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Regulation of spermatogonia

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ABSTRACT: The progression of spermatogenesis relies on the precise regulation of spermatogonial self-renewal and differentiation. Many genes/proteins have been identified with specific expression profiles in individual spermatogonial cell types but the molecular mechanism of their regulation is still obscure. In this review, an overview of current knowledge on the various factors essential for these two major steps of spermatogonial development is presented. Elucidating the function of these factors was typically made possible through either naturally occurring mutant mice or genetically modified mice, in which a specific and clearly defined spermatogonial arrest is seen. In addition, a few factors, which are potentially important but less clearly defined, are also included. Moreover, the relevance and postulated interactions between these factors is discussed.

Introduction

This article is focused on the genes/proteins, hormones and other physiological factors that are essential for the self-renewal of spermatogonial stem cells (SSCs) and spermatogonial differentiation. Most of the genes discussed have been shown in naturally occurring mutants or genetically modified mice to produce an infertile or sub-fertile reproductive phenotype. In addition, these genes have been shown to play a definitive role in one germ cell type or one particular step of spermatogonial development. Genes that have demonstrated effects on multiple organs, tissues and cell types or result in general hormonal changes will not be emphasized except for those with clear effects on a specific event of self-renewal and differentiation of spermatogonia. The majority of the experimental data presented here is based on the rodent model and the regulation of the two phases of spermatogonial development in adult male mice, self-renewal and differentiation, will be the focus. Numerous papers have been published in the past 15 years that describe the reproductive phenotypes of mutated genes in mice that result in male sterility. An even greater number of papers deal with the specific expression of genes/proteins in a subpopulation of spermatogonia. In this chapter, a restricted summary of recent progress in understanding the critically important regulators of spermatogonia using genetic models is presented.

Spermatogenesis: in general

Spermatogenesis is a complex process, in which spermatogonial stem cells form spermatozoa. This process is continuous throughout the adult life in most mammals. Sertoli cells play a crucial nurturing role in coordinating and supporting important events of spermatogenesis. In the

seminiferous tubules, adjacent Sertoli cells form a “blood-testis” barrier via junctional complexes and separate the intra-tubular germinal epithelium into two compartments: a basal compartment where cells are exposed to the extra-tubular environmental milieu and a luminal compartment where germ cells are subject to a unique environment produced by Sertoli cells as well as germ cells (see Figure 1) (Griswold, 1998).

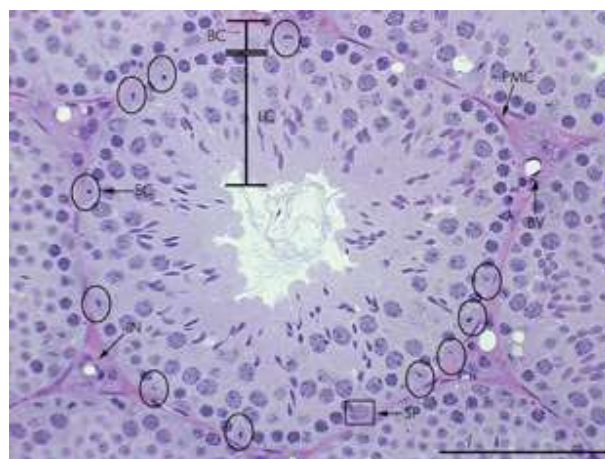


Figure 1: Histological structure of seminiferous tubules in the adult mouse testes.

Junctional complexes are formed between the adjacent Sertoli cells (Sertoli-Sertoli barrier or blood-testis barrier) and this barrier separates the space within the seminiferous tubule into basal and luminal compartments. A layer of peritubular myoid cells surrounds the tubules. The nuclei of Sertoli cells are circled and nuclei of spermatogonia are enclosed by a rectangle. Abbreviations: BC- basal compartment; LC- luminal compartment; IN- interstitium; BV- blood vessel; SC- Sertoli cell; SP- spermatogonia; PMC- peritubular myoid cell. Bar represents 100 μ m.

Spermatogenesis occurs in mitotic, meiotic and postmeiotic phases. In the mitotic (proliferative) phase, spermatogonia undergo either self-renewal or differentiation, both involving successive divisions (Eddy, 2002). Mitotic germ cells (spermatogonia) are located in the basal compartment, whereas meiotic and postmeiotic germ cells are found in the luminal compartment. In the meiotic phase, genetic materials are recombined and segregated in spermatocytes. In the postmeiotic phase, spermatids undergo a series of morphological and structural changes to become spermatozoa. These changes include acrosome and tail formation, chromosome condensation and the removal of the excessive cytoplasm at the time of spermiation. The entire process of spermatogenesis takes about 35 days in the mouse, with mitotic, meiotic, and postmeiotic phases lasting 11, 10 and 14 days, respectively (Eddy, 2002).

Spermatogonia

In rodents, spermatogonial stem cells (SSCs) are single cells located on the basement membrane of the seminiferous tubules. The SSC either divides into two new single cells or into a pair of spermatogonia (A_{pr}) that do not complete cytokinesis and stay connected by an intercellular bridge (de Rooij and Russell, 2000; de Rooij, 2001). The A_{pr} spermatogonia divide further to form chains of 4, 8, and occasionally, up to 32 aligned (A_{al}) spermatogonia. The individual A_s , A_{pr} , and A_{al} cells are morphologically indistinguishable by light microscopy on a histologically stained cross-section of testis. Collectively, these three types of cells are called undifferentiated type A (A_{undiff}) spermatogonia. The A_{al} spermatogonia go through a differentiation step with morphological changes and become A_1 spermatogonia. In rodents, there are five divisions

following A₁ formation, forming successively A₂, A₃, A₄, In (intermediate) and B spermatogonia. The B spermatogonia divide into preleptotene spermatocytes. Each step of differentiation is associated with a mitotic division except for the transition of A_{al} to A₁. Spermatogonia from A₁ to B are termed differentiating spermatogonia. A_s spermatogonia are conventionally considered the only true SSCs even though A_s, A_{pr} and A_{al} are designated A_{undiff} (De Rooij, 1998). Recent studies suggest that A_{pr} and A_{al} may still possess stem cell characteristics under certain conditions (Yoshida *et al.*, 2007a).

