more than those of FSH at this early timepoint of testis development. During prepubertal testis formation, inhibin B activity strongly reflects Sertoli cell proliferation and FSH action. Afterwards inhibin B remains on a low basal level, reflecting Sertoli cell density. After puberty when spermatogenesis is completed, the germ cells become the major determinant of inhibin B production in adulthood. Changes in inhibin B levels are then correlated with the status of germ cell proliferation and depend only secondarily on FSH. Probably rising androgen levels are the driving force of this switch (Gromoll *et al*. 2000; Meachem *et al*. 2001). Interestingly inhibin B levels directly correlate to sperm counts, giving an indication of spermatogenic efficiency.

In summary, during puberty the control of inhibin B production switches from FSH action to spermatogenesis and the correlation with FSH flips from a positive to a negative regulation feedback loop (Andersson *et al*. 1997; Kumanov *et al*. 2006).

Regulation of inhibin B and determination of Sertoli cell numbers in the immature testis are only two of the numerous functions of the heterodimeric gonadotropin FSH. In adulthood, FSH, a member of the glycoprotein hormone family, plays a major role in the regulation of spermatogenesis (Simoni *et al*. 1997). Leydig cell production and maturation is also influenced, as was demonstrated by withdrawing the endogenous LH effects (Haywood *et al*. 2003). All these effects are mediated via G protein-coupled transmembrane FSH receptor (for review see Simoni *et al*.

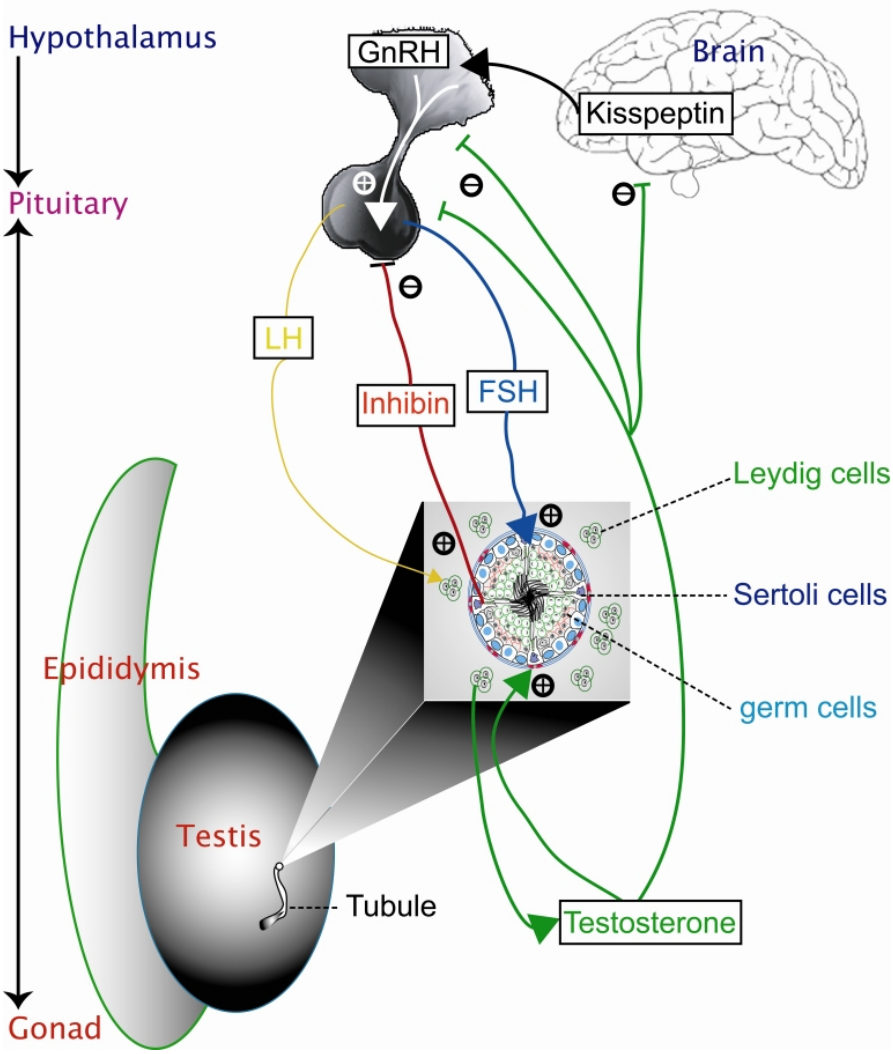

Fig. 5 Endocrine regulation of male functions along the hypothalamic-pituitary-gonadal axis: kisspeptin is secreted from the brain and stimulates the release of gonadotropin-releasing hormone (GnRH), secreted from the hypothalamus, which induces the release of both gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) from the pituitary. LH stimulates the Leydig cells of the testes to produce and release testosterone, whereas FSH stimulates Sertoli cells of the testes to support the germ cells to undergo spermatogenesis. Serum testosterone and inhibin down regulate the production of kisspeptin, GnRH and both gonadotropins in a negative feedback loop.

