Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli cell signaling

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Abstract

Spermatogenesis is a continuous and productive process supported by the self-renewal and differentiation of spermatogonial stem cells (SSCs), which arise from undifferentiated precursors known as gonocytes and are strictly controlled in a special 'niche' microenvironment in the seminiferous tubules. Sertoli cells, the only somatic cell type in the tubules, directly interact with SSCs to control their proliferation and differentiation through the secretion of specific factors. Spermatocyte meiosis is another key step of spermatogenesis, which is regulated by Sertoli cells on the luminal side of the blood–testis barrier through paracrine signaling. In this review, we mainly focus on the role of Sertoli cells in the regulation of SSC self-renewal and spermatocyte meiosis, with particular emphasis on paracrine and endocrine-mediated signaling pathways. Sertoli cell growth factors, such as glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2), as well as Sertoli cell transcription factors, such as ETS variant 5 (ERM; also known as ETV5), nociceptin, neuregulin 1 (NRG1), and androgen receptor (AR), have been identified as the most important upstream factors that regulate SSC self-renewal and spermatocyte meiosis. Other transcription factors and signaling pathways (GDNF–RET–GFRA1 signaling, FGF2–MAP2K1 signaling, CXCL12–CXCR4 signaling, CCL9–CCR1 signaling, FSH–nociceptin/OPRL1, retinoic acid/FSH–NRG/ERBB4, and AR/RB–ARID4A/ARID4B) are also addressed.

Reproduction (2015) **149** R159–R167

Introduction

Spermatogenesis is precisely controlled by intrinsic gene expression and extrinsic stimuli, as shown by many loss- and gain-of-function studies. Spermatogonial stem cells (SSCs) are undifferentiated spermatogonia that maintain the potential to self-renew and differentiate into committed progenitors, which sustains spermatogenesis (Kanatsu-Shinohara & Shinohara 2013). Although self-renewal through cell division continues through the life of the organism, little is known about how germline cells acquire and maintain their self-renewal activity. In mice, SSCs (A_{single}) and committed progenitor spermatogonia (A_{paired} and A_{aligned}) are collectively described as undifferentiated A-spermatogonia based on morphological analyses (Clermont & Bustos-Obregon 1968). These undifferentiated A-spermatogonia undergo a series of cell divisions to form differentiating spermatogonia (A1, A2, A3, A4, intermediate, and B spermatogonia) before entering meiosis (de Rooij 2001; see Fig. 1). The SSCs are defined by their ability to self-renew, and it is difficult to distinguish them from the more abundant committed progenitors based on only morphology alone. Several well-defined markers can be used to identify SSCs and other undifferentiated spermatogonia

(see Fig. 1). Although PLZF (Costoya *et al.* 2004), SALL4 (Gassei & Orwig 2013), and CDH1 (Tokuda *et al.* 2007) are expressed in all undifferentiated A-spermatogonia at all stages, GFRA1 (Grasso *et al.* 2012), LIN28 (Zheng *et al.* 2009), NANOS2 (Suzuki *et al.* 2009), and NGN3 (Yoshida *et al.* 2004) are primarily expressed in specific types of undifferentiated A-spermatogonia. In addition, ID4 and PAX7 are specific to type A_{single} spermatogonia (Oatley *et al.* 2011, Aloisio *et al.* 2014). NANOS3 is detectable in most undifferentiated type A-spermatogonia and differentiating type A1 spermatogonia (Suzuki *et al.* 2009). c-KIT, a tyrosine kinase receptor for KITL, is expressed in some type A_{aligned} spermatogonia, differentiating spermatogonia, and the earliest preleptotene spermatocytes (Yoshinaga *et al.* 1991). SOHLH1 and SOHLH2 are expressed in both c-KIT-negative and c-KIT-positive spermatogonia, with the exception of GFRA1⁺ spermatogonia (Suzuki *et al.* 2012). PLZF is the first transcription factor to be identified as being involved in SSC self-renewal. SALL4 functions by removing PLZF from its cognate targets (e.g. c-KIT). Thus, the competition between PLZF and SALL4 is an important topic of investigation. It has been recently shown by Liao *et al.* (2014) that reduced levels of PLZF in *Dnmt3l*-knockout Thy1⁺ cells results in the releases of the PLZF antagonist