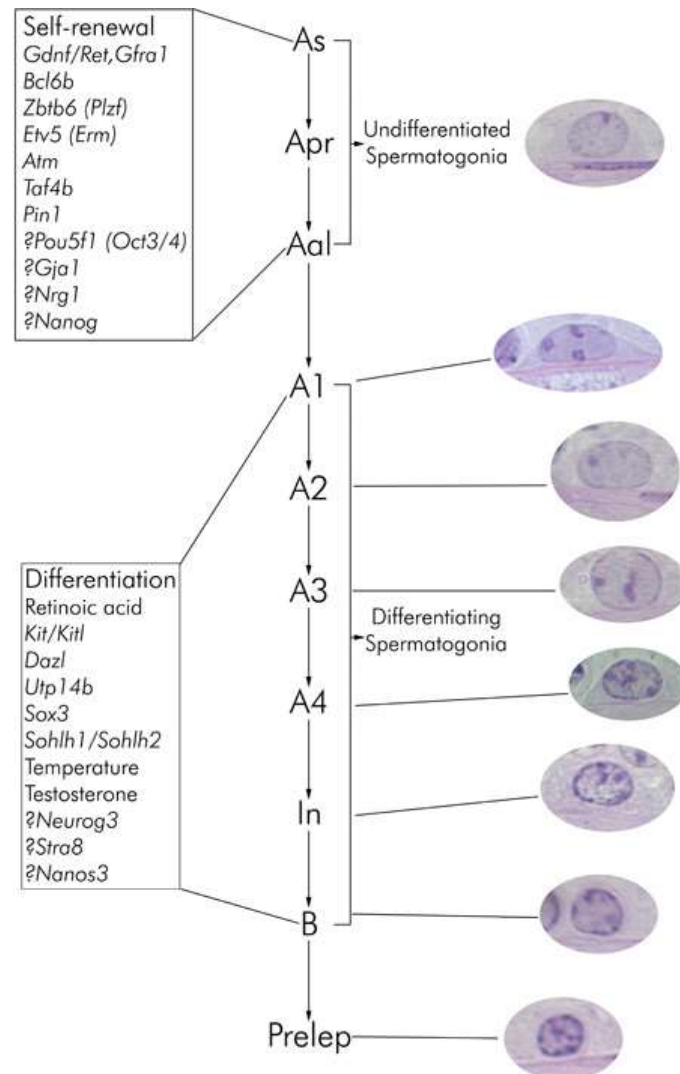


Figure 2: Scheme of spermatogonial self-renewal and differentiation in the mouse, showing the subsequent spermatogonial cell types.

Light photomicrographs show the main morphological features of individual spermatogonial cell type based on the criteria described in previous studies (Chiarini-Garcia *et al.*, 2001b; Chiarini-Garcia *et al.*, 2002; Chiarini-Garcia *et al.*, 2008). The function of various factors regulating spermatogonial self-renewal and differentiation is indicated.

Table 1: Overview of the genes essential for the regulation of mitotic phase of spermatogenesis

Gene/hormone/others	Animal model	Male fertility	Spermatogenesis anomaly	References
Self-renewal of A_{undif}				
<i>Gdnf</i>	Heterozygous KO	Initially fertile then infertile in older mice	Germ cell depletion in older mice	Meng <i>et al.</i> (2000)
	Whole testes transplantation of homozygous testes	NA	Germ cell depletion	Naughton <i>et al.</i> (2006)
	Overexpression	infertile	Clusters of A _{undif} , Atrophy of ST, and testicular tumors in older mice	Meng <i>et al.</i> (2000)
<i>Gfra1</i>	Whole testes transplantation of homozygous testes	NA	Germ cell depletion	Naughton <i>et al.</i> (2006)
<i>Ret</i>	Whole testes transplantation of homozygous testes	NA	Germ cell depletion	Naughton <i>et al.</i> (2006)
	Point mutation Knockin testes	infertile	Germ cell depletion	Jijiwa <i>et al.</i> (2008)
<i>Etv5</i>	Homozygous KO	infertile	Germ cell depletion	Chen <i>et al.</i> (2005)
<i>Zbtb16 (Plzf)</i>	Homozygous KO	infertile	Germ cell depletion	Costoya <i>et al.</i> (2004)
	Natural mutant (luxoid)	infertile	Germ cell depletion in older mutant mice	Buaas <i>et al.</i> (2004)
<i>Taf4b</i>	Homozygous KO	Initially fertile then infertile in older mice	Germ cell depletion in older mice	Falender <i>et al.</i> (2005)
<i>Atm</i>	Homozygous KO	infertile	Progressive depletion of A _{undif} , accumulation of DNA damage and cell-cycle arrest in A _{undif}	Takubo <i>et al.</i> (2008)
<i>Bcl6b</i>	Homozygous KO	Subfertile	Partial depletion of germ cells	Oatley <i>et al.</i> (2006)
<i>Pin1</i>	Homozygous KO	Initially subfertile then infertile in older mice	Reduced number of PGCs and progressive depletion of spermatogonia	Atchison and Means (2003)
Other potentially important factors <i>Pou5f1, Nrg1, Nanog, Gja1</i>				Lolicato <i>et al.</i> (2008); Hamra <i>et al.</i> (2007); Yoshida <i>et al.</i> (2006) Brehm <i>et al.</i> (2007)
Spermatogonial differentiation				
Retinoic acid	Vitamin A deficient testes	Infertile	Arrest of A _{al} spermatogonia	Griswold <i>et al.</i> (1989) van Pelt <i>et al.</i> (1995)