1997). In a transgenic mouse model lacking a functional FSH receptor (FSHRKO mice; Krishnamurthy *et al*. 2001), the lack of FSH stimulation in the target tissues resulted in decreasing serum testosterone levels. In primates, evidence has emerged that FSH is the most important driving force for the renewal of type A spermatogonia, for the multiplication of the spermatogonia and is supported by testosterone action for the proliferative activity in the germ line as such (Luetjens *et al*. 2005; Wistuba *et al*. 2005). Furthermore, the quality of spermatogenesis is remarkably dependent on FSH (Krishnamurthy *et al*. 2000). However, data has been reported that support a major role of testosterone in this process (McLachlan *et al*. 2002). The final testicular maturation of spermatozoa, the spermiation, clearly depends on the interplay of both hormones FSH and testosterone, respectively. The progression of germ cells through meiosis, i.e. from the spermatocyte stage to the spermatid stage is not regulated by gonadotropin but depends on androgen function alone, mediated by the Sertoli cells (Tsai *et al*. 2006). Additionally, similar to testosterone, FSH protects the germ line cells against apoptosis (Krishnamurthy *et al*. 2000; Wistuba *et al*. 2005; Luetjens *et al*. 2005). The expression of several genes is also regulated by FSH; e.g. the transcription factors glia cell line-derived neurotrophic factor (GDNF) or cAMP response element modulator (CREM), essential for spermatogenesis, are under control of its action (de Cesare *et al*. 2000).

The numerous functions of FSH have to fulfil highly regulated routes of signal transduction in the Sertoli cells. Therefore, it is of no surprise that many pathways are involved in the intracellular transduction of the FSH signal, as the cAMP-PKA pathway, the MAP kinase pathway, the phosphatdylinositol 3 kinase pathway, the phospholipase A2 pathway and the Calcium pathway (reviewed by Walker and Cheng 2005). It is also very likely that there is major crosstalk between all these transduction pathways.

In the male sex, the action of another gonadotrope glycoprotein hormone, LH is also essential to successfully produce mature gametes. LH is the key signal for the Leydig cells to produce testosterone. The regulation of androgen levels through the release of pituitary LH is responsible for the constitution and maintenance of the male phenotype, as mentioned before. During perinatal development, LH levels are elevated for a short period before they decrease and remain low until puberty. During puberty LH levels again rise and stimulate matured Leydig cells to produce androgens. After initialization this endocrine relationship is maintained during male adulthood in a feedback loop. The endocrine axis is regulated by testosterone on several levels, starting with the periventricular regions of the brain, the GnRH releasing neurons in the hypothalamus and the LH and FSH releasing cells in the pituitary.

Spermatogenesis is stimulated indirectly by LH-driven testosterone secretion of the Leydig cells (Weinbauer *et al*. 2001). The importance of normal testosterone levels for spermatogenesis can be drawn from infertility patients with low serum testosterone levels accompanied by high LH levels. The pituitary increases LH release to stimulate testosterone production and thereby increase sperm production. So far it is still not possible to disrupt spermatogenesis effectively without simultaneous inhibition of androgen production (Kamischke and Nieschlag 2004). Unfortunately this inhibition leads to a deficiency of extratesticular androgens and has to be compensated by administering testosterone to maintain the male phenotype (Kamischke and Nieschlag 2004; Luetjens *et al*. 2007).

The testicular effects of LH are mediated by the Leydig cells via the LH-receptor (LHR). The action of the LHR and LH on male reproduction was impressively demonstrated using a knockout (KO) mouse model. If the receptor was disrupted in the region of the first exon, infertility in both sexes was the main effect. In male mice the endocrine profile was altered: LH serum levels were increased, testosterone levels were remarkably decreased and estradiol levels were slightly elevated. In addition, malformations of the reproductive tract as abdominal testes, micropenis and decreased weight of the organs of the reproductive tract were observed in these mice (LH/CG receptor KO mouse, Lei *et al*. 2001).

In another LH KO mouse model, in which exon 11 was disrupted (LuRKO mouse, Zhang *et al*. 2001; Huhtaniemi *et al*. 2002), male and female mice were born with a normal phenotype of the reproductive tract and morphological abnormalities developed only postnatally. In the male sex, Leydig cell numbers and volume were reduced and gonadotropin levels (LH and FSH) were elevated, whilst androgens were decreased. Interestingly, it was found that spermatogenesis could be initiated and meiosis completed in the male germ line. It was concluded that these processes are driven, at least in part, by FSH. The differences between these two models might be due to the different strategies of gene disruption. In the first model, LHR translation is not possible because of the disruption site, situated in the first exon, whilst in the LuRKO mouse, exon 1 to 10 of the LHR gene are translated.

In primates, LHR function is more complex. The receptor in Old World monkeys and Great Apes interacts with two hormones, the chorionic gonadotropin (CG) and LH, respectively. CG maintains pregnancy and is involved in sexual differentiation only during intrauterine life in males; the main expression of the receptor is situated in the Leydig cells. LH is crucial for androgenization and complete gametogenesis. In the female primate, the receptor is mainly expressed in the ovarian granulosa cells. The LHR is part of the G protein-coupled glycoprotein hormone receptor family, comprising the closely related FSH receptor (FSHR) and thyroidea-stimulating-hormone receptor (TSHR). All these receptors present a large extracellular binding domain and a seven transmembrane domain. Only one exon encodes the transmembrane domain in all members of the family but the hinge region of the LHR, which is highly conserved among all mammals analyzed so far, differs genomically from the FSHR and the TSHR by an additional exon, namely exon 10 (Gromoll *et al*. 1996; Ascoli *et al*. 2002). Whilst in the LuRKO mouse model prenatal sex differentiation is normal and only postnatal development is disturbed (Lei *et al*. 2001; Zhang *et al*. 2001), sex differentiation of primates requires regular interaction between this gonadotropin and its receptor (Gromoll *et al*. 2003). This is supported by the analysis of some rare receptor mutations found in the human that result in disturbed hormone-receptor interaction and in a phenotype of sex reversal (Huhtaniemi *et al*. 2002).

