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Review

Molecular control of rodent spermatogenesis ☆

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ABSTRACT

Spermatogenesis is a complex developmental process that ultimately generates mature spermatozoa. This process involves a phase of proliferative expansion, meiosis, and cytodifferentiation. Mouse models have been widely used to study spermatogenesis and have revealed many genes and molecular mechanisms that are crucial in this process. Although meiosis is generally considered as the most crucial phase of spermatogenesis, mouse models have shown that pre-meiotic and post-meiotic phases are equally important. Using knowledge generated from mouse models and *in vitro* studies, the current review provides an overview of the molecular control of rodent spermatogenesis. Finally, we briefly relate this knowledge to fertility problems in humans and discuss implications for future research. This article is part of a Special Issue entitled: Molecular Genetics of Human Reproductive Failure.

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1. Introduction

The process of spermatogenesis takes place in the testis within the seminiferous tubules, which contain germ cells and Sertoli cells. The somatic Sertoli cells are essential for the creation of a microenvironment that enables the sustained generation of spermatozoa throughout life. The interstitial tissue in between the seminiferous tubules contains blood and lymphatic vessels, macrophages and Leydig cells, which produce growth factors and testosterone. Peritubular myoid cells surround the tubules, provide structural support, supply growth factors and facilitate the movement of fluid and sperm through the tubule lumen [1].

Spermatogenesis is a complex developmental process during which spermatogonial stem cells enter the differentiation pathway and ultimately give rise to spermatozoa. This whole process can be divided in a mitotic phase, a meiotic phase and the so-called phase of spermiogenesis in which haploid round spermatids elongate and are released into the lumen of seminiferous tubules as spermatozoa [1].

The spermatogonial stem cells are considered to be single cells located at the basement membrane of the seminiferous tubules. In rodents, these cells are called A single (A_s) spermatogonia and regularly undergo mitotic divisions. A_s spermatogonia either undergo self-renewal divisions, that generate new spermatogonial stem cells to ensure maintenance of the stem cell pool, or divide into differentiating spermatogonia (reviewed in ref. [2]). Starting from the initiation of spermatogonial differentiation and onwards cytokinesis is incomplete. As such, one A_s spermatogonium gives rise to two differentiating spermatogonia that remain connected via an intercellular bridge. These A paired (A_{pr}) spermatogonia subsequently expand clonally by mitotic division resulting in the formation of chains of 4, 8, 16 and sometimes 32 A aligned (A_{al}) spermatogonia. A_s, A_{pr} and A_{al} spermatogonia are frequently referred to as undifferentiated spermatogonia (reviewed in ref. [3]). Currently, there are no molecular markers available to distinguish these different types of A spermatogonia [4,5]. A_{al} spermatogonia in mouse and rat differentiate without division into A_1 spermatogonia and start the strictly time regulated part of spermatogenesis consisting of successive mitotic divisions to form chains of A_2, A_3, A_4, Intermediate and finally B spermatogonia (reviewed in ref. [3,6]). These B spermatogonia undergo the last mitotic division to give rise to primary spermatocytes. Subsequently, primary spermatocytes undergo the first meiotic division (M-I) which generates secondary spermatocytes. Thereafter, the second meiotic division (M-II) follows rapidly giving rise to haploid round spermatids (reviewed in ref. [7]) that transform into spermatozoa during the process of spermiogenesis (reviewed in ref. [8]). Based on morphological criteria, spermiogenesis in the mouse is subdivided in 16 steps, the first 12 of which span the cycle of the seminiferous epithelium. Steps 1–8 include round spermatids, whereas steps 9–16 include elongating spermatids, which are finally released into the lumen of the seminiferous tubule (reviewed in ref. [1,9]).

Spermatogenesis occurs in a time regulated fashion, resulting in a co-ordinated spatial organization of cell types within the tubule that can be recognized as stages. Once A_{al} spermatogonia differentiate into A_1 and onwards, the next cohort of A_{al} spermatogonia will follow, within a fixed time interval, before the previous cohort completes spermatogenesis. As a result, fixed association of four to five generations of developing germ cell types exist, which can be recognized

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as areas along the seminiferous tubules. These associations of developmental germ cell types are used for the staging of the seminiferous epithelium which, in the mouse and rat, are subdivided into twelve (I–XII) and fourteen (I–XIV) stages, respectively [10,11].

2. Molecular control of spermatogenesis

Animal models are widely used to study the molecular control of spermatogenesis. A large number of mouse models have been generated that relate gene ablation or overexpression to spermatogenic failure. Spermatogenic failure occurs at different levels, including defective migration of primordial germ cells, loss of spermatogonial stem cells, arrest during spermatogenesis, inadequate spermiogenesis or a disrupted micro-environment. All these disruptions can result in azoospermia, severe oligozoospermia, asthenozoospermia or teratozoospermia. Additionally, many *in vitro* studies have proven the role of specific genes in the regulation of proliferation, differentiation and survival of spermatogonia. In the current review, we provide an overview of the major mechanisms involved in rodent spermatogenesis. Moreover, we describe candidate genes of which animal models and/or *in vitro* studies indicate an indispensable role at one or more steps of spermatogenesis.

2.1. Fetal germ cells

2.1.1. Germ cell fate decision

Germ cell fate is acquired in mammalian epiblast cells and results in the formation of primordial germ cells (PGCs) (reviewed in refs. [12,13]). Obviously, failure to properly induce germ cell fate is detrimental for spermatogenesis and fertility.

At embryonic day (E) 6 in the mouse, a single layer of epiblast cells located adjacent to the extra-embryonic ectoderm, is positive for the transcriptional regulator *Prdm1*, also known as *Blimp1*. Loss of *Prdm1* in mouse embryos causes a severe reduction in the appearance of PGC-like cells and the few PGC-like cells that remain in these embryos display an aberrant gene expression profile. These experiments indicate that *Prdm1* is essential for PGC specification [14].

Germ cell fate acquisition requires signalling from the extraembryonic ectoderm as well as the visceral endoderm and involves bone morphogenetic proteins (BMPs). Loss of *Bmp4*, which is normally expressed in the extraembryonic ectoderm, completely abrogates the appearance of PGCs in mouse embryos [15]. Similarly, genetic ablation of *Bmp2*, *Bmp8b* or the BMP receptor *Alk2* inhibits the formation of mouse PGCs [16–18]. Interestingly, expression of constitutively active ALK2 in the visceral endoderm of *Bmp4*-deficient embryos rescues the formation of PGCs, confirming that BMP signalling is required for PGC specification [18]. In conclusion, both cell intrinsic factors, such as *Prdm1*, and cell extrinsic factors, such as BMPs, are required for the acquisition of germ cell fate.