Gene/hormone/others	Animal model	Male fertility	Spermatogenesis anomaly	References
<i>Dazl</i>	Homozygous KO	Infertile	Failure of A _{al} -A ₁ transition	Schrans-Stassen <i>et al.</i> (2001)
<i>Kitl</i>	Natural mutant (S117H)	Infertile	Failure of A _{al} -A ₁ transition	de Rooij <i>et al.</i> (1999)
<i>Utp14b</i>	Natural mutant (Jsd)	Infertile	Failure of A _{al} -A ₁ transition	de Rooij <i>et al.</i> (1999)
<i>Sohlh1</i>	Homozygous KO	Infertile	Block of A _{al} -A ₁ transition leaky in prepubertal testes but becomes complete in adults	Ballow <i>et al.</i> (2006)
<i>Sohlh2</i>	Homozygous KO	Infertile	Block of progression from A ₁ to B spermatogonia	Hao <i>et al.</i> (2008)
<i>Sox3</i>	Homozygous KO	Infertile	Nearly agametic with only A _{undif}	Raverot <i>et al.</i> (2005)
Body temperature	Cryptorchidism in C57BL6	Infertile	Failure of A _{al} -A ₁ transition	de Rooij <i>et al.</i> (1999)
Testosterone level	In W/W ^v and Jsd Mutants	Infertile	Restoration of A _{al} -A ₁ transition by lowering testosterone level;	Matsumiya <i>et al.</i> (1999); Tohda <i>et al.</i> (2001); Ohta <i>et al.</i> (2003)
Other potentially important factors: <i>Stra8</i> , <i>Neurog3</i> , <i>Nanos3</i> ,				Zhou <i>et al.</i> (2008); Lolicato <i>et al.</i> (2008); Yoshida <i>et al.</i> (2004)

Self-renewal of SSCs

A continuous supply of differentiating germ cells is essential for spermatogenesis. Therefore, SSCs must have the capacity for self-renewal and maintenance of the undifferentiated state. SSCs constitute only a small fraction of the adult testicular germ cells. Approximately 30,000 SSCs exist in each adult mouse testis and are located in the most peripheral region of the seminiferous tubule (De Rooij, 1998). The only available technique to evaluate spermatogonial stem cell character is the transplantation of cells to recipient males and determination of testis colonization activity (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). In practice, certain surface markers (integrin- $\alpha 6^+$, INTEGRIN- $\beta 1^+$, THY-1⁺, CD24⁺, CD9⁺, KIT⁺, INTEGRIN- αv^- , MHC-Ia/ $\beta 2M^-$, SCA-1⁺ and CD34⁺) have been used to sort and enrich SSCs (Shinohara *et al.*, 1999; Kanatsu-Shinohara *et al.*, 2003). These surface markers are present in other stem cells as well.

Gdnf-Gfra1/Ret-Bcl6

GDNF is produced and secreted by Sertoli cells and a subset of type A spermatogonia express the GDNF receptor complex including ret proto-oncogene (RET) and its co-receptor glial cell line derived neurotrophic factor family receptor alpha 1 (GFRA1) (Golden *et al.*, 1999). GDNF promoted the maintenance and proliferation of SSCs under in vitro culture conditions and has become an essential component in the medium used to culture murine SSCs (Nagano *et al.*, 1998; Kanatsu-Shinohara *et al.*, 2003; Kubota *et al.*, 2004). *Gdnf*^{-/+} mice exhibited a depletion of SSC reserves with age whereas mice over-expressing GDNF accumulated spermatogonia that were

unable to differentiate, leading to the formation of germ cell tumors in older mice (Meng *et al.*, 2000).

GDNF binding to GFRA1 induced RET activation and in turn resulted in the binding of various molecules to the RET intracellular domain. Both *Gfra1*- and *Ret*-null mice die early in life (Pichel *et al.*, 1996; Sanchez *et al.*, 1996; Schuchardt *et al.*, 1994). Whole-testis transplantation techniques demonstrated that *Gdnf*-, *Gfra1*- and *Ret*-null testes showed severe depletion of SSCs by 7 dpp (Naughton *et al.*, 2006). This was presumably due to the lack of proliferation of SSCs and the inability of these cells to maintain an undifferentiated state (Naughton *et al.*, 2006). Homozygous RET Y1062F knock-in mice, in which RET tyrosine 1062 was replaced with phenylalanine, showed atrophic testes and almost undetectable germ cells by 21 dpp (Jijiwa *et al.*, 2008).

BCL6, a transcription repressor, is a *Gdnf*-responsive gene in cultured SSCs (Oatley *et al.*, 2006). SiRNA suppression of its mRNA level resulted in the loss of SSCs under culture condition. Analysis of *Bcl6*-null testes revealed degeneration of spermatogenesis in some of the seminiferous tubules (Oatley *et al.*, 2006).