LH is functionally comprised of two proteins, the LHα and LHβ subunits. About 40 million years ago, a LHβ gene duplication accompanied with a reading frame shift occurred in an ancestral primate, giving rise to the new subunit CGβ (Talmadge *et al*. 1984; for review of different species see Ben-Menahem and Grotjan 2007). This new hormone was integrated into the endocrine regulation processes and assumed its above mentioned functions (Maston and Ruvolo 2002; Wistuba *et al*. 2005; Luetjens *et al*. 2005). The previous assumption of a clear distinction between the function of both hormones – CG establishes and maintains pregnancy whilst LH is necessary for male sexual differentiation and regulation of androgen production – was recently disproved. In the neotropical common marmoset (*C. jacchus*, platyrrhini), an alternative role for CG in the male sex was shown. In all New World monkeys studied, exon 10 of the LH receptor is not expressed and has become a part of the introns (Zhang *et al*. 1997; Gromoll *et al*. 2003). The New World monkey receptor isoform was described as LHR type II (Gromoll *et al*. 2003). Hence, while the expression of this exon is essential for LH binding in the human (Gromoll *et al*. 2002), it appears to be unnecessary in New World monkeys. This became possible because of an evolutionary phenomenon that had occurred in all analyzed platyrrhine primates (Gromoll *et al*. 2003). There is evidence that the pituitary of New World monkeys does not produce LH but CG (Müller *et al*. 2004). Therefore, the Leydig cells of these species are driven by CG rather than by LH. Moreover, marmoset puberty is marked by a rise in serum CG levels (Chandolia *et al*. 2006; Wistuba *et al*. 2006) that remarkably parallels the pubertal LH secretion reactivation at puberty onset present in Old World monkeys (catarrhini; e.g. Plant *et al*. 2005). To date it remains unclear which mechanism on the female side distinguishes between the CG function for pregnancy and for the reproductive processes. First clues show that the promotor region of CGβ has changed and, depending on the expressing organ (pituitary or placenta), different amounts of CGβ are produced. So far, the newly evolutionized CG is explained as a consequence of geographic isolation that preserves a state of the LH/CG endocrine system reflecting an ancestral situation existing when the neotropical primate taxa split off from the Old World monkey line. There must be a strong evolutionary drift in the LH-CG system because other mammals have generated alternate LH-CG types (Sherman *et al*. 2001). Therefore in terms of animal experimental design, the Old World monkey species resemble the endocrine physiology of the human much better than do the New World species.

Androgens and gestagens

After the early androgen actions such as the production of testosterone by the neonatal testis, and its stimulating effect on Wolffian duct development to form the vas deferens and epididymis and the virilisation of the urogenital sinus, testosterone becomes a major player in spermatogenesis.

The androgens, such as testosterone and DHT, can initiate complete spermatogenesis without the help of gonadotropins (Singh *et al*. 1995). FSH alone leads only to meiotic stages of spermatogenesis (Singh and Handelsman 1996). Classic hormone-withdrawal experiments in rats provide evidence that an androgen is necessary for the completion of meiosis and the differentiation of round spermatids into spermatozoa (Ghosh *et al*. 1991). Recent publications clearly demonstrated that the androgen receptor is essential for the completion of meiosis and the development of spermatozoa in knockout mice (Yeh *et al*. 2002; Chang *et al*. 2004).

In an attempt to distinguish the differential functions of testosterone and its metabolites in the testis, Tsai *et al*. (2006) generated mice in which the androgen receptor was differentially knocked out in the Leydig, Sertoli or in the germ cells. Mice lacking the AR in their germ cells have quantitatively full spermatogenesis, whereby Sertoli androgen receptor knock-out mice show spermatogenesis only up to meiosis I. The lack of a functional AR in Leydig cells has a major influence on Leydig cell steroidogenic function and leads to spermatogenic arrest, predominately at the round spermatid stage. This demonstrates that Sertoli cells, as the major target of testosterone, transmit the signal and initiate and maintain spermatogenesis.

Testosterone is also the regulator for the Sertoli cell function to provide cell structure support and maintain the seminiferous tubular fluid. After pubertal maturation, the expression of the Sertoli cell AR follows in a seminiferous-tubule-stage-specific manner (Al-Attar *et al*. 1997). The androgen signals in Sertoli cells help maintain cell morphology, basement membrane development, and seminiferous epithelial integrity (Wang *et al*. 2006). Sertoli cells are highly specialized with well-elaborated cytoskeletons maintaining cell shape, position, and transport of organelles within the cell. The cytoskeleton also stabilizes the cell

Table 1 Short compilation of the main hormones and their receptors in the male.