2.1.2. PGC migration and survival

At E7.5–E8, alkaline-phosphatase positive mouse PGCs are located at the posterior primitive streak from where they start to migrate into the allantois and the embryonic endoderm [19] and can subsequently be detected in the hindgut at E9.0 [20]. Live cell imaging experiments have demonstrated that PGCs move from the hindgut into the body wall at E9.5 before the formation of the mesentery that separates the hindgut from the body wall [20]. Migration continues towards the genital ridges and is complete by E13.5 [21] During this period, PGCs proliferate with a doubling time of approximately 16 hours [21,22], whereas PGCs that migrate to the wrong place or do not arrive in time, are removed by apoptosis [23].

Both migration itself and proliferation/apoptosis during migration, are directed by interactions between receptors, expressed by PGCs, and ligands, secreted by somatic cells (reviewed in ref. [12]). The tyrosine kinase receptor c-KIT and its ligand, stem cell factor (SCF) [24] are particularly important during this process. SCF is expressed at high levels along the migratory route until E10.5. The role of SCF in migration is demonstrated by the phenotype of $Scf^{-/-}$ embryos, in which PGCs display reduced velocity and altered directionality [24]. Around E10.5, SCF becomes specifically downregulated in the midline of the embryo, and the PGCs that are still present in this area, become apoptotic [23]. Since *Scf*-deficient embryos display elevated BAX-dependent apoptosis [23,24], this implies that SCF is also involved in regulation of apoptosis. Proliferation of PGCs during migration is regulated by SCF in a stage dependent manner: PGCs are able to proliferate in the absence of SCF before migration out of the hindgut, however, loss of SCF blocks PGC proliferation during migration in the mesentery and genital ridges [25]. In conclusion, the requirement of *c-Kit*/SCF during PGC migration occurs at multiple levels.

Another receptor–ligand complex that is involved in PGC migration is the G-protein-coupled receptor CXCR4 and its ligand SDF1. SDF1 is expressed at and around the genital ridges, while PGCs express CXCR4. Ablation of either SDF1 or CXCR4 in mice does not disrupt early stages of migration up to the hindgut, but causes a severe reduction in the amount of PGCs that reach the genital ridges. Thus, CXCR4–SDF1 interaction is required for PGC migration from the hindgut to the genital ridges and the colonization of the genital ridges [26,27] reviewed in ref. [12].

In addition to receptor–ligand complexes, there are several proteins expressed by PGCs that function in migration and survival of PGCs. For example, PGC-expressed adhesion molecules are required for proper migration: antibody-mediated blocking of the adhesion molecule CDH1, also known as E-CADHERIN, in mice impairs the migration of PGCs into the genital ridges [28,29]. Similarly, loss of *Cdh1* impairs PGC colonization of the genital ridges [30]. Also germ cell progenitor- and pluripotency-associated genes are required for maintenance of PGCs during migration: loss of *Oct-4* or *Nanos3* in mice causes a decrease in the total number of PGCs during migration and results in the absence of germ cells in male adults [31,32]. Whereas enhanced apoptosis is detected in *Oct-4* deficient PGCs, this is not the case for *Nanos3*-deficient PGCs. On the other hand, PGCs deficient in *Nanos2* undergo apoptosis shortly after their arrival in the gonadal ridge [31]. Further studies are required to determine the exact mechanism of PGC elimination upon loss of these genes.

2.1.3. Gonocytes

Once PGCs have reached the genital ridges, they become surrounded by the somatic Sertoli cells and seminiferous cords are formed. At this time, PGCs are called gonocytes. Masculinizing signals originating from the somatic cells in the gonad direct the gonocytes towards the male developmental fate (reviewed in refs. [33,34]). Recent experiments demonstrated that execution of the male or female developmental program in response to these sexual cues requires germ cell expression of *Dazl* [35].

Male gonocytes continue to proliferate until they enter quiescence at around E15–16 in the mouse [36–38] and E17–E18 in the rat [39]. This period is associated with changes in expression of various cell cycle proteins. For example, PP2A is expressed in proliferating gonocytes and becomes downregulated during quiescence [40]. In addition, several signalling pathways are involved in inhibition of gonocyte proliferation during quiescence. For example, loss of a subunit of Activin A induces proliferation during the quiescent period and results in a higher number of gonocytes. TGFβ is also involved in regulating proliferation and additionally, is essential for counteracting apoptosis in gonocytes [38,41].

After birth, gonocytes start to move from the centre of the tubule towards the basement membrane, which involves c-KIT/SCF signalling [42]. At the same time, proliferation resumes and A spermatogonia are formed [36,37,43–45].

2.2. Spermatogonia

2.2.1. Balance between stem cell self-renewal and differentiation

A_s spermatogonia are considered to be the spermatogonial stem cells. These cells undergo self -renewal divisions, thereby maintaining the stem cell population, or they can divide to produce A_{pr} spermatogonia that enter the differentiation pathway. A tightly regulated balance between self-renewal and differentiation is crucial to maintain spermatogenesis throughout life. When the balance shifts towards differentiation, the pool of stem cells will ultimately become depleted. On the contrary, a shift towards self-renewal will cause an accumulation of stem cells and a reduced production of differentiated cells. Studies using mutant mice have revealed a number of genes that are involved in regulating this balance, such as ID4 [46], ZBTB16 [47,48] and NANOS2 [49] (Fig. 1). Strikingly, the inhibitor of DNA binding 4 (ID4) is, to our knowledge, the only gene involved in this balance that is specifically expressed in mouse A_s spermatogonia. Downregulation of ID4 *in vitro* inhibits the proliferation of spermatogonial stem cells and deletion of ID4 in mice causes progressive germ cell depletion, resulting in male sterility [46].

Although the formation of A_{pr} and subsequently A_{al} spermatogonia seems to be a prerequisite for spermatogonial differentiation, it was recently proposed that cells within the A_{pr} and A_{al} population also possess stem cell potential [50]. As such, several proteins expressed by A_s–A_{al} spermatogonia have been implicated in regulating the balance between self-renewal and differentiation. One of these proteins is NANOS2, which is expressed in A_s and A_{pr} spermatogonia. Postnatal deletion or overexpression of *Nanos2* causes depletion or accumulation of mouse undifferentiated spermatogonia, respectively [49]. Similarly, loss of *Zbtb16*, which is normally expressed by A_s–A_{al} spermatogonia, causes a reduction in the number of undifferentiated spermatogonia [47,48,51]. The percentage of differentiating spermatogonia is higher in *Zbtb16* deficient testis compared to wild type, indicating that ZBTB16 promotes stem cell self-renewal over differentiation [51]. In line with this, ZBTB16 has been shown to transcriptionally repress the early spermatogonial differentiation marker *c-Kit* [52].