Figure 1 Characteristics of mammalian spermatogonial development and protein markers of SSCs. Whether SSC division is a symmetric or asymmetric process in mammals remains a topic of debate. Regardless of division pathways, SSCs undergo either self-renewal, to maintain a pool of SSCs, or differentiation, committing to differentiating into spermatogonia and then entering meiosis. A number of well-defined markers of SSCs in the mammalian germline have been summarized, of which ID4 and PAX7 selectively mark type A_{single} spermatogonia. Undifferentiated type A-spermatogonia may be basically classified into three categories: $\text{GFRA1}^+ \text{NANOS2}^+ \text{LIN28}^- \text{NGN3}^-$, $\text{GFRA1}^+ \text{NANOS2}^+ \text{LIN28}^+ \text{NGN3}^-$, and $\text{GFRA1}^- \text{NANOS2}^- \text{LIN28}^- \text{NGN3}^+$ (Suzuki *et al.* 2009). The dashed and colored arrows indicate the period of expression. The succession of the various types of germ cells during the early stage of mouse spermatogenesis is represented in the image: A_{single} , a single spermatogonia; A_{paired} , a paired spermatogonia; A_{aligned} , an aligned spermatogonia; A_1 , A_2 , A_3 , A_4 , types A_1 – A_4 spermatogonia; Int, intermediate spermatogonia; B, type B spermatogonia.

SALL4, which is associated with maintaining the delicate balance of cycling and quiescent SSCs/progenitor cells. Further study is required to investigate whether $\text{SALL4}^+ / \text{PLZF}^- A_{\text{single}}$ and A_{paired} spermatogonia are destined to differentiate. The human ortholog of the mouse SSC maintenance factor PLZF is expressed in spermatogonia, and its biallelic loss in humans is associated with genital hypoplasia (Fischer *et al.* 2008). To our knowledge, PAX7 is the only protein known to be expressed in SSCs in a variety of mammals, including human. Further studies are needed to identify additional markers of human SSCs and other undifferentiated spermatogonia. It is also important to test the classic studies of SSCs through spermatogonial transplantation techniques and other functional SSCs assays.

Meiosis is essential for the production of haploid gametes from spermatocytes and maintaining genome integrity (Li *et al.* 2005). Prophase of meiosis I is unique in that it is elongated and further broken down into leptotene, zygotene, pachytene, diplotene, and diakinesis. During these substages of prophase I, DNA double-strand breaks, alignment and synapsis of homologues, and crossover formation ensure homologous recombination between non-sister chromatids (review in Pawlowski & Cande (2005)). Meiosis is precisely controlled by intrinsic and extrinsic factors, as shown in numerous gene knockout studies.

Scrotal hypothermia is essential for normal spermatogenesis (Paul *et al.* 2009); therefore, it is important to

understand why spermatogenesis is negatively affected by heat. Our previous studies demonstrated that temporal heat stress in the testes resulted in a dramatic reduction in the expression of upstream factors, such as androgen receptor (AR), Wilms' tumor gene 1 (WT1), and liver receptor homolog 1 (LHR1) in monkey (Zhang *et al.* 2006, Guo *et al.* 2007, Chen *et al.* 2008), rat (Guo *et al.* 2007), and mouse (Cai *et al.* 2011); however, these effects were reversible. The reversible dedifferentiation of Sertoli cells is followed by a temporary reversible disruption of the blood–testis barrier (BTB), which leads to dramatic germ cell apoptosis in the testis (Lue *et al.* 2006). Importantly, heat-induced changes in Sertoli cells and the failed spermatogenesis were found to recover over time. Upstream factors may act as transcription factors to regulate the reversible change in Sertoli cell differentiation and the process of spermatogenesis via BTB-associated proteins. For example, when the AR was over-expressed, the heat-induced downregulation of BTB-associated proteins was rescued. AR knockdown by RNA interference (RNAi) or treatment with an AR antagonist (flutamide) inhibited the recovery of BTB-associated proteins. Furthermore, the co-localization and interactions of components of partitioning-defective protein 6 (PAR6)-PAR3-aPKC-Cdc42 polarity complex components were disrupted in both AR-knockdown and heat-treated Sertoli cells (see Fig. 2; Li *et al.* 2013). Our study suggests that a small number of undifferentiated spermatogonia survival in the disrupted niche after heat treatment and the SSC niche and BTB are re-established after the heat stress is withdrawn. Sertoli cells, which are the principal contributors to the SSC niche and the BTB structure, play a central role in both the disruption and recovery process. Absalan *et al.* (2011) demonstrated that spermatogonia isolated from bilateral cryptorchid mice have the ability to regenerate spermatogenesis. Based on these findings, we support the notion that spermatogenesis is regulated by a complex paracrine and endocrine system within a structurally well-organized tissue. Central to this system are the Sertoli cells, which provide nutrient and structural support for the differentiating germ cells.