Hormones	Abbr.	Structure	Tissue	Cell types
Kisspeptin	**Kiss-1**	Kiss1 family (54 amino acids)	Forebrain, hypothalamus	Kisspeptin-secreting neurons
Gonadotropin-releasing hormone	**GnRH**	Decapeptide	Hypothalamus, midbrain, testis	Terminal nerve (TN) cells
Follicle-stimulating hormone	**FSH**	Glycoprotein hormone	Pituitary	Folliculo-stellate cells
Luteinsing/Chorionic gonadotropes/ hormone	**LH/CG**	Glycoprotein hormone	Pituitary	Anterior pituitary gonadotrope secretory cells
Testosterone	**T**	Steroid	Testis	Leydig cells
Anti-mullerian hormone	**AMH**	Transforming growth factor B, GDNF subfamily	Fetal testis, neonatal testis	Sertoli cells
Progesterone	**P**	Steroid	Pituitary	Gonadotrope secretory cells
Inhibin/Activin		Transforming growth factor B, GDNF subfamily	Testis	Sertoli cells, Germ cells
Insulin-like factor 3	**INSL3**	Insulin family	Testis	Fetal Leydig cells
Estrogen	**E**	Steroid	Testis, epididymis	Immature Sertoli cells, Leydig cells, germ cells, epithelial cells
Receptors				
G protein-coupled receptor GPR54	**GPR54**	G-protein coupled receptor 1	Brain	GnRH neurons
GnRH Receptor	**GnRHR**	G-protein-coupled receptor (GPCR) superfamily	Pitiutary, testis, prostate	FSH and LH producing cells
FSH Receptor	**FSHR**	G-protein coupled receptor 1 FSH/LSH/TSH subfamily	Testis	Sertoli cells, germ cells
LH/CG Receptor	**LHR**	G-protein coupled receptor 1 FSH/LSH/TSH subfamily	Testis, epididymis	Leydig cells, epithelial cells
Androgen Receptor	**AR**	Nuclear hormone receptor NR3 subfamily	Testis, epididymis, Wolffian ducts, forebrain, larynx	Sertoli cells, Leydig cells, germ cells, neurons, epithelial cells, muscle cells
Progesterone Receptor	**PR**	Nuclear hormone receptor NR3 subfamily	Hypothalamus, pituitary, testis, prostata, epidydimis, spermatozoa	Peritubular cells, neurons
Anti-mullerian hormone receptor	**AMHR**	Transmembrane receptors	Fetal testis, Mullerian ducts	Leydig cells, epithelial cells
Estrogen receptor	**ER**	Nuclear hormone receptor NR3 subfamily	Hypothalamus, forebrain, testis, epididymis	ER neurons, epithelial cells, Sertoli cells, Leydig cells, germ cells
Inhibin/Activin Receptor			Pituitary	
Insulin-like factor 3 Receptor	**INSL3R**		Gubernaculum	

membrane at sites of cell-cell contact, adheres and aids in the movement of developing germ cells and in the release of mature spermatids during spermiation. In the testes, the morphological relationship between tight junctions and anchoring junctions is remarkably different from other epithelia. Tight junctions are located at the basolateral region of the Sertoli cells. The occluding inter-Sertoli cell tight junctions are the major elements of the blood-testis barrier at the basal compartment of the seminiferous epithelia. A loss of testosterone action in the Sertoli cells impairs functional tight junction formation (Wang *et al*. 2006). Testosterone also plays a role in Sertoli cell secretion of functional proteins and peptides to nourish germ cell development, and to cooperate with germ cells in germ cell movement and spermiation. Androgen binding to the AR in Sertoli cells activates a transcriptional reaction leading to changes in signaling transduction, but how testosterone supports germ cell differentiation is largely unknown.

Gestagens such as progesterone and the expression of its genomic receptor (PR) in males have come to attention in the course of attempts to inhibit spermatogenesis in male hormonal contraception with a gestagen (Kamischke and Nieschlag 2004; Wenk and Nieschlag 2006). In females, progesterone is known to be associated with reproductive functions in the ovary, uterus, mammary gland and brain but in males knowledge of the function and expression pattern is limited (Oettel and Mukhopadhyay 2004). PR is expressed in two major isoforms, called PR-A and PR-B, which are the product of two different transcriptional start sites and differ by an N-terminal extension (Conneely *et al*. 2001) and expression is stimulated by estrogens. PR expression in males was found in the pituitary, hypothalamus, in smooth muscle cells of the epididymis and prostate and scarcely in the testicular peritubular cells (Luetjens *et al*. 2006). Male progesterone receptor knockout mice (PRKO) are phenotypically normal and fertile (Lydon *et al*. 1995). Such male mice have elevated LH concentrations, suggesting a function of progesterone in the control of LH secretion (Schneider *et al*. 2005). Recent experiments with a human neuronal medulloblastoma cell line suggested that PR-A and PR-B have different functions in the progesterone-mediated regulation of GnRH receptor promoter activity (An *et al*. 2005). Intriguingly, in these cells progesterone stimulated GnRH mRNA transcription, showing the complexity of PR involvement in the regulation of the human GnRH system (An *et al*. 2005). Tissue culture experiments demonstrated that smooth muscle cells are a major source of PR mRNA, independent of gender (Hodges *et al*. 1999). Here, the PR could exert a function similar to its role in other tissue smooth muscle cells, i.e. inhibition of calcium-ATPase and regulation of calcium influx, thereby hindering cell contraction and fluid transport (Crews and Khalil 1999; Fomin *et al*. 1999; Toshima *et al*. 2000; Saner *et al*. 2003). The peritubular cells of the testis and the epididymal smooth muscle cells are needed to maintain a constant flow of tubular and epididymal fluid with maturing spermatids and sperm. PR might be involved in retarding this movement. However, in the testis, the number of PR positive cells is very low so that such effect may be minor and in a contraceptive trial in the cynomolgus monkey with norethisterone enanthate, another gestagen, a direct testicular effect was not found (Junaidi *et al*. 2005).

The hormones involved in male reproduction and their related receptors are summarized in **Table 1**.

TRANSPLANTATION OF THE GERM LINE: MATURATION OF MALE GAMETES AWAY FROM HOME

Recent experiments transplanting testicular cells or tissues resulted in novel insights into testis biology, spermatogonial stem cell fate and regulative mechanisms of spermatogenesis. Although, originally intended as a new technique of germ line preservation for experimental and therapeutic purposes (for review see Brinster 2002; Wistuba and Schlatt 2002; Orwig and Schlatt 2005; Dobrinski 2006), testicular germ cell transplantation and testicular grafting also led to much information on testicular development and plasticity.