An important signalling network functioning in A_s–A_{al} spermatogonia is the GDNF signalling network [50,53–55]. The growth factor GDNF is secreted by the somatic Sertoli cells and peritubular cells ([56,57] reviewed in ref. [58]) and is a well-known inducer of SSC proliferation *in vitro* ([54,59–61]; reviewed in ref. [4]). Binding of GDNF to the GDNF-family receptor α1 (GFRα1) catalyses the activation of the c-RET receptor, which activates several signalling cascades, such as PI3K/AKT, MEK and SCR kinases ([62,63]; reviewed in ref. [64]). Strikingly, GDNF induces the A_s-expressed and self-renewal associated gene ID4 and the self-renewal associated gene *Bcl6b* [46,65]. Overexpression of GDNF induces the accumulation of undifferentiated spermatogonia in mice, whereas heterozygous ablation causes loss of SSCs and progressive germ cell depletion [53,66]. Similarly, deletion of the GDNF co-receptors *Gfrα1* or *c-Ret* causes a reduction in the number of undifferentiated spermatogonia and also leads to germ cell depletion [55,67,68]. Interestingly, *Zbtb16* deficient spermatogonial cells display reduced responsiveness to GDNF and show downregulation of *Gfrα1*/*c-Ret* receptors. This is caused by reduced transcriptional activation of *Redd1*, resulting in hyperactivation of mTORC and subsequent downregulation of *Gfrα1*/*c-Ret*. Inhibition of mTORC re-establishes wild type expression levels of *Gfrα1*/*c-Ret*, implying a link between ZBTB16, GDNF/mTORC and maintenance of the undifferentiated state [51] (Fig. 1).

Opposed to its role in self-renewal, GDNF has been reported to work synergistically with NEUREGULIN-1 to induce the *in vitro* formation of A_{al} spermatogonia [69]. Similarly, studies using *Pdk1*- and *Pten*-deficient mice implied that enhanced signalling of the PI3K/AKT pathway impairs stem cell self-renewal via the inhibition of FOXO transcription factors, that subsequently results in downregulation of the GDNF receptor *c-Ret* [70]. As PI3K forms one of the signalling pathways downstream of GDNF [62], this suggests a negative feedback loop that inhibits stem cell self-renewal. In conclusion, GDNF signalling is crucial in regulating the balance between SSC self-renewal and differentiation, whereby stimulation of self-renewal is the most well established effect. However, to which side the balance is shifted might be influenced by other cell intrinsic or extrinsic factors, such as the magnitude of PI3K signalling or the presence of NEUREGULIN-1.

Another set of proteins implicated in spermatogonial self-renewal are the PIWI proteins. These proteins are predominantly expressed in germ cells and bind PIWI-interacting RNAs (piRNAs): 24–40 nt long small RNAs (reviewed in ref. [71]). In mammals, PIWI proteins have been mainly linked to meiosis and spermiogenesis. However, experiments in Drosophila have shown a role for PIWI in proliferation of germ line stem cells [72]. In line with this and in addition to meiotic defects, loss of the PIWI protein MIWI2 in mice also causes progressive germ cell loss [73]. Together, this implies that also in mammals, PIWI proteins might function in self-renewal of germ cells.

Clearly, the somatic cells of the testis are involved in regulating the balance between SSC self-renewal and differentiation via the production of growth factors, such as GDNF, CSF1, SCF, Activin A and BMP4 ([56,57,74–78], reviewed in ref. [58]) (Fig. 1). However, the precise molecular pathways determining the spermatogonial stem cell niche still need to be further unravelled. One factor known to be involved is *Itgb1*, also known as *Integrin β1*. This adhesion molecule is expressed by Sertoli cells ([79]) and by a subset of spermatogonia that is enriched for SSCs [80,81] and is required for SSC homing [80–82]. In line with the importance of surface proteins in SSC behaviour, experiments using a rat spermatogonial cell line show that BMP4-induced spermatogonial differentiation correlates with altered expression levels of a series of proteins involved in cell-adhesion, including E-cadherin. Possibly, these growth factor-induced changes in adhesion molecules are required for the migratory capacities of differentiating spermatogonia [83].

In addition to adhesion molecules, the interstitium and vascular network are critical for the spermatogonial stem cell niche (reviewed in ref. [58]). The location of undifferentiated and differentiating mouse and rat spermatogonia is related to the position of the interstitium and vascular network, suggesting that secreted factors such as CSF1 from Leydig cells are involved in establishing the spermatogonial stem cell niche [84–86]. These secreted factors as well as their signalling pathways require further elucidation.

2.2.2. Spermatogonial differentiation

The transition of A_{al} into A_1 spermatogonia is marked as the start of the strictly time regulated part of spermatogenesis. Knowledge about the mechanisms that steer spermatogonial differentiation is, however, still limited. A block in differentiation into A_1 spermatogonia is observed in vitamin A deficient animals, demonstrating that this step is dependent on retinoic acid [87–91]. However, the retinoic acid target genes involved in this process are still largely unknown. The tyrosine kinase receptor c-KIT, which is expressed from late A_{al} spermatogonia onwards, is induced upon the differentiation of A_{al} into A_1 spermatogonia [92,93]. Moreover, heterozygous mutation of the *c-Kit* encoding white spotting (w) locus or mutation of its ligand stem cell factor (SCF) causes a block in the differentiation of A_{al} into A_1 spermatogonia [93–95]. Similar to c-KIT, the expression of CYCLIN D2 is induced during the A_{al}-A_1 transition and is maintained up to the spermatocyte level. Therefore, the induction of this cell cycle-regulated protein possibly reflects an upcoming requirement for CYCLIN D2 during meiosis [96].

Other factors, such as the spermatogenesis and oogenesis specific helix–loop–helix (SOHLH) proteins, have been hypothesized to play a role during early spermatogonial differentiation [97–99]. These basic HLH proteins can form homo- or heterodimers [99,100] and are involved in transcriptional regulation. Mouse SOHLH1 is expressed in A_{al}, A_1–A_4, Intermediate and B spermatogonia [97], whereas