In this review, we summarize findings regarding the paracrine regulation of SSC self-renewal and spermatocyte meiosis by Sertoli cells. The well-defined glial cell line-derived neurotrophic factor (GDNF) is a soluble factor that is secreted by Sertoli cells and influences the self-renewal of SSCs while inhibiting their differentiation (Meng *et al.* 2000). The generation and characterization of male total and conditional AR knockout mice from different laboratories demonstrate the exceeding necessity of Sertoli cell-expressed AR signaling for meiosis (review in Wang *et al.* (2009)). Significant progress has recently been made by us and others in exploring the effects of Sertoli cells on the regulation of SSC self-renewal and spermatocyte meiosis. Several authoritative reviews have recently been presented that describe the

Figure 2 Diagram summarizing the hypothesized mechanism underlying the reversible effects of scrotal heat stress on Sertoli cell function. The disruption of spermatogenesis and its reversible recovery can be divided into six steps (Li *et al.* 2013). Heat stress perturbs the BTB structure, which may be mediated via effects on upstream transcription factors (WT1, AR, and LHR1) and the PAR3/PAR6 polarity complex within Sertoli cells and the subsequent effects on BTB-associated proteins between Sertoli cells. Thus, Sertoli cells are considered to be the main cellular mediators of the effects of heat stress on spermatogenesis.

transcriptional regulation of spermatogonial differentiation and spermatocyte meiosis by intrinsic factors (Rossi & Dolci 2013, Hai *et al.* 2014, Song & Wilkinson 2014). Thus, in this review, we focus on Sertoli cell-controlled paracrine signaling during early stages of spermatogenesis.

GDNF signaling

Niche factor GDNF, secreted by Sertoli cells, is a well-defined paracrine factor that promotes SSC self-renewal and maintenance, as shown both *in vivo* and *in vitro*. *Gdnf*^{-/-} mice die at birth with renal and neuronal abnormalities (Meng *et al.* 2000). *Gdnf*^{+/-} mice present an exhausted SSC pool and progressive germ cell loss, whereas mice overexpressing GDNF show an accumulation of undifferentiated spermatogonia (Meng *et al.* 2000). Subsequent studies have described the long-term culture of SSCs based on this finding (Kubota *et al.* 2004, Hofmann *et al.* 2005). This culture system allows a large number of SSCs to be collected; thus, the investigation of SSCs at the molecular level is now possible using these techniques. Human and mouse spermatogonia use the same extrinsic factor, GDNF, for their maintenance and growth *in vitro* (Wu *et al.* 2009). GDNF signaling acts via the RET tyrosine kinase present on undifferentiated type A-spermatogonia (Naughton *et al.* 2006) and requires a ligand-specific co-receptor GFRA1 (Jing *et al.* 1996) to promote spermatogonial self-renewal in mice (see Fig. 3). Studies in RET Y1062F knockin mice suggest that GDNF–RET signaling is essential for the self-renewal of SSCs via tyrosine 1062 (Jijiwa *et al.* 2008).

Further studies have been undertaken to identify the signaling pathways through which these molecules function. The molecular machinery of the GDNF

signaling pathway in SSC self-renewal has been investigated in *in vitro* SSC culture experiments with specific inhibitors. Using this approach, it was shown that the PI3K/AKT-dependent pathway (Lee *et al.* 2007) or the SRC family kinase (SFK) pathway (Oatley *et al.* 2007) mediate GDNF function in SSCs. A microarray analysis of cultured Thy1⁺ SSCs was conducted to identify GDNF-inducible genes in SSCs, which show decreased expression upon GDNF withdrawal and increased expression upon GDNF replacement (Oatley *et al.* 2006). The transcription factors B cell CLL/lymphoma 6 member B (BCL6B), ETS variant 5 (ERM; also known as ETV5), DNA-binding protein 4 (ID4), LIM homeobox 1 (LHX1), BRACHYURY (T), and POU class 3 homeobox 1 (POU3F1) were reported to be essential for SSC self-renewal (Oatley *et al.* 2006).