In general, there are two possibilities to transplant the male germ line: (i) intratubular transplantation of isolated spermatogonial cells into a recipient's testis depleted of endogenous spermatogenesis. (ii) ectopic or orthotopic grafting of immature testicular tissue pieces or cells in a host animal.

These two routes of germ line transplantation provide different conditions. Testicular germ cell transplantation, first performed by Brinster and Zimmermann (1994) is conducted by enzymatic digestion of testicular tissues to obtain isolated spermatogonial cells. These are microinjected into the seminiferous tubules of an aspermatogenic host via the rete testis or the efferent ducts. In the environment of the recipient the donor cells colonize the stem cell niches offered by the recipients' seminiferous epithelium. When colonization is successful, the spermatogonial cells start to establish donor-derived spermatogenesis. Apart from phylogenetic distance, the age of donor and/or recipient seems to play an important role for the success of germ cell transplantation. The immature mouse testis provides an excellent environment for donor cell colonization compared to the adult one. This methodology was effectively utilized in germ cell transplantation experiments in non-rodent species, e.g. rabbits, dogs, and pigs (for review see Dobrinski 2005).

Grafting of testicular tissue fragments transplants the entire germ line of the donor into another organism (Honaramooz *et al*. 2002) but endocrine control of maintaining local cellular and hormone environment is altered. Both techniques have been shown to be suitable to achieve full spermatogenesis when intra-species transplantation was performed and in numerous experiments, successful germ cell transplantation was achieved even between species.

However, testicular germ cell transplantation fails when the phylogenetic distance between donor and host increases. So far, it was only possible to transplant spermatogonia from rodents into recipient mice, ending up with complete gamete maturation. Transplanting germ cells from large domestic animals or even primates and humans failed to overcome more than an early state of spermatogonial colonization. The spermatogonia fail to undergo differentiation of the germ line in the host (Dobrinski *et al*. 1999; Reis *et al*. 2000; Nagano *et al*. 2001a, 2002; Schlatt *et al*. 2006). Obviously the properties of spermatogonial stem cell niches, essential for spermatogonial settlement, differ among the mammalian phylogenetic groups. This may also be due to significant differences in testicular morphology (see above). Moreover, although the transplanted spermatogonia are "addressed" to their new niches, they cannot fit properly into the host niche provided by the Sertoli cells due to alterations of the cell-cell binding receptors.

Genetic modification of germ line stem cells was used for studies on physiological events in male reproduction. For the generation of transgenic animals it is necessary to become familiar with various factors influencing maturation processes. To generate these animals, retroviral vectors have been used to introduce genes into different cell types. *In vitro*, the delivery of retroviral-mediated genes into SSCs has been demonstrated (Nagano *et al*. 2001b). In contrast to other stem cells, such as haematopoietic stem cells or even embryonic stem cells the expression of retroviral vectors in SSCs is not silenced (Brinster 2002). Nagano *et al*. (2001b) observed stable integration and expression of a transgene in SSCs obtained between 2 and 20% of stem cells in adult and immature mice. After transplantation these SSCs resulted in approximately 4.5% transgenic progeny. The combination of retroviral vector transfection and transplantation provides a powerful approach to generate gain-of-function and/or loss-of-function transgenic animals for studies on stem cell biology and spermatogenic processes (Brinster 2002).

Unfortunately, further evaluation of the methodology revealed two main obstacles for the use of testicular germ cell transplantations in terms of germ cell preservation.

First, when the mouse model was used as a host for human cells (Reis *et al*. 2000; Nagano *et al*. 2002) it became apparent that the evolutionary distance between primates and rodents is too great to allow more than spermatogonial survival after cross-species transplantation. This problem might be solved by development of cryopreservation protocols that would make storage and later autologous re-transplantation possible. However, transplantation of tissue or cells from cancer patients always bears the risk of reintroducing cancer cells to the patient after successful treatment, although the tissue may exhibit no tumour *in situ*. A leukaemic rat model was utilized but re-transplantation of testicular cells after a successful treatment of the donors resulted in a complete malignant relapse in the experimental animals (Jahnukainen *et al*. 2001). Extremely efficient and proven separation techniques are needed to sort malignant cells from SSCs before re-transplantation after cancer treatment can become reality.

Against this background, two routes were suggested to improve the methodology for possible therapeutic use. Experiments were performed in rodents eliminating cancer cells by various cell sorting methods such as fluorescent activated cell sorting (FACS) or magnetic activated cell sorting (MACS) (Fujita *et al*. 2005; Geens *et al*. 2007). Both methods prove the principle but are still at an experimental stage.

Grafting of testicular tissue resulting in successful formation of mature gametes was first published in 2002 (Honaramooz *et al*. 2002). Apart from possibly maintaining the germ lines of endangered or transgenic animals, this method was motivated by the increasing need to offer possibilities for germ line preservation to prepubertal patients suffering from malignancies and facing the loss of fertility due to cytotoxic therapies. Because testicular germ cell transplantation of human cells into mouse host failed and cell sorting techniques remain experimental, a new concept was tested. Grafting testicular tissue into castrated immunodeficient mouse hosts might bypass the problem of disturbed interspecies stem cell – niche communication by transplanting the germ cells within their own somatic microenvironment. Spermatozoa matured in the host would then be available for assisted reproductive technology without the risk of re-transplanting malignancies to the cured patient.

Following the first successful proof of principle (Honaramooz *et al*. 2002), grafted testicular tissue was matured from various species as mouse, rat, pig, bovine, goat and even macaques (for review see Dobrinski 2005). In addition, experiments performed by Ohta and Wakayama (2004) in mice demonstrated that the age of the host seems to be of greater importance than the androgenisation, since it was even possible to mature spermatids in female animals transplanted with testicular fragments. Obviously the pituitary status and the gonadotropin levels are the endocrinological variables that control graft maturation. Apart from the recipients' age and state, the developmental age of the tissue grafted is decisive. So far, whenever adult tissue was grafted, successful completion of spermatogenesis in the transplants was not achieved (Schlatt *et al*. 2002).