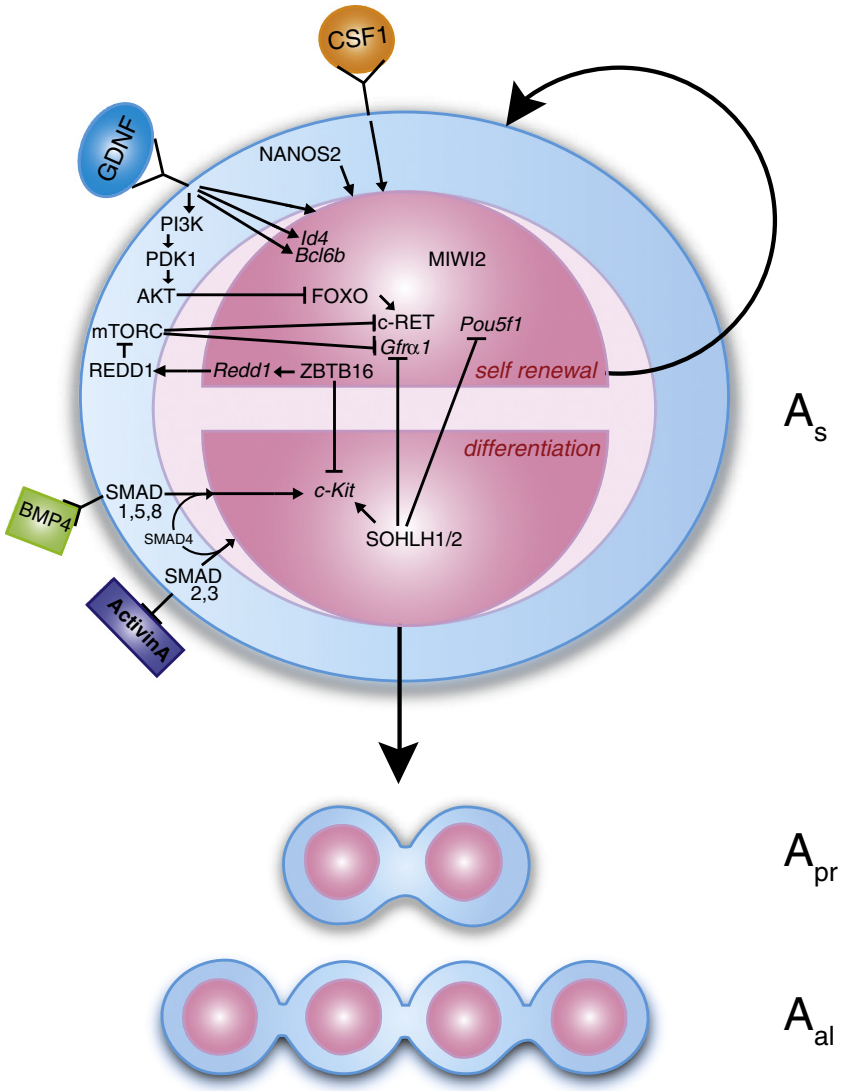

Fig. 1. Schematic illustration of key pathways that are involved in the regulation of self-renewal and differentiation in rodent spermatogonial stem cells. ↓ and ⊥ represent stimulation and repression, respectively, and can be either direct or indirect. Blue areas correspond to the cytoplasm, pink areas to the nucleus.

SOHLH2 is expressed in all A spermatogonia (A_s–A_{al} and A_1–A_4) and is absent in B spermatogonia [99]. Genetic ablation of *Sohlh1* and/or *Sohlh2* interferes with spermatogonial differentiation and causes impaired generation of spermatocytes and infertility [97–100]. In both *Sohlh1* and *Sohlh2*-deficient testes, spermatogonia are maintained, but the expression of the early spermatogonial marker *c-Kit* is reduced [97,98]. Degenerating A_2–A_4 spermatogonia are detected in *Sohlh2* deficient testis, suggesting a defect during the differentiation of A_1–A_4 into B spermatogonia [98]. However, the absence of early differentiating, c-KIT positive spermatogonia in *Sohlh2* deficient testis [99] implies that defects already occurred before the differentiation of A_{al} into A_1 spermatogonia. Consistently, it has recently been shown that SOHLH1 and SOHLH2 are co-expressed in subsets of undifferentiated, but not the most primitive, spermatogonia and are involved in repression of stem cell self-renewal genes [100]. Thus, SOHLH proteins seem to be involved during the first steps of spermatogonial differentiation by opposing self-renewal.

2.3. Spermatocytes

When type B spermatogonia finally divide into pre-leptotene spermatocytes, these germ cells are ready to enter meiosis, a crucial process in spermatogenesis during which diploid germ cells divide and differentiate into haploid spermatids. This is achieved by one round of DNA duplication followed by two consecutive rounds of chromosome segregation: meiosis I (movement of the homologous chromosomes, each consisting of one pair of sister chromatids, to opposite poles) and meiosis II (separation of the sister chromatids into haploid cells).

2.3.1. The meiotic prophase I

Meiosis starts in so called pre-leptotene spermatocytes with a prolonged S-phase, during which the DNA is duplicated, followed by a highly regulated and prolonged G_2-phase called the meiotic prophase I. Initiation of the meiotic prophase I depends on the RNA-binding protein DAZL, whose presence enables germ cells to respond to retinoic acid that, in turn, induces expression of the protein STRA8 [101,102]. STRA8 is required for the initiation of the meiotic prophase I and consequently, *Stra8*$^{-/-}$ mice are devoid of all later spermatocytes and spermatids, whereas spermatogonia and pre-leptotene spermatocytes are present at normal numbers [102]. The meiotic prophase I can be subdivided in four cytological stages: leptonema (chromatin condensation, initiation of DNA double-strand breaks (DSBs) and meiotic recombination), zygonema (initiation of synapsis of homologous

chromosomes), pachynema (full synapsis, development of recombination sites into at least one crossover per homologous chromosome pair) and diplonema (de-synapsis and visible crossover sites, the so-called chiasmata) (reviewed in refs. [7,103]) (Fig. 2). Proper positioning and subsequent segregation of the homologous chromosomes during the first meiotic division depends on the formation of these chiasmata. Therefore, the processes that lead to the formation of chiasmata, including the formation of DNA double-strand breaks (DSBs), homologue recognition and synapsis and meiotic recombination, are amongst the most critical events in spermatogenesis. During the meiotic prophase I, the sister chromatids are held together by cohesin complex proteins, whereas synapsis of the homologous chromosomes is achieved by formation of a large zipper-shaped protein complex known as the synaptonemal complex (SC) (reviewed in ref. [104]).

Synapsis of the homologous chromosomes and meiotic recombination are two highly intertwined events. The introduction of DSBs and subsequent meiotic recombination starts prior to and is essential for the initiation of synapsis of the homologous chromosomes [105–107]. Likewise, synapsis is required for recombination sites to develop into meiotic crossovers [108]. Since DSBs are required to enable homologue recognition and pairing and subsequent meiotic recombination, one could say that the preparation for synapsis already starts in leptotene spermatocytes with the deliberate introduction of DSBs by the enzyme SPO11 [105,109]. At about the same time, the telomeres attach to the nuclear envelope in order to form a cluster of telomeres in zygonema (called bouquet formation) that is additionally thought to facilitate homologue recognition and pairing (reviewed in ref. [110]).

In leptotene spermatocytes, the proteins SYCP2 and SYCP3 initiate the formation of fibrous cores alongside the homologous chromosomes, called the axial elements of the SC [111,112]. This occurs in accordance with meiosis specific cohesin complex proteins, including SMC1ß, RAD21L, REC8 and STAG3, that form a core structure to hold the sister chromatids together [113–118]. During zygonema, the axial elements, now referred to as lateral elements, are joined by transverse filaments shaped by SYCP1 molecules that interact in a dense region in the middle of the SC called the central element [119–121]. Further progression to full chromosome synapsis in pachynema and proper meiotic DSB repair depend on the formation of this central element structure [122–126].