Etv5 (Erm)

ETV5, a transcription factor, is mainly expressed in Sertoli cells (Chen *et al.*, 2005). *Etv5*-null testes had deficient SSC self-renewal and thus had progressive germ cell depletion and a Sertoli-cell-only syndrome. Microarray analyses of primary Sertoli cells of *Etv5*-null mice showed alterations in secreted factors known to regulate the haematopoietic stem cell niche. Genes with the greatest reductions included the chemokines, *Cxcl12* (*Sdf-1*), *Cxcl15* (*Lix*) and *Ccl7* (*Mcp3*). These chemokines are possibly the niche signaling molecules regulating SSC self-renewal (Chen *et al.*, 2005). The expression of the *Etv5* gene was highly regulated by GDNF in cultured SSCs (Oatley *et al.*, 2006). Reciprocal germ cell transplantation revealed ETV5 appeared to be required in both germ cells and Sertoli cells for normal spermatogenesis (Morrow *et al.*, 2007).

Zbtb16 (Plzf)

ZBTB16 is a transcription repressor whose expression is restricted to A_{undiff} spermatogonia (Costoya *et al.*, 2004; Buaas *et al.*, 2004). *Zbtb16*-null testes underwent a progressive loss of spermatogonia with age, and an increased apoptosis with no apparent major defects in somatic cells (Costoya *et al.*, 2004). The mouse mutant luxoid, in which there is a nonsense mutation in the gene encoding ZBTB16, exhibited a similar defective spermatogenic self-renewal (Buaas *et al.*, 2004). Germ cell transplantation experiments showed luxoid mutant germ cells could not colonize the recipient testes and showed that the defect is intrinsic to SSCs (Buaas *et al.*, 2004). Recent data suggested ZBTB16 modulated the histone lysine methylation state in the ZBTB16-expressing spermatogonia (Payne and Braun, 2006).

Taf4b

TAF4B RNA polymerase II, TATA box binding protein (TBP)-associated factor (TAF4B) is a gonad-specific component of TFIID complex (Verrijzer and Tjian, 1996). This complex contains the TATA-binding protein (TBP) and TBP-associated factors critically important for core promoter recognition and activator-dependent RNA Polymerase II recruitment (Verrijzer and Tjian, 1996; Freiman *et al.*, 2001). In the developing and adult testes, TAF4B protein is specifically expressed in spermatogonia and spermatids. While young *Taf4b*-null males aged between 28–58 days were initially fertile, *Taf4b*-null males became infertile by 3 months of age (Falender *et al.*,

2005). The null testes appeared histologically normal but protein expression of RET and ZBTB16 was reduced at this age. There was no difference between heterozygotes and null mutants in TUNEL staining, suggesting apoptosis was not the cause of low germ cell number in null testes. Serum follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (T) levels in nulls appeared normal (Falender *et al.*, 2005). Germ cell transplantation experiments showed a functional somatic environment in null testes that could support the spermatogenesis of Rosa26 donor SSCs. The testis weights of null mice showed a significant decrease at 3 wk of age and were reduced to 40% that of heterozygotes at 12 wk. By 8 months, the seminiferous tubules of null mice with few exceptions were completely devoid of germ cells (Falender *et al.*, 2005). Together, these data suggest that TAF4B is obligatory for the maintenance of a normal SSC population in adult testes.

Atm

The protein kinase ataxia telangiectasia-mutated (ATM) regulates apoptosis and cell cycle checkpoint responses after DNA double-strand breaks (DSBs), telomere erosion and oxidative stress (Shiloh, 2003). *Atm*-null males and females were infertile (Hamer *et al.*, 2004). *Atm*-null testes showed a progressive depletion of undifferentiated spermatogonia accompanied by cell-cycle arrest (Takubo *et al.*, 2008). Total protein levels of ATM showed no difference in both undifferentiated and differentiating spermatogonia but the activation of ATM preferentially occurred in undifferentiated spermatogonia. BrdU pulse labeling and Pyronin Y staining demonstrated an accumulation of cells at G₁ phase and a block of S phase in *Atm*-null undifferentiated spermatogonia (Takubo *et al.*, 2008). A defective self-renewal ability of *Atm*-null undifferentiated spermatogonia was observed in culture and the capability of colonization was significantly impaired in germ cell transplantation assay. *Atm*-null undifferentiated spermatogonia exhibited accumulation of DNA damage and activation of the p19^{Arf}–p53–p21^{Clp1/Waf1} pathway. Moreover, suppression of p21^{Clp1/Waf1} in the *Atm*-null background restored the transplantation ability of undifferentiated spermatogonia (Takubo *et al.*, 2008). Collectively, ATM plays an essential role in self-renewal of SSC by suppressing DNA damage-induced cell-cycle arrest and this represents a novel mechanism for the regulation of SSC self-renewal.

Pin1

Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1 (PIN1) is a phosphorylation-directed peptidyl-prolyl isomerase that regulates cell cycle progression. Many critical cell signaling phosphoproteins are modulated by PIN1 in a variety of ways, including CYCLIND1, c-JUN, CDC25, p53 and β -CATENIN (Atchison and Means, 2004). Primordial germ cells (PGCs) in *Pin1*-null mice exhibited a prolonged cell cycle and decreased proliferation leading to a severe reduction in PGC number at birth (Atchison *et al.*, 2003). Germ cells in postnatal *Pin1*-null testis were able to initiate and complete spermatogenesis although young *Pin1*-null male mice had a reduced fertility. However, there was a progressive and age-dependent degeneration of spermatogenic cells leading to a complete germ cell loss by 14 months of age (Atchison and Means, 2003). This depletion of germ cells was not due to increased apoptosis. A depletion of spermatogonia was found to be the first step of the degenerative process. Immunohistochemistry studies show that PIN1 is localized to both germ cells and Sertoli cells (Atchison and Means, 2003). Indirect evidence suggested that within germ cells PIN1 was preferentially expressed in A_{undiff} spermatogonia. Taken together, these results reveal an essential role of PIN1 in maintaining SSCs in the adult testis (Atchison and Means, 2003).