In general, this transplantation technique can be performed (graft is localized ectopically at a different body position, e.g. under the back skin as well as orthotopically:

graft is localized in the scrotum) using various approaches: xenologously between individuals of different species, heterologously (between two different individuals of the same species) and autologously (within an individual functioning simultaneously as donor and host).

To date, grafting of testis fragments has failed with transplanted human and neotropical common marmoset (*Callithrix jacchus*) tissue. Interestingly, the latter exhibits a testicular organization very similar to the human (Luetjens *et al*. 2005).

The xenotransplantation experiments in marmosets (Wistuba *et al*. 2004) resulted in graft survival but spermatogonial arrested tubules, probably due to the different gonadotrope endocrinology in the host – mice produce LH and the neotropical monkeys need CG. This observation was supported by an autologous ectopic transplantation of testicular tissue in these primates achieving a graft development up to meiotic stage (Wistuba *et al*. 2006). Ongoing studies indicate that here also the site of transplantation might influence the success of transplantation.

In contrast, the developmental failure of human transplants to mature when grafted xenologously into immunodeficient mouse recipients is very likely caused by the use of material from adult men whose tissue is not able to restore spermatogenesis after transplantation (Schlatt *et al*. 2006; Geens *et al*. 2007). To our knowledge there is only one study published so far reporting the transplantation of fetal human testis tissue into mice. This report demonstrated spermatogenic maturation up to a pre-/peripubertal stage in the recipient (Yu *et al*. 2006). Human testicular grafting still suffers from very limited access to testicular material. Ethically it has to be carefully evaluated, in particular also because of the risk for zoonosis by host donor gene transfer, i.e. through retrovirus infections. In a very recent study in mice and rats, Hou *et al*. (2007) transplanted material contaminated with leukaemic cells and found a full transmission of the malignancy into the recipient animals, in mice as well as in rats, indicating that the transferred tissue also kills recipients of another species. So far xenografting for germ line preservation is still far from any therapeutic application. The most realistic use for this technique is cytotoxic drug testing or assessing the malignant potential of grafted material (Jahnukainen *et al*. 2006).

Summarizing the state of the art, the success of testicular grafting depends on

i) The age of donor material must be immature. Immature material obviously survives periods of ischemia better than material from mature donors.

ii) The age of the recipient should be before and around puberty because of the fully active pituitary in puberty. High gonadotropin levels, rather than a steady androgenic state seem to support graft development.

iii) The location of the transplants can be crucial. In marmosets transplanting testis tissue into the scrotum leads to spermatogenesis but not onto the back skin.

iv) Therapeutic options will become not available before problems of zoonosis and malignant relapse have been solved. The latter requires sophisticated methods to sort out malignant cells from the graft tissue.

Cell sorting represents a novel approach. Effective cell sorting requires isolation (and possibly the culture) of testicular cells, selective killing of cancer cells and subsequent rearrangement of testicular structures. Very recently the first breakthroughs on this route have been achieved. These studies describe the rearrangement of seminiferous tubules after grafting single cell pellets of testicular cells under the skin of immunodeficient mice (Gassei *et al*. 2006; Honaramooz *et al*. 2007). Honaramooz and colleagues observed *de novo* generated tubules with full spermatogenesis after implantation of isolated neonatal porcine testis cells. The rearranged tubules showed morphogenic and physiologic similarities to normal testis tissue and demonstrated the enormous plasticity of testicular somatic and germ cells.

While, to date, germ line transplantation is far from being a therapeutic option in humans, in terms of experimental access to germ line and testicular environment, testicular germ cell transplantation represents a technique to analyse testis development and spermatogenesis. In foreign language journals such as Russian and Chinese, clinicians claim to have succeeded in transplanting human testicular tissue. Although far from germ line transplantation experiments this scientific curiosity was reported to have resulted in at least one life birth of a child.

UNDERSTANDING SPERMATOGENESIS *IN VITRO*: LESSONS FROM GERM CELL CULTURE

Separated from the animal and brought into the culture dish, *in vitro* expansion, differentiation and manipulation of male germ cells have become increasingly important to understand the premeiotic, meiotic and postmeiotic physiology of the germ line (Hue *et al*. 1998; Shinohara *et al*. 2000; Hasthorpe 2003; Izadyar *et al*. 2003; Kanatsu-Shinohara *et al*. 2003; Nagano *et al*. 2003; Kubota *et al*. 2004). These experiments used a large variety of *in vitro* conditions and a broad range of different supporting factors but also demonstrated the limitation of approaches to complete spermatogenesis *in vitro* due to the lack of an appropriate optimal culture system (Sofikitis *et al*. 2005).

The current hypothesis is that successful approach must combine essential growth factors (e.g. SCF, GDNF, LIF and hormones) with a structural environment that mimics the stem cell niche of the testis (Spradling *et al*. 2001).