Finally, during diplonema, the SC is disassembled. This process is also crucial for meiotic progression since spermatocytes that fail to disassemble the SC display a pachytene/diplotene arrest [127,128].

2.3.2. Male specific meiotic arrest at prophase I

Disruption of pairing and synapsis triggers a male specific meiotic arrest at a stage of the seminiferous epithelium that would normally contain pachytene spermatocytes [129], in the mouse known as the stage IV pachytene checkpoint. A separate pachytene checkpoint for synapsis was first described for the nematode *C. elegans* and found to be regulated by the protein PCH2 [130]. Interestingly, the mammalian homolog of PCH2, TRIP13, is involved in the timely removal of HORMAD1 and HORMAD2 [131], two recently described proteins that specifically localize to unsynapsed chromosomes [131–133]. Spermatogenesis of *Hormad1*$^{-/-}$ mice also arrests at epithelial stage IV and *Hormad1*$^{-/-}$ spermatocytes fail to pair and align their homologous chromosomes and initiate meiotic recombination. Additionally, HORMAD1 appears to play a role in recruiting the protein ATR to unsynapsed chromosomes, which is required in order to achieve meiotic silencing of unsynapsed chromosomes (MSUC) [134].

Apart from the pseudo-autosomal regions (PARs), the X and Y chromosomes remain unsynapsed due to a lack of homology. This leads to formation of the XY-body in which the sex chromosomes

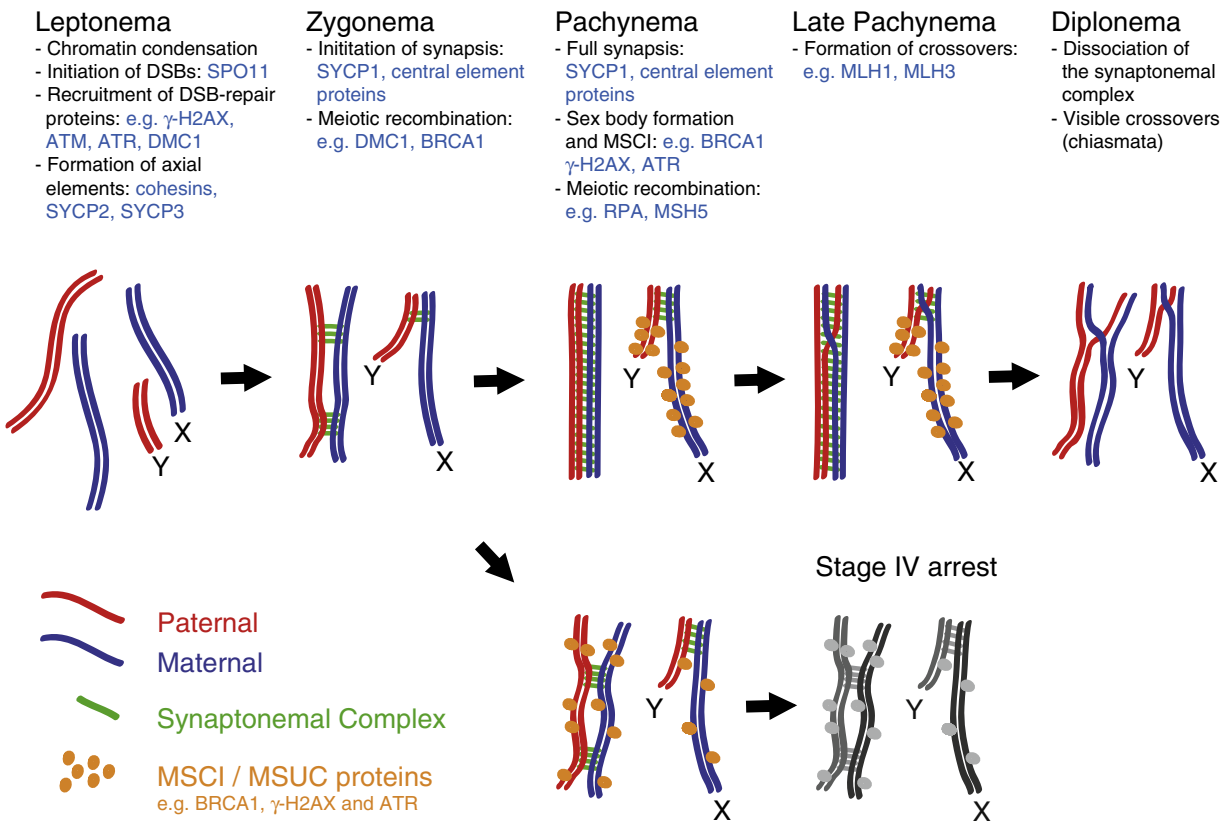

Fig. 2. Schematic illustration of the cytological stages of the meiotic prophase I. Disturbed DSB processing or insufficient pairing and synapsis of the homologous chromosomes interferes with meiotic sex chromosome silencing (MSCI) and causes stage IV meiotic arrest in the mouse.

are marked by several proteins, including BRCA1, γ-H2AX and ATR [135]. These proteins cause silencing of the X and Y-chromosomes by a mechanism called meiotic sex chromosome inactivation (MSCI) (reviewed in ref. [135]) (Fig. 2). However, when the autosomal chromosomes fail to synapse in time, they are also silenced, in this case by MSUC [136,137]; a process that also requires BRCA1, γ-H2AX and ATR and possibly sequesters these proteins away from the XY-body [138]. Consequently, in case of extensive autosomal asynapsis, this will lead to failure of sex chromosome silencing leading to stage IV pachytene arrest [138] (Fig. 2). Interestingly, a meiotic prophase arrest is not seen in female germ cells, a difference that can be explained by the absence of the Y-chromosome and MSCI in these cells [139,140]. In line with this idea, it is recently shown that stage IV pachytene arrest in mouse spermatocytes is caused by failure to silence Y-chromosome located genes [141]. Even more so, the insertion of the normally Y-located paralogs Zfy1/2 on non-silenced autosomal chromosomes already appears sufficient to trigger stage IV pachytene arrest in otherwise normal spermatocytes [141].