BCL6B has been shown to be important for maintenance of the self-renewal of SSCs *in vitro* based on results obtained using siRNA; the degeneration of spermatogenesis or a Sertoli cell-only phenotype was observed in the *Bcl6b*-null testes (Oatley *et al.* 2006). ETV5 was

Figure 3 Current understanding of signaling pathways regulating SSC self-renewal in mouse testes. (1) GDNF/RET/GFRA1 activate PI3K/AKT and SFK intercellular signaling mechanisms, influencing transcription factors that have been shown to promote SSC self-renewal, such as BCL6B, ETV5, LHX1, ID4, T, and POU3F1. (2) FGF2 relies on MAP2K1 activation to drive SSC self-renewal via the upregulation of the *Etv5*, *Bcl6b*, and *Lhx1* genes. (3) Sertoli cell-secreted CXCL12 signals via the CXCR4 receptor present on SSCs to regulate self-renewal through an undefined intercellular signaling pathway. (4) *Ccl9* encodes a chemokine that facilitates Sertoli cell chemoattraction of undifferentiated type A-spermatogonia through its receptor CCR1, which is present on SSCs. ERM is predominantly expressed within Sertoli cells and promotes SSC self-renewal via the regulation of CXCL12/CXCR4 and CCL9/CCR1 signaling.

expressed both in Sertoli cells (Chen *et al.* 2005) and in SSCs (Oatley *et al.* 2007, Tyagi *et al.* 2009). *Etv5*-null germ cells fail to initiate spermatogenesis after transplantation into the testes of W/W^v mice. Specifically, a positive feedback loop involving ETV5 and GDNF/RET/GFRA1 regulates SSC self-renewal (Tyagi *et al.* 2009). Interestingly, a recent study has suggested that ETV5 directly activates the expression of microRNA-21 to regulate the self-renewal of mouse SSCs (Niu *et al.* 2011). MicroRNA signaling in testicular Sertoli and germ cells may comprise another level of regulation of the SSC niche (reviewed in van den Driesche *et al.* (2014)). siRNA-mediated reduction in ID4 abolished the ability of SSCs to expand *in vitro*, and *Id4*-mutant mice exhibit progressive loss of the undifferentiated spermatogonial population (Oatley *et al.* 2011). Similarly, knockdown of LHX1 in cultured SSCs reduced their ability to form colonies in functional SSC transplantation assays (Oatley *et al.* 2007). Functional transplantation of SSCs following the silencing of T significantly reduces the number of donor cell-derived colonies formed within the testes of busulfan-treated recipient mice (Wu *et al.* 2011). In addition, transient depletion of POU3F1 reduces the number of SSCs via the induction of apoptosis, and transplantation analyses revealed impaired SSC maintenance (Wu *et al.* 2010).

To thoroughly explore the downstream gene network, microarray analysis of Thy1⁺ spermatogonial cell cultures depleted of ETV5, BCL6B, and POU3F1 by RNAi showed that several genes and microRNA integral for SSC self-renewal are regulated by these upstream transcriptional factors (reviewed in Song & Wilkinson (2014)). The functional interactions and feedback loops of GDNF signaling with other SSC self-renewal regulators will be the focus of future studies and will be useful for dissecting this unique process. Human orthologs of mouse SSC maintenance genes, such as *RET*, *GFRA1*, *ETV5*, *ID4*, and *BCL6B*, are highly enriched in human spermatogonia relative to somatic cells (Wu *et al.* 2009, Sachs *et al.* 2014). Whether these genes contribute to SSCs self-renewal in humans will require further investigation via *in vitro* studies.

Recent studies have concentrated on the impact of GDNF on SSCs rather than molecular control of *GDNF* expression in Sertoli cells. Most recently, an interesting study has characterized the promoter region of the mouse *Gdnf* gene in Sertoli cells, and deletion analysis revealed the involvement of the three CRE sites in the basal and cAMP-induced expression of *Gdnf* in Sertoli cells (Lamberti & Vicini 2014).