Experiments in the 1960s and 70s on testis tissue culture in the intact testicular microenvironment were pivotal for germ cell maintenance and development *in vitro*. In these experiments, the tubular structure formed by elements of the basal membrane and by the Sertoli cells was maintained for a culture period of up to eight months (Steinberger *et al*. 1970a, 1970b). In further studies, organ culture performed with rodent testicular tissue was addressed to explore regulation processes by varying *in vitro* conditions, adding hormones and/or growth factors controlling somatic and premeiotic germ cell proliferation and differentiation (Schlatt *et al*. 1999; Meehan *et al*. 2000). Exposing fragments of immature testicular tissue to activin and FSH maintained Sertoli cell proliferation and germ cell expansion was initiated during three days of organ culture. Erkillä *et al*. (1997) cultured seminiferous tubules to examine the influence of testosterone on apoptosis. Testosterone was found to suppress apoptosis, and seems to be crucial for germ cell survival.

The next step of germ line culture dealt with isolated cells separated from the immature testicular tissues, obtained by enzymatic digestion protocols similar to those applied in testicular germ cell transplantation. Suspensions of SSCs and more differentiated germ cells from mature and immature prepubertal tissue were investigated in many studies and revealed that juvenile germ cells survived much better than adult cells (Creemers *et al*. 2002; Nagano *et al*. 2003). Thus, to establish SSC lines and/or to study spermatogenic progress starting at the stage of SSC use of juvenile tissue is appropriate. To date, various methods for the isolation and enrichment of spermatogonia have been successfully established, such as fluorescence activated cell sorting (FACS) (Shinohara *et al*. 2000; Guan *et al*. 2006), gravity-sedimentation in percoll (Koh *et al*. 2004), the STAPUT technique (Dirami *et al*. 1999), magnetic activated cell sorting (MACS) (von Schönfeldt *et al*. 1999) and magnetic sorting with dynabeads (Hofmann *et al*. 2005). For most of these SSC enrichment methods, the availability of highly specific cell surface markers is crucial. In mice, some proven spermatogonial markers are GFRα-1 (glia cell line-derived neurotrophic factor (GDNF) family receptor-alpha;), Notch-1 (*Notch* superfamily; encodes cell-surface receptors involved in cell-fate decisions) c-kit (transmembrane tyrosine kinase, expressed on differentiating cells; von Schönfeldt *et al*. 2004), CD-9 (tetraspanin transmembrane protein; Kanatsu-Shinohara *et al*. 2004a) and CDH-1 (previously known as E-cadherin; Tokuda *et al*. 2007). So

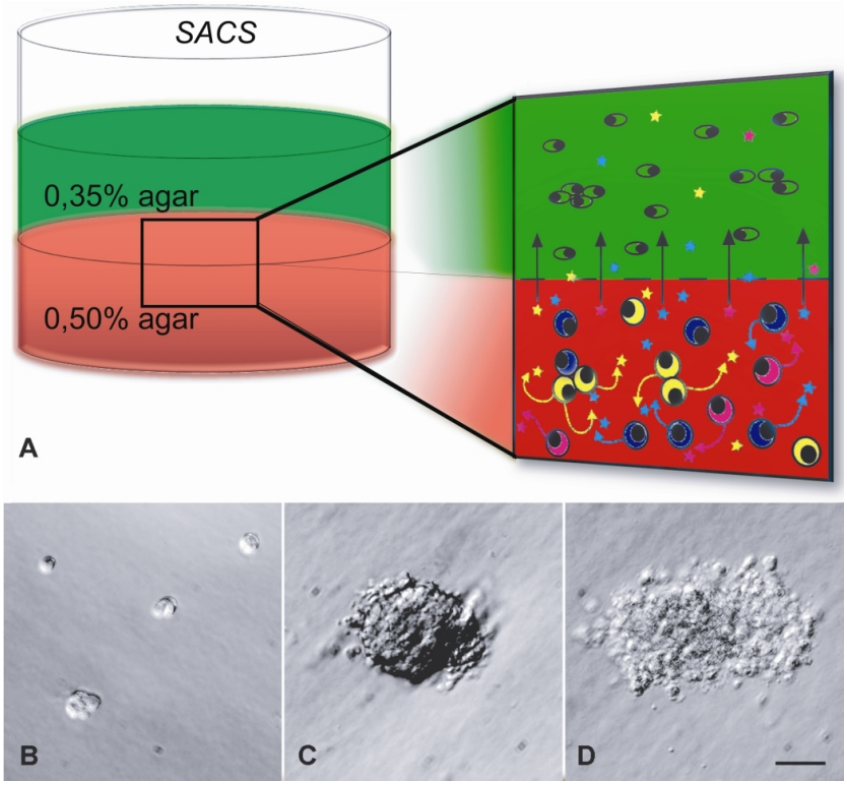

Fig. 6 Schematic illustration of the Soft-Agar-Culture-System (SACS). The two phases (gel phase (0.35% agar; green) and solid phase (0.5% agar; red)) are shown in higher magnification on the right side. Soluble factors (e.g. stem cell factor (SCF); stars) produced by supporting cells (circles) in the solid phase diffuse through the solid into the gel phase (arrows). Cultured cells (ovals) in the gel phase absorb these factors without direct physical cell-cell contact between germ and somatic cells and differentiate. Images of clonal expansion of SAC-cultured spermatogonia after different culture periods (B (day 0), C (day 3), D (day 30)) found in the gel phase. Scale bar = 25 µm.

far, all these markers are described in rodents. No reliable SSC marker is known for primates. This is probably related to the differing spermatogonial cell populations mentioned above. All the SSC markers used in approaches dealing with mouse cells detect SSCs – but also spermatogonial cells that are even slightly further differentiated. Momentarily the protein GFRα-1 appears to be the cell surface marker that selects the population of spermatogonia in which the proportion of undifferentiated "real" SSCs is the largest.