Niche for A_{undiff} spermatogonia and blood vessel formation

A study from Chiarini-Garcia and colleagues showed A_{undiff} spermatogonia are preferentially localized to the basal area of the tubules adjacent to interstitium and vasculature rather than to regions adjacent to other tubules (Chiarini-Garcia *et al.*, 2001a). Recently, Yoshida *et al.* recorded a real-time imaging of A_{undiff} spermatogonia during migration and cell division (Yoshida *et al.*, 2007b). With three-dimensional reconstructions of serial sections, they validated the results of Chiarini-Garcia obtained by two-dimensional thin cross-sections of adult testes. The images clearly showed the expansion of A_{pr} spermatogonia to 8- and 16-cell chains in the regions close to the interstitium and blood vessels, and in turn, these cells in chains migrated laterally along the inner tubular surface and became uniformly distributed along the tubules when they became A₁-A₂ spermatogonia (Yoshida *et al.*, 2007b). In an isolated tubule following germ cell transplantation, it was also observed that A_{undiff} spermatogonia redistribute preferentially close to the interstitium and newly developed blood vessels outside the host tubule. These data collectively established a correlation between the physically intimate co-presence of A_{undiff} spermatogonia and interstitium/blood vessels (Yoshida *et al.*, 2007b).

Other factors potentially involved in regulation of SSC self-renewal

Pou5f1 (Oct3/4)

POU domain, class 5, transcription factor 1 (POU5F1) is a transcription factor required to maintain the pluripotency and self-renewal of embryonic stem (ES) cells (Pesce and Scholer, 2001). During gastrulation, POU5F1 expression was progressively repressed in the epiblast and by 7.5 dpc was confined exclusively to primordial germ cells (PGCs) (Scholer *et al.*, 1990) and was required for their survival (Kehler *et al.*, 2004). Expression of POU5F1 was detected in gonocytes throughout embryonic testis development. In postnatal testes, POU5F1 was localized in proliferating gonocytes and later in A_{undiff} spermatogonia (Pesce *et al.*, 1998). *Pou5f1*-null homozygous embryos died around the time of implantation (Nichols *et al.*, 1998), thus precluding further analysis of gene function during postnatal spermatogonial development.

Gjal (Cx43)

Gap junction protein, alpha 1 (GJA1), which is also known as connexin 43 (CX43), is the predominant testicular gap junctional protein located between neighboring Sertoli cells and between Sertoli cells and germ cells (Brehm and Steger, 2005). Total deletion of the *Gjal* gene led to perinatal lethality (Reaume *et al.*, 1995). Adult Sertoli cell-specific *Gjal*-null (*Scx43*-null) mice had a normal testis descent but also had a dramatic reduction in size and weight which resulted in sterility (Brehm *et al.*, 2007). Ninety five percent of seminiferous tubules in adult *Scx43*-null testes were arrested at the level of spermatogonia or with Sertoli cell-only syndrome and intra-tubular Sertoli cell clusters, whereas 5% of tubules showed qualitatively normal spermatogenesis. Numbers of spermatogonia per tubule were decreased and the number of Sertoli cells per tubule increased in *Scx43*-null testes (Brehm *et al.*, 2007). These results, for the first time, indicated that gap junctions might play a crucial role on the integrity of spermatogonial stem cell niche.

Spermatogonial differentiation

There are three important regulatory points for spermatogonial differentiation. The first key point occurs at the conversion of A_s to A_{pr} spermatogonia and the second key point commences during the transition of A_{al} to A_1 spermatogonia. The third key point is the survival and progression of A_1 to B spermatogonia (de Rooij, 2001). A_{pr} spermatogonia do not complete cytokinesis and stay connected by an intercellular bridge. Once A_{pr} spermatogonia are formed, these cells are committed to become haploid gametes. Cytokinesis in all subsequent divisions will also be incomplete, leading to the formation of an increasingly large syncytia of germ cells (see Figure 3). The intercellular bridge is considered to be the first visible sign of spermatogonial differentiation (Aponte *et al.*, 2005). The ratio of self-renewal and differentiation must be strictly maintained around 1:1, otherwise germ cell tumors or depletion of SSCs may occur when the balance skews either way (Meng *et al.*, 2000). At this point, the molecular mechanism underlying this decision toward incomplete cytokinesis and differentiation is largely unknown.

In a recent study, Greenbaum and colleagues reported that testis-expressed gene 14 (TEX14) is an essential component of germ cell intercellular bridges and the intercellular bridges could not be observed by electron microscopy among the *Tex14*-null spermatogonia. The proliferation and differentiation of *Tex14*-null spermatogonia appeared to be unaffected but spermatogenesis was halted before the completion of the first meiotic division (Greenbaum *et al.*, 2006). During each cycle of the seminiferous epithelium, most of the A_{al} spermatogonia differentiate into A_1 spermatogonia at about stage VII (De Rooij, 1998). Most of the known factors regulating spermatogonial differentiation are active in the transition of cells from A_{al} to B spermatogonia. A summary of the actions of these regulatory factors follows.