Fibroblast growth factor 2–MAP2K1/AKT signaling

Fibroblast growth factor 2 (FGF2, also known as basic FGF (bFGF)) is expressed and secreted by mammalian Sertoli cells and stimulates SSC self-renewal. *In vitro*, supplementation of FGF2 in combination with GDNF

allowed for long-term self-renewing expansion of SSCs (Kubota *et al.* 2004). In humans, FGF2 treatment increased the diameters of SSC colonies (Mirzapour *et al.* 2012). Although the role of the GDNF signaling pathway has been described in SSCs, little is known about the involvement of FGF2. A recent study has demonstrated that FGF2 relies on MAP2K1 activation to drive SSC self-renewal via upregulation of *Etv5*, *Bcl6b*, and *Lhx1* genes using a mouse germline stem (GS) cell culture system that allows for *in vitro* expansion of SSCs (Ishii *et al.* 2012; see Fig. 3). GS cells with activated MAP2K1 proliferate in the presence of GDNF, which indicates that MAP2K1 can substitute for FGF2. Thus, PI3K/AKT, SFK, and MAP2K1 are critical regulators of SSC self-renewal. Interestingly, Lee *et al.* (2009) used a SSC transplantation assay to show that the activation of H-RAS is sufficient to induce SSC self-renewal and that PI3K/AKT and MAP2K1 are RAS effectors.

A subsequent study indicated that FGF2 may regulate mouse SSC proliferation and stem cell activity *in vitro* via autocrine phosphorylation of AKT and ERK1/2 pathway (Zhang *et al.* 2012). Conditional knockout of the *Fgf2* gene in postnatal Sertoli cells and SSCs could help to elucidate the essential role of FGF2 in SSC self-renewal *in vivo*.

CXCL12–CXCR4 signaling

Cxcl12 encodes a chemokine that is expressed and secreted by Sertoli cells and binds to the CXCR4 receptor on SSCs to regulate self-renewal and maintenance (see Fig. 3). The reduction of CXCR4 signaling in primary cultures of undifferentiated mouse spermatogonia by treatment with a CXCR4-specific inhibitor and the injection of CXCL12-blocking-antibody results in SSC loss, and the inhibition of CXCR4 signaling in testes of adult mice by injection of CXCR4-specific inhibitor impairs SSC maintenance, leading to loss of the germline (Yang *et al.* 2013). Both GDNF and FGF2 regulate the expression of *Cxcr4* mRNA in Thy1⁺ spermatogonial cell cultures. Microarray profiling of gene expression revealed that the disruption of CXCL12–CXCR4 signaling leads to a significant decrease in the abundance of the *Fgf2* transcript (Yang *et al.* 2013), suggesting that CXCL12, FGF2, and GDNF form a cooperative network that influences SSC self-renewal. Moreover, CXCR4 participates in both primordial germ cell homing during embryogenesis and SSC homing to the germline niche in postnatal testes (Ara *et al.* 2003, Kanatsu-Shinohara *et al.* 2012). Sertoli cell-specific *Sin3a* deletion resulted in the formation of few undifferentiated spermatogonia. Chemokine CXCL12 and its receptor CXCR4 are not detected in neonatal Sertoli cell-specific *Sin3a*-knockout testes (Payne *et al.* 2010), indicating that CXCL12–CXCR4 signaling influences the establishment of the SSC niche.

Mclver *et al.* (2013) observed that the CXCL12 and its receptor CXCR4 are expressed in the human testis,

which led us speculate that CXCL12–CXCR4 signaling may be involved in human SSC biology.

ERM–chemokine signaling

Chen *et al.* (2005) demonstrated that ERM is expressed exclusively within Sertoli cells and is required for SSC self-renewal. Mice with targeted disruption of the ERM cannot maintain SSC self-renewal without a disruption in normal spermatogenic differentiation; thus, progressive germ cell depletion and Sertoli-cell-only syndrome were observed (Chen *et al.* 2005). Microarray analysis of purified wild type (WT) and *Erm*^{−/−} Sertoli cells indicates that GDNF expression was unchanged and that several chemokine genes, *Cxcl12*, *Cxcl5*, and *Ccl7*, were significantly reduced in *Erm*^{−/−} Sertoli cells, suggesting that these genes play a role in regulating the SSC niche (Chen *et al.* 2005). Simon *et al.* (2010) reported that recombinant CCL9 restored the chemoattractive ability of *Erm*^{−/−} Sertoli cells, suggesting that CCL9/CCR1 may be a direct target of ERM. Transient siRNA oligonucleotides targeting ERM in mouse SSC cultures further confirmed that ERM is a regulator of *Cxcr4* (Wu *et al.* 2011). Therefore, changes in chemokine production and the consequent decreases in chemoattraction by *Erm*^{−/−} Sertoli cells result in SSC loss in *Erm*^{−/−} mice (see Fig. 3). ERM appears to be one of the few transcription factors expressed by Sertoli cells that control SSC self-renewal through either CXCL12/CXCR4 or CCL9/CCR1 signaling. The intracellular mechanisms of CXCL12/CXCR4 and CCL9/CCR1 signaling require further investigation.