The physiological conditions needed to propagate and differentiate cultured germ cells were analyzed in conventional cultures (e.g. de Rooij and Grootegoed 1998; Feng *et al*. 2002; Izadyar *et al*. 2003; Kanatsu-Shinohara *et al*. 2003). To transfer the function of testicular somatic cells (i.e. Sertoli cells and Leydig cells, which *in situ* are deeply involved in the regulation of spermatogenesis and germ line dynamics, into the *in vitro* situation, testicular cell types were co-cultured as feeder cells, or media containing growth factors were provided (e.g. LIF leukaemia inhibiting factor; GDNF glia cell-line derived neurotrophic factor; Kubota *et al*. 2004; SCF, stem cell factor; Dirami *et al*. 1999). As recently shown, LIF seems to support the maintenance and differentiation of SSCs *in vitro* better than GDNF (Guan *et al*. 2006).

Apart from cellular support and the presence of conditioned media, one result of tissue culture analysis and germ cell transplantation experiments was the exigency of a niche for SSC settlement. This niche is also determined by its spatial structure. In conventional cell culture, dishes or flasks are commonly used that do not mimic these structural prerequisites. The availability of a three-dimensional culture environment improved normal germ cell progression (Hofmann *et al*. 1992) and supported *in vitro* meiosis with a higher success rate (Lee *et al*. 2006, 2007). A methodology first established to characterize clonal outgrowth of bone marrow cells and factors regulating their differentiation and expansion (Horowitz *et al*. 2000), has been modified for clonal expansion of cultured germ cells and presents a novel method to analyze germ cell development. The three-dimensional matrix in this approach is obtained by using agar layers, one soft gel phase (0.35% agar) and an underlying solid phase (0.5% agar) (**Fig. 6**). This combined arrangement of the Soft Agar Culture System (SACS) allows adding isolated somatic testicular cells (as some sort of specified feeder layer) and or certain growth factors in the solid phase separate from the isolated and enriched germ cells seeded in the gel phase of the SACS. While intimate physical contact between germ cells and Sertoli cells exists in the mammalian testis, *in vitro* these direct interactions seem to be of less importance for proliferation or differentiation of spermatogenic cells (Tesarik *et al*. 1998).

In rat germ cell culture, it was demonstrated that the crucial step for *in vitro* maturation of germ cells is the transition from middle to late pachytene spermatocytes. Here, the expression of regulatory proteins plays a key role in meiotic events (Perrard *et al*. 2003). Furthermore, testosterone and FSH have an enhancing effect on the two meiotic divisions and the postmeiotic expression of a germ cell-specific gene in cultured pachytene spermatocytes of rats. Testosterone and SCF have been suggested to improve Sertoli and germ cell survival in culture by inhibiting apoptosis (Tesarik *et al*. 2001). In principle, these results identify the initiation of meiosis in cultured cells as the critical period, depending on the presence or absence of endocrine and paracrine factors and on the support of Sertoli cells (Vigier *et al*. 2004). These factors involve several activating intracellular signalling molecules, including members of the Bcl-2 family, Fas/Fas ligand and tumour necrosis factor alpha-related apoptosis-inducing ligand (TRAIL), P53 and cyclic AMP responsive element modulator (CREM) (Gnessi *et al*. 1997; Tesarik *et al*. 2001; Gotaroli *et al*. 2004).

The *in vitro* completion of mammalian spermatogenesis remains a challenge, although remarkable progress has been achieved in identifying conditions for long-term culture of mouse spermatogonial stem cells (SSCs). These cells did not lose their original potential of establishing spermatogenesis, as proven by re-transplantation into a recipient testis in which settlement and maturation occurred (Kanatsu-Shinohara *et al*. 2003; Toyooka *et al*. 2003; Kanatsu-Shinohara *et al*. 2005; Guan *et al*. 2006; Kubota and Brinster 2006).When SSCs from neonatal mouse testes were cultured in a primary medium in the presence of GDNF, LIF, EGF and bFGF, occasionally colonies were

found exhibiting similarities to those formed by embryonic stem cells or cultured epiblast cells. When these colonies were selected and subsequently cultured in a secondary medium adapted for embryonic stem (ES) cells, they developed a profile of ES cells (Kanatsu-Shinohara 2004b). This result indicates that cultured mouse spermatogonia isolated from neonatal testes can give rise to pluripotent stem cells. Moreover, primordial germ cells (PGCs) obtained from the epiblast and transplanted into the testis differentiated into sperm (Chuma *et al*. 2005). These studies reveal a surprisingly high plasticity of the male germ line, as was also concluded from the transplantation experiments.

A consequent and exciting recent finding was that cells from adult testes exhibit the potential to give rise to differentiated cells from all of the three germ layers *in vitro* (Guan *et al*. 2006). These conclusions might open routes for novel therapeutic strategies, since the use of ES cells for therapeutic purposes suffers from ethical implications as well as from an observed genetic instability. A source of stem cells remains even in differentiated testicular tissue. The SSCs have to fulfil the requirements of a self-renewing stem cell pool life long and they are obviously only a very small distance away from pluripotency. Methods already available might allow directing these cells back to an undifferentiated state, thus enabling them to serve as starting material to build up tissues of all three germ layers without destroying embryonic life. In addition, it was shown that genetic imprinting and chromosomal balance of those cultured germ line stem cells are much more stable than they are in ES cells (Kanatsu-Shinohara *et al*. 2004b).

Studies aiming to generate sperm from various cell types *in vitro* claim an extremely rapid germ cell differentiation from ES cells (Nayernia *et al*. 2006a) and from bone marrow stem cells (Nayernia *et al*. 2006b) under the control of retinoic acid. These highly interesting conclusions need confirmation, but if these approaches can be established reliably, *in vitro* generation of male germ cells would be a very elegant tool to treat infertility.

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