Given the central role of DSB formation and repair during meiosis, it is of no surprise that several DNA-damage response proteins play key roles during meiotic prophase I. Their various functions during meiosis have been reviewed extensively [7,142–144] and disruption of these proteins has, in many cases, been described to lead to stage IV pachytene arrest (reviewed in ref. [145]). However, despite their elimination at the same stage of spermatogenesis, spermatocytes in different mouse mutants can display different cytological endpoints and phenotypes [146]. For instance, although all eliminated at stage IV, *Atm*$^{-/-}$ spermatocytes only reach leptonema [147,148], whereas *Dmc1*$^{-/-}$ [146,149], *Spo11*$^{-/-}$ [105,146] and *Msh5*$^{-/-}$ [150] spermatocytes reach zygonema. Proceeding even further, *Sycp1*$^{-/-}$ [108] and *Smc1β*$^{-/-}$ [114] spermatocytes, in which synapsis is disturbed, manage to reach pachynema before undergoing apoptosis at stage IV [129]. Hence, the cytological endpoint and the final elimination of spermatocytes from the seminiferous epithelium at stage IV seem to be two separate events.

Interestingly, the tumour-suppressor p53, normally induced in response to DSBs, is not essential for stage IV meiotic arrest [151–153]. This raises the question whether wrongly processed DSBs actually form a direct trigger for meiotic prophase I arrest. Because disturbed DSB formation or processing leads to defective pairing and synapsis of the homologous chromosomes (reviewed in ref. [135]), it could also be that DSBs, instead of directly triggering a checkpoint, cause extensive MSUC [138]. Extensive MSUC then interferes with the required silencing of the Y chromosome, which subsequently leads to stage IV pachytene arrest [141]. This would additionally explain why wrongly processed DSBs only lead to meiotic prophase arrest in spermatocytes and not in oocytes: a similar DSB surveillance mechanism may be lacking in oocytes just because these cells do not have to silence a Y chromosome.

Interestingly, also the piRNA interacting proteins MILI and MIWI2 of the PIWI family are required for male meiotic progression [73,154]. Both *Mili*$^{-/-}$ and *Miwi2*$^{-/-}$ spermatocytes do not reach full synapsis and, as a consequence, are eliminated from the testes [73,154]. Moreover, although this has not been exactly determined for *Mili*$^{-/-}$, *Miwi2*$^{-/-}$ spermatocytes are eliminated exactly at stage IV [73]. Despite the fact that piRNAs are also present in female germ cells, deletion of PIWI family members only leads to male sterility [73,154,155]. Therefore, it may well be that piRNAs play a crucial role in silencing the Y-chromosome and thus the pachytene stage IV meiotic checkpoint.

2.3.3. The meiotic divisions

After meiotic prophase I, when the SC has been dismantled at diplonema, proper alignment and segregation of chromosomes during the first meiotic division depends on the presence of at least one chiasmata per homologue chromosome pair (reviewed in ref. [7]). During eukaryotic cell divisions a spindle assembly checkpoint (SAC) monitors whether chromosomes are properly bi-oriented, by pulling at the centromeres and sensing whether appropriate tension can be established between the chromosomes that need to be separated (reviewed in ref. [156]). Bi-orientation and tension between the homologous chromosomes during meiotic metaphase I depend on the presence of chiasmata (reviewed in ref. [156]). It is therefore that ablation of the MutL homologs MLH1 and MLH3 in mice, two proteins required for the development of recombination sites into crossovers during pachynema, leads to activation of the SAC and arrest at metaphase I [157].

Whereas homologous chromosomes are connected by chiasmata, the sister chromatids are held together by cohesin complex proteins (reviewed in ref. [156]). During anaphase I, the meiotic cohesin subunit REC8 is cleaved off from the chromosome arms but protected at the centromeres by the protein SHUGOSHIN-2 in order to prevent premature separation of the sister chromatids [158,159]. Similar to chiasmata between homologous chromosomes during metaphase I, centromeric cohesin is required for bi-orientation and tension between sister chromatids in order to satisfy a second SAC during metaphase II. Finally, at the metaphase II/anaphase II transition, the remaining REC8 molecules at the centromeres are cleaved off, thereby allowing segregation of the sister chromatids and the eventual generation of haploid round spermatids (reviewed in ref. [156]).

2.4. Spermatids

At the completion of meiosis round spermatids are formed. These then undergo dramatic morphological and cytological changes during the process of spermiogenesis. Morphologically, spermatids develop a distinct head, midpiece and tail region. These changes result in slender elongated mature spermatids that are released into the lumen of the seminiferous tubule during spermiation. Cytologically, they undergo chromatin remodelling, develop an acrosome and remove almost all of their cytoplasm. A schematic overview of these processes is depicted in Fig. 3.

2.4.1. Axoneme

The sperm tail or flagellum starts to develop in round spermatids during step 1 of spermiogenesis (reviewed in ref. [1]), (Fig. 3a). The flagellum develops from a centriole at one pole of the round spermatid and is composed of a microtubular structure known as the axoneme, which is required for motility (reviewed in ref. [1,160]). The development of the flagella is a continuous process and is completed near the end of spermiogenesis [1]. Numerous mutant studies have shown that defects in axoneme formation lead to infertility (reviewed in ref. [161]). Deletion of proteins involved in axoneme formation such as HOP, SPAG6 and TEKSTIN-T lead to loss of crucial axoneme structures and subsequent loss of flagellar motility [162–164]. Interestingly, apart from immotility, loss of axonemal structures also causes head anomalies suggesting a co-ordinated interaction between head and tail formation in developing spermatids. This may be due to two connecting microtubular structures, known as the IMT (intramanchette transport) and IFT (intraflagellar transport) pathways, that are used for transport of proteins from head to tail for axoneme formation (reviewed in refs. [165,166]).

2.4.2. Manchette

The manchette is a transient microtubular structure that forms during nuclear elongation (steps 8–14) (reviewed in ref. [165]), (Fig. 3b). A ring-like part of the manchette, the perinuclear ring, surrounds the base of the elongating nucleus and plays a key role in shaping the nucleus and the sperm head (reviewed in ref. [165]). One of the most well characterized cases of infertility is caused by a defective manchette formation, and is observed in *Azh* null (abnormal spermatozoon head) mice [167]. Spermatids from *Azh* null mice often show a displacement of the manchette and have bent and coiled tails