Role of follicle-stimulating hormone–nociceptin/OPRL1 signaling in meiosis

Total serum immunoactive follicle-stimulating hormone (FSH) levels increase in mice beginning at 8 days *post-partum* onward (Barakat *et al.* 2008). FSH directly activates the intracellular signaling pathway and leads to the secretion of paracrine factors that indirectly promote spermatogenesis. Recently, the paracrine factors have involved in the signaling between Sertoli cells and germ cells to mediate the effect of FSH on spermatocyte meiosis have been investigated. Nociceptin was found to be upregulated by a surge in FSH levels in mouse Sertoli cells (Eto *et al.* 2012), and the nociceptin receptor OPRL1 is exclusively expressed on the plasma membrane of spermatocytes (Eto *et al.* 2013). Nociceptin induces and maintains REC8 phosphorylation, which is responsible for meiotic chromosome dynamics in spermatocytes during meiosis (Lee *et al.* 2003, Eto *et al.* 2013; see Fig. 4). Although the mechanism by which nociceptin stimulates REC8 phosphorylation in spermatocytes remains unclear, nociceptin/OPRL1 is a novel extrinsic signaling pathway that plays a crucial role in the progression of meiosis.

Figure 4 Schematic diagram of the Sertoli cell-controlled paracrine mechanisms acting on meiosis. (1) RA is synthesized by retinaldehyde dehydrogenases (RALDH) within the Sertoli cells, and it binds to nuclear RA receptors (RAR), which stimulates *Stra8* expression in pre-spermatocytes. (2) FSH and RA induce spermatocyte meiosis indirectly through Sertoli cells when NRG1 and NRG3 are upregulated and bind to their receptor ERBB4 in pre-spermatocytes. (3) OPRL1 localizes to the surface of spermatocytes and responds to nociceptin, which is expressed and secreted by Sertoli cells and functions in spermatocytes to induce REC8 phosphorylation and subsequent meiotic chromosome dynamics. (4) Androgen–AR binds to the ARE on androgen-responsive genes to regulate the expression of unknown signaling factors that may interact with specific receptors in spermatocytes to trigger the expression of *Stra8* gene and/or effector molecules. ARID4A/B also functionally links the AR and RB pathways in meiotic regulation.

Effect of retinoic acid/FSH–neuregulin signaling in meiosis

In murine juvenile testes, Sertoli cell-synthesized retinoic acid (RA) serves as a fundamentally positive regulator in the commitment of spermatocytes to meiosis and it also regulates progression of the early stages of meiotic prophase via the stimulated RA gene 8 (*Stra8*) (Mark *et al.* 2008). However, the molecular pathway by which RA induces meiosis requires further elucidation. Neuregulin 1 (NRG1), a member of the epidermal growth factor family, has only been found to be present in Sertoli cells, and its receptor, ERBB4, localizes to the surface of germ cells (Falls 2003, Zhang *et al.* 2011). Recently, Zhang *et al.* (2011) have demonstrated that RA and FSH act on Sertoli cells to promote the expression of NRG1 and NRG3, directly inducing meiosis in pre-spermatocytes, based on the analyses of *Nrg1*^{Ser−/−} mutant mice and of re-aggregated cultures consisting of spermatogonia and Sertoli cells (see Fig. 4). However, the mechanisms by which RA promotes *Nrg1* and *Nrg3* mRNA expression in Sertoli cells and how NRG1/3 stimulates *Stra8* gene expression remain to be elucidated.