Retinoic acid (RA)

RA, a biologically active metabolite of vitamin A (retinol), is essential for male fertility (Griswold *et al.*, 1989); (Livera *et al.*, 2002). In vitamin A deficient (VAD) mice, only A_{undiff} spermatogonia could be found in the seminiferous tubules (van Pelt and de Rooij, 1990). At 24–48 hours after the injection of either retinol or RA, arrested A_{al} spermatogonia re-entered the cell cycle and differentiate into A_1 spermatogonia with the subsequent increased expression of kit oncogene (*Kit*) in type A spermatogonia (Schrans-Stassen *et al.*, 1999; van Pelt and de Rooij, 1990). In long-term experiments with VAD mice or rats after the replacement of retinol or RA, several rounds of synchronous spermatogenesis occurred (van Pelt and de Rooij, 1990; Griswold *et al.*, 1989). It is difficult to determine whether RA induction of spermatogonial differentiation is a direct action in germ cells or an indirect regulation via Sertoli cells, since receptors for retinoids are expressed in both somatic and germ cells (Dufour and Kim, 1999). Recent studies show a robust induction by RA of a gene named *Stra8*, together with *Kit*, in the cultured A_{undiff} spermatogonia without the presence of somatic cells (Zhou *et al.*, 2008). This result suggests that RA regulation of differentiation is a direct action on spermatogonia.

RA is a general differentiating factor for many cells and tissues at multiple developmental stages (Clagett-Dame and DeLuca, 2002). There is some evidence that RA interacts with several other factors involved in the regulation of spermatogonial differentiation. Firstly, RA significantly increased the expression of *Kit* in A spermatogonia of VAD rodent testes (Schrans-Stassen *et al.*, 1999). Secondly, RA induced spermatogonial differentiation in cultured cryptorchid testes (Haneji *et al.*, 1983). More importantly, expression of *Stra8* was significantly up-regulated in cryptorchid testes 24 hours after the injection of RA to these animals (unpublished data). Thirdly, the level of deleted in azoospermia-like (*Dazl*) was significantly up-regulated by RA treatment in VAD mouse

testes (unpublished data). Finally, RA treatment dramatically down-regulates genes coding for two key enzymes involved in testosterone production, cytochrome P450, family 17, subfamily a, polypeptide 1 (*Cyp17a1*) and cytochrome P450, family 11, subfamily a, polypeptide 1 (*Cyp11a1*), in VAD mice testes (unpublished data). If this inhibition of *Cyp17a1* and *Cyp11a1* by RA can be translated to a regulation of testosterone synthesis under physiological conditions, it suggests that RA and testosterone have reciprocal actions in the testis. Collectively, it indicates that RA might constitute one of the initial factors in the regulatory chains/networks in spermatogonial differentiation.

Kit/Kitl

KITL, which is also known as stem cell factor (SCF), is secreted by Sertoli cells in the testis, while protein of KIT, the receptor of KITL, is localized on the surface of germ cells (Loveland and Schlatt, 1997). The signaling of KITL/KIT has extensive roles in multiple steps of germ cell development. Investigations of several different mutants of *Steel* (encoding KITL) and the *White spotting* (encoding KIT) reveal that depending upon the specific site of the mutation, defects of KITL/KIT signaling lead to variety in the severity of spermatogenic failure. De Rooij *et al.*, chose to study *Sl17h/Sl17h* mice with a splicing defect in the cytoplasmic tail of the protein encoded by *Kitl* (Brannan *et al.*, 1992; de Rooij *et al.*, 1999). The most advanced germ cells present in *Sl17h* seminiferous tubules were actively proliferating A_{undiff} spermatogonia revealed by their topological arrangement and clonal sizes. These cells failed to differentiate into A₁ spermatogonia. Accumulation of A_{al} spermatogonia was not observed due to the apoptosis in spermatogonia (de Rooij *et al.*, 1999).

Dazl

Dazl encodes an RNA-binding protein essential for both male and female fertility (Ruggiu *et al.*, 1997). A genetic defect, microdeletions of the long arm of the Y-chromosome, is the cause of some cases of human oligozoospermia or azoospermia (deKretser, 1997). One of the human Y-chromosome genes identified within this region is deleted in azoospermia (*Daz*) (Ruggiu and Cooke, 1999). However, *Daz* only exists in the human and some primates. In other mammals, it is represented by a single-copy, autosomal gene *Dazl* (Cooke *et al.*, 1996). *Dazl*-null murine seminiferous tubules contained spermatogonia with very few tubules containing pachytene spermatocytes. The clonal composition study using tubule whole mounts indicated that the spermatogonia that remained in the null tubules were A_{undiff} spermatogonia (Schrans-Stassen *et al.*, 2001). BrdU incorporation and the mitotic index of cells in the null testes showed that these A_{undiff} spermatogonia were actively proliferating but were also undergoing active apoptosis. Immunohistochemistry staining revealed approximately 80% of the spermatogonia in null testes were KIT positive, indicating that KIT/KITL signaling was either upstream or independent of the DAZL regulatory pathway in spermatogonial differentiation (Schrans-Stassen *et al.*, 2001).