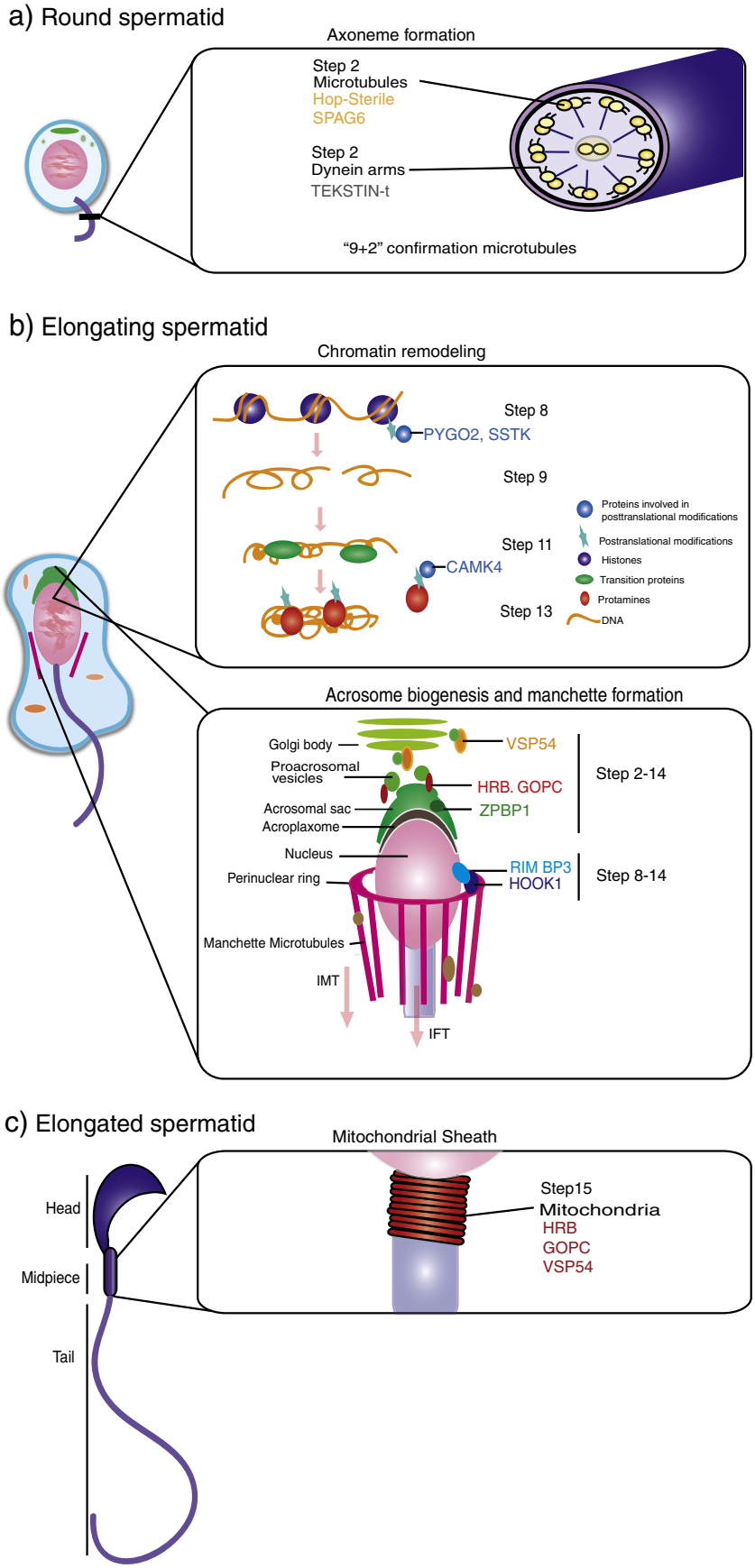

Fig. 3. Schematic illustration of key genes involved in processes related to morphological and cytoplasmic changes in rodent spermatids during spermiogenesis. a) axoneme formation, b) manchette formation, formation of acrosomal sac and chromatin remodelling c) formation of mitochondrial sheath.

and decapitated sperm [167]. It is now known that a mutation in the *Hook1* gene is responsible for these spermiogenic defects [168]. HOOK1 plays a role in connecting the manchette to the nucleus and possibly stabilizes this structure [168,169]. RIM-BP3, a manchette-associated protein, interacts with HOOK1 and may be involved in linking manchette-bound HOOK1 to the nucleus [170]. Consequently, *Rim-bp3* null mice are infertile.

2.4.3. Cytoplasmic removal

Cytoplasmic removal is an important process ensuring the development of compact and slender spermatozoa. Removal of cytoplasm from the spermatids occurs in three phases. Most of the cytoplasm is removed by the tubulobulbar complexes (TBCs) which are cytoplasmic protrusions of the spermatid head leading into the Sertoli cells [1,8]. These protrusions are lined with F-actin filaments (reviewed in ref. [165]). In normal mice, CAPZA3 interacts with F-actin in the TBC and plays a role in cytoplasmic removal. *Repro32* null mutant mice, which contain a mutation in the *Capza3* gene, are infertile with abnormal spermatid morphology and motility problems resulting from aberrant removal of cytoplasm [171].

Under normal circumstances, the remainder of the cytoplasm is removed via the residual body that pinches off at spermiation and the cytoplasmic droplet, a small pocket of cytoplasm, situated near the neck of the spermatid. Although most of the cytoplasm is shed by the TBC, the residual body and cytoplasmic droplet are also crucial in spermiogenesis as shown by *Spem1* mutant mice [172]. Although the function of SPEM1 is not yet known, preliminary studies have shown that *Spem1* mutant spermatids retain their cytoplasm [172]. It is hypothesized that SPEM1 inhibits the detachment of cytoplasm from the spermatid, consequently, cytoplasmic retention hinders the straightening of the spermatids and causes severe structural defects leading to malformed spermatozoa [172].

2.4.4. Acrosome biogenesis

The acrosome is a granular vesicle that encases the top of the nucleus and contains hydrolytic enzymes required for oocyte penetration during fertilization (reviewed in ref. [173]). Acrosome biogenesis is a progressive process that starts at step 2 and finishes at step 12 of spermiogenesis (reviewed in ref. [1]), (Fig. 3b). During steps 2–4, proacrosomal vesicles originating from the Golgi apparatus are transported to the upper pole of the spermatid head where they fuse, to form an acrosomal sac on top of the nucleus. An F-actin structure, the acroplaxome, connects the acrosome to the nucleus and is involved in transport of proacrosomic vesicles to form an acrosomal sac (reviewed in refs. [165,174]. The acrosomal sac flattens, condenses and elongates during steps 5–12 [1]. Defects in acrosome formation can cause globozoospermia, a condition characterized by severe head and acrosomal malformations leading to infertility. Deletion of proteins involved in vesicle-to-vesicle fusion, transport and sorting such as HRB [175], GOPC [176] and VSP54 [177] that cause spermiogenic defects in acrosome biogenesis lead to globozoospermia in mice. In addition to acrosomal defects, these mutants lack a mitochondrial sheath around the midpiece and display impaired motility. The mitochondrial sheath is the power supply of the sperm flagellum (reviewed in ref. [178]). Part of the mitochondria migrate to the midpiece, condense, elongate and wrap around the neck of the sperm at step 15 to form this sheath (Fig. 3b). The IMT and IFT pathways are proposed to play a role in mitochondrial localization (reviewed in ref. [178]). Thus, it is possible that proteins involved in molecular transport via the IMT/IFT pathways could have an effect on both acrosome biogenesis and mitochondrial sheath formation.

In addition to vesicle formation defects, acrosome condensation has been shown to play an important role in acrosome biogenesis. ZPBP1 is believed to play a role in acrosome condensation during step 6. Deletion of *Zpbp1* in mice causes a phenotype that is similar to deletion of *Hrb* and *Gopc*, and results in male infertility [179].