The function of androgen–AR signaling in meiosis

Genetic studies in the mouse demonstrate that meiosis is one of the most important processes controlled by androgen–AR signaling. Currently, more than 1029 different AR missense mutations have been reported, contributing to ~2% of cases of non-specific male infertility (Gottlieb *et al.* 2012). When bound by

androgen, cytoplasmic AR in Sertoli cells quickly translocates to the nucleus and binds to the androgen response elements (ARE) on androgen-responsive genes (Zhou *et al.* 2002). In addition to Sertoli cells, testicular AR is also expressed in Leydig cells and peritubular myoid (PM) cells. In the absence of AR in Sertoli cells in mice, spermatogenesis does not progress beyond the pachytene or diplotene stages of meiosis (Chang *et al.* 2004, De Gendt *et al.* 2004), while the deletion of the *Ar* gene in mouse germ cells does not affect spermatogenesis or male fertility (Tsai *et al.* 2006). Germ cells themselves lack AR function, but they depend on androgen to progress beyond meiosis, indicating that androgens regulate meiosis indirectly by acting on Sertoli cells. Therefore, the molecular mechanism by which Sertoli cells transduce the androgenic stimulus to germ cells is of great significance and should be addressed.

Retinoblastoma (RB) has been reported to interact with AR and functions as a co-activator to induce AR transcriptional activity. A recent study has identified several AR- and RB-responsive genes acting as downstream targets of ARID4A and ARID4B (Wu *et al.* 2013). ARID4A and ARID4B are expressed mainly in Sertoli cells. An investigation of germ cell development in *Arid4a*^{-/-}*Arid4b*^{+/-} mice revealed spermatogenic arrest in the meiotic spermatocytes and post-meiotic haploid spermatids (Wu *et al.* 2013). This study thus indicates that ARID4A/B functionally links the AR and RB pathways in the regulation of spermatogenic meiosis. The signaling pathways of spermatocyte meiosis regulated by Sertoli cells are illustrated in Fig. 4.

Perspectives and concluding remarks

In summary, we have systematically addressed the roles of Sertoli cell-derived soluble factors and Sertoli cell-expressed transcription factors in the regulation of SSC self-renewal and spermatocyte meiosis. ID4 and PAX7 are two proteins known to be expressed in A_{single} spermatogonia (SSCs), and *Id4*-GFP is a candidate reporter line that preferentially labels SSCs (Oatley *et al.* 2011). Special attention should be paid to determine whether the cessation of spermatogenesis is accompanied by a deficiency in SSCs. Analyses of cells that retain the label long-term may be a useful approach for detecting an SSC defect. In the future, it will be crucial to identify SSC-specific markers and reporter lines that preferentially label SSCs to distinguish between SSC and spermatogonial progenitors.

Microarray analysis can be used to identify the genes and microRNA that are regulated by GDNF-inducible transcription factors involved in SSC self-renewal, such as BCL6B, ETV5, ID4, LHX1, T, and POU3F1 (review in Song & Wilkinson (2014)). Similar studies suggest that GDNF and FGF2 share common target genes. However, microarray data cannot distinguish between direct and indirect targets, and further analysis of the transcriptional

regulation is required. Very recently, Kanatsu-Shinohara *et al.*, have reported that F-box and WD-40 domain protein 7 (FBXW7) play an important role in negatively regulating self-renewal. To our knowledge, this is the first report of increased SSC activity in knockout mice. Investigation of other negative regulators that may counteract self-renewal signals will deepen our knowledge of SSC biology (Kanatsu-Shinohara *et al.* 2014).

Human SSCs share some but not all phenotypes with rodent SSCs, and long-term culture of SSCs has not yet been achieved in humans (Guo *et al.* 2014). Most of the knowledge related to the paracrine regulation of SSC fate has been derived from rodents. To our knowledge, no transcription factors have yet been identified that have roles in either SSC self-renewal or differentiation in humans. Because of the significant differences between rodent and human spermatogonia, human orthologs of mouse transcription factors that control SSC events in mice have yet to be further investigated. It will also very important to explore the regulation of human SSC self-renewal by Sertoli paracrine signaling to provide insights into male infertility.