Sox3

The SRY-box containing gene 3 (*Sox3*) is a single-exon gene located on the X-chromosome, that belongs to a family of the high mobility group (HMG) of transcription factors (Foster and Graves, 1994). The co-localization of SOX3 with Neurogenin 3 (NEUROG3) in the testes indicated a specific expression of SOX3 in A_{undiff} spermatogonia (Raverot *et al.*, 2005). Both females and males of *Sox3*-null mice are sterile and the nature of male infertility in C57/BL6 background has been characterized (Raverot *et al.*, 2005; Weiss *et al.*, 2003). Testicular defects

became evident in 10 dpp null testes as type A spermatogonia were the only germ cells present within the seminiferous tubules. Proliferation of the germ cells seemed normal and TUNEL assay performed at 1, 2 and 3 weeks of age did not detect any significant differences in apoptosis between WT and null testes. However, it was found that mRNA and protein expression of *Neurog3* was reduced, whereas the *Pou5f1* mRNA level was increased and *Ret* level remained the same in null testes. Collectively, these data suggest that there is an inability of *Sox3*-null A_{undiff} spermatogonia to differentiate and the regulation of differentiation by *Sox3* may involve NEUROG3 (Raverot *et al.*, 2005).

Sohlh1

Spermatogenesis and oogenesis specific basic helix-loop-helix 1 (SOHLH1) is a transcription factor preferentially expressed in type A spermatogonia (Ballow *et al.*, 2006a). SOHLH1 is initially detected in A_{al} spermatogonia in Stage IV and its expression is most significant in differentiating spermatogonia. *Sohlh1*-null males are sterile. TUNEL assays revealed the greatest difference in apoptosis between wild types and nulls occurred at 15 dpp (Ballow *et al.*, 2006a). This appeared to be due to dying spermatocytes, and the apoptotic index declined with age due to reduced numbers of spermatocytes observed in the tubules of older animals. The mitotic index revealed by BrdU incorporation in *Sohlh1*-null spermatogonia was very close to that of juvenile spermatogonial depletion (*Jsd*) mice. A few spermatocytes were observed in a low percentage of the seminiferous tubules of prepubertal *Sohlh1*-null testes. This observation suggested a “leaky” initial block of spermatogonial differentiation, but this block became complete in sexually mature animals. Null testes of 7 dpp mice still expressed ETV5, TAF4B, ZBTB16 and showed reduced expression of NEUROG3 (Ballow *et al.*, 2006a). However, the exact step of spermatogonial differentiation in which this block occurred was uncertain.

Sohlh2

Spermatogenesis and oogenesis specific basic helix-loop-helix 1 (SOHLH2) is a germ cell specific transcription factor (Ballow *et al.*, 2006b). Male *Sohlh2*-null mice were infertile (Hao *et al.*, 2008). Postnatal *Sohlh2*-null mice had reduced numbers of intermediate and type-B spermatogonia, seemingly normal undifferentiated spermatogonia and degenerating colonies of differentiating spermatogonia. Degenerating cells resembled A_2 spermatogonia, and accumulated in M-phase prior to death. In adult *Sohlh2*-null testes, the ratio of A_{undiff} /differentiating spermatogonia was twice the normal level. In culture, *Sohlh2*-null A_{undiff} spermatogonia proliferated normally but failed to express KIT (Hao *et al.*, 2008). Thus, SOHLH2 seems to be required for progression of differentiating type-A spermatogonia into type-B spermatogonia.

Utp14b (Jsd)

In mice that are homozygous for the *Jsd* mutation, a single wave of spermatogenesis is followed by failure of type A spermatogonia to differentiate, rendering male animals sterile, whereas female animals are apparently normal (Boettger-Tong *et al.*, 2000). Hormonal profiles of *Jsd* homozygous males indicated that the circulating testosterone level was normal and the serum FSH level was elevated in young animals but returned to normal at 1 year of age. Germ cell transplantation studies showed spermatogenic failure in *Jsd* was due to a defect in germ cells but not in the intra-tubular somatic environment (Boettger-Tong *et al.*, 2000). De Rooij and his colleagues reported spermatogenic phenotypes in adult *Jsd* mutant testes resembled what is observed in *Sli 7h* mutants, including actively proliferating A_{undiff} spermatogonia accompanied by

spermatogonial apoptosis (de Rooij *et al.*, 1999). Occasionally, a few B spermatogonia were observed in *Jsd* adult seminiferous tubules.

Recent studies identified a small rearrangement within a gene named UTP14, U3 small nucleolar ribonucleoprotein, homolog B (yeast) (*Utp14b*) underlying the *Jsd* phenotype. *Utp14b* represents a testis-specific retroposed copy of the ubiquitously expressed X-linked gene *Utp14a* (Rohozinski and Bishop, 2004; Bradley *et al.*, 2004). Because yeast *Utp14*, the homologue of mouse *Utp14a*, is an essential component of a large ribonucleoprotein complex containing the U3 small nucleolar RNA, Rohozinski and Bishop proposed the autosomal retroposon *Utp14b* has been selected for in evolution due to its stability to increase the efficiency of protein production during spermatogenesis (Rohozinski and Bishop, 2004). Bradley *et al* proposed that it may imply a strong selective pressure to enable ribosome assembly in male meiotic cells (Bradley *et al.*, 2004). The rate and severity of spermatogonial depletion in mice with different genetic backgrounds were studied and it was concluded that the source of Y-chromosome was a major factor in determining the severity of spermatogonial depletion in *Utp14b^{isd}* mutant mice (Bolden-Tiller *et al.*, 2007).