2.4.5. Chromatin remodelling

The elongation phase of spermiogenesis involves extensive chromatin remodelling which results in nuclear condensation and cessation of transcription (Fig. 3b). Crucial for this process is the replacement of histones by protamines. In mammals, starting at steps 11–12, histones are first replaced by transition proteins (TPs) which are subsequently replaced by protamines (PRMs) (reviewed in ref. [180]). Studies have shown that histone modifications such as hyperacetylation [181–184] and phosphorylation are crucial steps mediating removal of these proteins and subsequent TP incorporation [185,186]. Mutant mice with deletion of *Pygo2* or *Sstk*, genes involved in histone acetylation and phosphorylation, respectively, show morphological abnormalities accompanied by defects in chromatin condensation in spermatids, resulting in male infertility [185,186].

TP1 and TP2, encoded by *Tnp1* and *2*, are believed to play a role in nuclear condensation [180]. Depletion of either TP proteins shows only modest spermatogenic abnormalities whereas concomitant ablation causes infertility [187,188]. This implies that both TPs have partially overlapping functions. Interestingly, *Tnp1* null mice display a more severe phenotype when compared to their *Tnp2* null counterparts. This is in line with the fact that in wild type mice, TP1 is more abundantly expressed and thus, ablation causes more severe defects [180]. Curiously, in addition to chromatin abnormalities, these mutants also display head and tail anomalies [188]. In addition, *Tnp* mutants also show obscured PRM2 processing, leading to an imbalance in the ratio of PRM1:PRM2 [180]. In accordance with this, haploinsufficiency of PRM1 or PRM2 results in male infertility [189]. This suggests that the infertility observed in *Tnp* null mice may be caused, partially, by an indirect effect on the PRM ratio.

The importance of nuclear condensation in spermiogenesis is further confirmed by various studies that have shown that disruption of proteins required for transcription of *Tnps* and *Prms*, such as CREM and TRF2, results in male infertility [190,191]. Interestingly, it is not only the transcription of *Prms* but also their post-translational modifications that are important for their incorporation into chromatin. PRM2 is synthesized as a precursor that requires phosphorylation of its mature protein before it can displace TP2 and becomes incorporated into chromatin [192]. It has been shown that kinase CAMK4 is involved in PRM2 phosphorylation as loss of CAMK4 inhibits PRM2 phosphorylation and subsequently prevents its incorporation into chromatin leading to male infertility [193].

DNA attains a supercoiled formation when wrapped around histones. As a result, removal of histones results in torsional stress which can be relieved by DNA strand breaks [194]. Widespread DNA strand breaks occur during steps 9–11 in elongating spermatids [195,196]. Interestingly, a few studies have shown that TP or PRM deficient spermatids display persistent DNA strand breaks [194]. Therefore, it is conceivable that the repair of these breaks, which might require TP or PRM, may be important for genomic integrity and possibly male fertility.

2.4.6. Spermatid–Sertoli cell junction and spermiation

The apical ectoplasmic specialization (aES) is an important spermatid–Sertoli cell junction that starts to form in the Sertoli cell cytoplasm in step 8 [8]. The aES is composed of various cell adhesion molecules including nectins, integrins–laminins and cadherins–catenin complexes [197]. This junction is crucial for proper sperm development during late spermiogenesis and holds spermatids in place until spermiation. Disruption of proteins involved in aES formation, such as NPHP1 [198], NECTIN-2 and NECTIN-3 [199], and integrin α6β1-laminin333 [197] results in elongation defects and premature detachment of spermatids from the surrounding Sertoli cell.

Timely disassembly of the aES is of utmost importance for the completion of spermiation and is postulated to be a prerequisite for spermatid disengagement [8]. Spermatids from mutant mice lacking EHD1, a protein involved in endocytic recycling, show maintenance

of the aES due to defective integrin β1 recycling leading to retention of mature spermatids within the seminiferous epithelium [200]. In wild type mice, following aES disassembly and spermatid disengagement, elongated spermatozoa have completed the process of spermiation and are released into the lumen of the seminiferous tubule at step 16 [8].

3. Conclusions

The numerous animal studies mentioned above have provided major insights into the molecular control of spermatogenesis. However, the translation of animal studies to the clinic has been difficult [201] and only a few studies have found a possible link between candidate genes found in animals and genetic aberrations in infertile male patients [202–204]. This can have both a biological and an epidemiological cause. Biologically, meiosis and spermiogenesis are quite similar when comparing humans to rodents. However, clear differences are visible in the spermatogonial compartment. In primates, the spermatogonial compartment contains A_{pale} and A_{dark} spermatogonia, which represent the active and quiescent spermatogonial population, respectively. Quiescent spermatogonia have, so far, not been detected in the rodent testis (reviewed in refs. [205–207]). Furthermore, rodent spermatogonia undergo massive clonal expansion via the formation of A_{pr}, A_{al} and subsequently differentiating A_1–A_4, Intermediate and B spermatogonia [6]. In contrast, clonal expansion in the differentiating human spermatogonial compartment is limited and one generation of B spermatogonia is the only distinguishable differentiating type of spermatogonia. Additionally, the arrangement of stages of the seminiferous epithelium is different between rodents and humans. Whereas longitudinal arrangements of stages are found in rodents, such arrangement areas are much smaller in humans and results in several stages in each single tubular cross section. Possibly, this is caused by the difference in kinetics of spermatogonial expansion between rodents and humans [207]. Concerning gene expression, most spermatogonial markers are similarly expressed in primates and rodents. However, for some genes distinct expression has been identified, for example for TSPY and POU5F1 (reviewed in refs. [206,208]). Whether these differences in gene expression have functional consequences remains to be investigated. In addition to biological differences, epidemiological causes could also explain the difficulty in translating animal studies to the clinic. It might be that genetic causes of human spermatogenic failure are so diverse that common causes are hardly found in small cohorts of patients. Contrary to mouse models, homozygous deletions causing spermatogenic failure have not yet been detected in humans, with the exception of Y-chromosome deletions [209,210]. To elucidate the molecular control of human spermatogenesis, whole genome studies of large sample collections of men with spermatogenic failure are required and will hopefully provide more knowledge on genetic causes of male infertility. In addition, detailed molecular studies that focus on the gene expression patterns of individual human germ cell types during normal sperm development are required to determine the molecular control of human spermatogenesis. Finally, more insight into the molecular regulation of specific steps in spermatogenesis is expected to be gained from *in vitro* systems. Although not yet efficient, *in vitro* spermatogenesis with mouse testicular cells [211] and testis organ cultures [212] are promising tools that can be used to more rigorously study the effect of genetic aberrations. Obviously, the development of *in vitro* systems for human spermatogenesis will be of great importance and will open up new possibilities to develop treatments for human spermatogenic failure.

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