Stem cell niches are formed on the basis of both architectural support and external stimuli produced by niche cells. In this study, we summarize the recent understanding of Sertoli paracrine regulation of SSC function. However, contributions from other somatic cell populations surrounding the seminiferous tubules, such as PM cells, interstitial Leydig cells, and blood vessels, are also possible. Yoshida *et al.* (2007) demonstrated a vascular and interstitial tissue-associated niche for undifferentiated spermatogonia in the mouse. However, the role of the vasculature by layers of appears to be indirect because spermatogonia are separated from the vasculature by the layers of basement membrane. Colony-stimulating factor 1 (CSF1) has been identified as an extrinsic stimulator of SSC self-renewal that implicates Leydig and selects PM cells as contributors of the testicular stem cell niche in mammals (Oatley *et al.* 2009). However, whether *in vivo* SSCs express the CSF1 receptor remains to be investigated. A recent study has reported that testosterone-dependent regulation of GDNF expression in PM cells has a significant influence on the microenvironment of the niche and SSC maintenance (Chen *et al.* 2014). Future studies should investigate how the activity of the SSC niche is regulated by interstitial tissues, hormones, spermatogenic cycles, and aging. Although *in vivo* analysis of the niche is difficult owing to the three-dimensional structure of seminiferous tubules, *in vitro* reconstitution of the SSC niche has recently become possible (Kanatsu-Shinohara *et al.* 2012). Characterization of the *in vitro* reconstituted niche will provide a unique tool to study the interactions between SSCs and their microenvironment.

Azoospermia has been observed in 10–15% of cases of male infertility and in 1% of the general population, and non-obstructive azoospermia has been diagnosed in 60% of azoospermic men. The ability of generating

mature and functional male gametes from SSCs could enable us to understand the precise etiology of male infertility and offer an invaluable source of autologous male gametes for treating male infertility in azoospermic patients. From this viewpoint, the development of *in vitro* differentiation methods will be the next important step. In addition, spermatogonia transplantation is inefficient in nonrodent animals because of the difficulties related to preparing the recipients and the low concentrations of SSCs. Thus, the development of *in vitro* differentiation approaches will overcome these problems. Currently, considerable progress has been made in generating male germ cells, including spermatogonia, spermatocytes, and spermatids, from various types of stem cells (reviewed in Hou *et al.* (2014)). Of note, haploid spermatids were generated in human SSCs from cryptorchidism patients by treatment with RA and stem cell factor (Yang *et al.* 2014). We developed an efficient system by co-culturing GFR α 1-positive gonocytes/undifferentiated spermatogonia with autologous testicular cells isolated from mice around 2.5 days old, creating a functional 'testis' *in vitro* which undergoes spermatogenesis to produce haploid sperm (Y Zhang, SL Deng, SR Chen, CY Cheng and YX Liu, unpublished data). This culture system will provide an invaluable source of autologous male gametes for treating male infertility in azoospermic patients.

Other candidate molecules in Sertoli cells that may be involved in SSC self-renewal and meiosis include WNT5a, doublesex and MAB 3-related transcription factor 1 (DMRT1), and WT1. Yeh *et al.* (2011) have identified WNT5a as an extrinsic regulator of SSC self-renewal (Yeh *et al.* 2011). WNT5a was shown to be present in the Sertoli cells of the mouse testis, and the WNT5a receptor was detected on the SSC cell surface. It remains unknown how WNT5a influences SSCs. Germ cells in Sertoli cell-specific knockout of DMRT1 testes efficiently enter meiosis, but the majority exhibit arrest at or before the pachytene stage (Kim *et al.* 2007). In our study, mice with a deletion of *Wt1* exhibited clear meiotic arrest and undifferentiated spermatogonia accumulation in the seminiferous tubules (Zheng *et al.* 2014). However, it remains to be defined how DMRT1 and WT1 regulate meiosis in a paracrine manner.

The results of studies carried out in Sertoli-*Ar* knockout mice clearly demonstrate that AR function in Sertoli cells is essential for the maintenance of fully competent Sertoli cell functions that support the completion of meiosis I during spermatogenesis. The next study will focus on which signaling events mediate AR function in spermatogenesis. Genetic screening of infertile males has revealed many AR mutations that are associated with male infertility. The restoration of spermatogenesis in *Ar*-knockout mice via alteration of candidate paracrine signals will be a powerful tool for demonstrating male infertility caused by AR mutation.

We expect that further investigation of Sertoli cell-specific genes and cell signaling in the regulation of self-renewal and spermatocyte meiosis in SSCs will yield useful insights into the treatment of male infertility and the development of safe and effective male contraception.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

Funding

Supported by the Major Research Plan '973' Project (2011CB944302 and 2012CB944702), the National Technology Support Project (2012DAI131B08), and the National Natural Science Foundation of China (31471352, 31471400, 31171380, 31071018, 31071271, and 81270662).

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Received 15 September 2014

First decision 20 October 2014

Revised manuscript received 25 November 2014

Accepted 11 December 2014