Testosterone

Testosterone has no direct impact on germ cell development but indirectly regulates spermatogenesis through Sertoli cells (Johnston *et al.*, 2001; Zhou *et al.*, 2002). A deficiency in testosterone production or defects in androgen receptor results in a halt of spermatogenesis in the middle of meiosis, indicating testosterone action in Sertoli cells is essential for the completion of meiosis in males (Holdcraft and Braun, 2004a). Numerous studies also showed testosterone was required for spermiogenesis and spermiation (Holdcraft and Braun, 2004b). There is no current evidence to suggest spermatogonial differentiation and self-renewal requires the presence of testosterone. On the contrary, the presence of a normal testosterone level is suggested to be inhibitory to spermatogonial differentiation in three different animal models, including irradiated rats (Shetty *et al.*, 2006a), *Jsd* (Matsumiya *et al.*, 1999; Shetty *et al.*, 2006b) and *Sl17h* homologous mutants (Ohta *et al.*, 2003). Suppression of the testosterone level by antagonists of GnRH/LH or androgen receptor successfully reversed the spermatogonial arrest and induced spermatogonial differentiation in these models (Shetty *et al.*, 2006a). It seems that the beneficial effects of testosterone are exhibited in mid-meiosis and later stages of spermatogenesis, while the detrimental effects reside in differentiating spermatogonia. It is also worth noting that the destructive role of testosterone can be only observed either under the pathological condition or in naturally mutated rodents. We speculate that a delicate fine-tuning of focal testosterone action during the seminiferous epithelial cycle in normal testis is essential to coordinate the regulatory requirements for a successful spermatogenesis.

Temperature

In the human and many other mammalian species, successful spermatogenesis requires a temperature below 37°C. Cryptorchidism, where testes fail to descend into the scrotum and reside in an environment of body temperature, results in a spermatogenic arrest and infertility. The severity of this arrest highly correlates to the genetic backgrounds of the mice being studied. The most severe phenotype is observed in the cryptorchid C57/BL6 testes, in which the most advanced germ cells are A_{undiff} spermatogonia (Haneji *et al.*, 1983). The nature and cellular activity of these A_{undiff} spermatogonia resemble what was observed in *Sl17h* and *Jsd* homologous mutant testes (de Rooij *et al.*, 1999). Cryptorchid testes showed many similarities with those from *Jsd* mice as both models contained seminiferous tubules with A_{undiff} spermatogonia but with few B spermatogonia.

However, elevation of temperature closer to 37°C under certain conditions exhibits a beneficial effect on spermatogonial differentiation. For instance, artificial cryptorchidism generated by surgery rescued the spermatogonial arrest observed in *Jsd* mutants (Shetty and Weng, 2004). It is not clear why a temperature below 37°C is required for the completion of meiosis. Moreover, additional studies are required to understand why normal testosterone levels and scrotum temperatures are destructive to spermatogonial differentiation when *Utp14b* is mutated.

Other factors potentially involved in regulation of spermatogonial differentiation

Neurog3

NEUROG3, a class B basic helix-loop-helix (bHLH) transcription factor, is specifically expressed in A_{undiff} but not KIT-positive spermatogonia (mostly differentiating spermatogonia) (Yoshida *et al.*, 2004). Lineage tracing, using the Cre-loxP system, showed essentially all spermatogenesis in the adult testes was derived from NEUROG3-positive germ cells (Yoshida *et al.*, 2006). Recently, Nakagawa *et al.* showed the A_s spermatogonia with a high probability of steady-state self-renewal have low expression levels of NEUROG3 (Nakagawa *et al.*, 2007). Oatley *et al.* demonstrated that removal of GDNF/GFRA1 from the culture medium for SSCs up-regulated expression of *Neurog3* and postulated the *Neurog3* could be an early/initial signal for differentiation of SSCs (Oatley *et al.*, 2006).

Stra8

Stimulated by retinoic acid gene 8 (*Stra8*) was first identified as a gene under RA regulation in several carcinoma cell lines (OuladAbdelghani *et al.*, 1996). Recent studies showed it is a required down-stream mediator of RA action on initiation of meiosis in both male and female germ cells (Koubova *et al.*, 2006). *Stra8*-null animals were sterile in both males and females (Baltus *et al.*, 2006). Spermatogenesis was halted in *Stra8*-nulls around preleptotene spermatocytes (Baltus *et al.*, 2006). It was also demonstrated that *Stra8* induced by RA was closely correlated to spermatogonial differentiation and proliferation in vitro and in vivo (Zhou *et al.*, 2008); (unpublished data). Whether *Stra8* is required for the differentiation process and the exact mechanism of *Stra8* regulation in spermatogonia is yet to be determined.

Nanos3

Nanos homolog 3 (*Drosophila*) (*Nanos3*) encodes for a zinc-finger protein with putative RNA-binding activity. The NANOS3 protein is expressed in both male and female gonads of early embryo and, after birth, is expressed only in A_{undiff} spermatogonia. Up-regulation of *Nanos3* caused the accumulation of the cells in the G_1 phase and treatment of A_{undiff} spermatogonia with retinoic acid (RA) resulted in a dramatic down-regulation of *Nanos3*. *Nanos3* targeted disruption resulted in the complete loss of germ cells in both sexes. Future studies are needed to define the function of NANOS3 in the self-renewal and differentiation of spermatogonia (Lolicato *et al.*, 2008).

Conclusion

Phenotype analyses and mechanistic studies using genetic models have resulted in several major advances in our understanding of spermatogonial self-renewal and differentiation. The summary depicted in Figure 2 represents the present knowledge about the regulation of

spermatogonia. It is important to note that many genetically modified mice with spermatogenic failure have yet to be studied in enough detail to fully understand the mechanisms of regulation.

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