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Signaling molecules and pathways regulating the fate of spermatogonial stem cells

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Abstract

Spermatogenesis is the process that involves the division and differentiation of spermatogonial stem cells (SSCs) into mature spermatozoa. SSCs are a subpopulation of type A spermatogonia resting on the basement membrane in the mammalian testis. Self-renewal and differentiation of SSCs are the foundation of normal spermatogenesis, and thus a better understanding of molecular mechanisms and signaling pathways in the SSCs is of paramount importance for the regulation of spermatogenesis and may eventually lead to novel targets for male contraception as well as for gene therapy of male infertility and testicular cancer. Uncovering the molecular mechanisms is also of great interest to a better understanding of SSC aging and for developing novel therapeutic strategies for degenerative diseases in view of the recent work demonstrating the pluripotent potential of the SSC. Progress has recently been made in elucidating the signaling molecules and pathways that determine cell fate decisions of SSCs. In this review, we first address the morphological features, phenotypic characteristics, and the potential of SSCs. And then we focus on the recent advances in defining the key signaling molecules and crucial signaling pathways regulating self-renewal and differentiation of SSCs. The association of aberrant expression of signaling molecules and cascades with abnormal spermatogenesis and testicular cancer are also discussed. Finally we point out potential future directions to pursue in research on signaling pathways of SSCs.

Keywords

spermatogonial stem cells; signaling pathways; renewal; differentiation

INTRODUCTION

Spermatogenesis is a complex process by which the male germ-line stem cells (spermatogonial stem cells) self-renew and differentiate to produce sperm. Spermatogonial stem cells (SSCs) constitute one of the most important stem cell systems in the body, not only because they produce sperm which transmit genetic information across the generations, but because it has recently been demonstrated that SSCs from mouse and human testis can convert to embryonic stem-like (ES-like) cells thus acquiring pluripotency (Kanatsu-Shinohara et al., 2004a; Guan et al., 2006; Seandel et al., 2007; Conrad et al., 2008; Kanatsu-Shinohara et al., 2008; Kossack et al., 2008). Within the microenvironment (the niche) of the seminiferous tubules, SSCs can be directed to one of two cell fate decisions: they self-renew to produce new stem cells in order to maintain the stem cell pool or

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differentiate into more advanced germ cells. When removed from their niche, SSCs can be reprogrammed biochemically to pluripotency. In physiological conditions, the SSCs self-renew slowly, however, they can divide rapidly in response to damage such as chemicals or radiation (Dym and Clermont, 1970; Meistrich, 1986; Dym, 1994; de Rooij and Russell, 2000). The fate decisions of SSCs are mainly regulated by paracrine and potential autocrine molecules, which are produced by somatic cells and germ cells, respectively. A thorough understanding of the molecular mechanisms, particularly the signaling molecules and pathways, regulating self-renewal, differentiation, and the conversion of SSCs to pluripotent ES-like cells is essential for the regulation of spermatogenesis and has important implications in offering new therapeutic targets for male infertility and testicular cancers as well as for the conversion of SSCs to ES-like cells for patient-specific therapy.

This review mainly focuses on the key signaling molecules and pathways regulating self-renewal and differentiation of SSCs based on the work of our group and others. Aberrant expression of the signaling molecules and the intracellular signaling pathways may lead to male infertility and testicular cancer. As a result, the signaling molecules and cascades now known or yet to be identified would become novel attractive targets for male contraception as well as for the treatment of male infertility and testicular cancer. Also we present an overview of the unique characteristics and the potential of SSCs and underscore the important questions awaiting investigation in studies on signaling pathways regulating SSC fate decisions.

1. Morphological features, phenotypic characteristics, and potential of SSCs

The term “stem cell” was first introduced more than one century ago in the context of self-renewal to describe spermatogonia in the testis (Regaud, 1901), but the origin of the term “stem cell” may be tracked to 1868 to an eminent German biologist Ernst Haeckel who used “stammzelle” (stem cell in German) to describe the ancestor unicellular organism [referenced in (Ramalho-Santos and Willenbring, 2007)]. Stem cells, by definition, are primitive cells that have the capacity to both self-renew and differentiate into one or more cell lineages (Weissman, 2000). There are two main types of stem cells, namely embryonic stem (ES) cells and adult stem cells. While ES cells derived from the inner cell mass of a blastocyst are capable of producing all the cell types in the body, the availability of these stem cells is limited due to ethical issues. Adult stem cells reside in various tissues and they can self-renew to maintain the pool of stem cells and differentiate into mature cells with particular functions. One of the advantages of using adult stem cells is that there are no ethical issues compared to using ES cells, and most importantly, several adult stem cells also demonstrate multipotency or pluripotency and are able to differentiate into a variety of cells that potentially can be used for regenerative medicine, such as cell transplantation and tissue engineering of human diseases.

There are several subtypes of A spermatogonia in rodents, including the A-single (A_s), the A-paired (A_{pr}), the A-aligned (A_{al}), and the A_1 - A_4 spermatogonia based on differences in their morphology and phenotype. Although the identity of true stem cells in the testis is still a subject of controversy, the A_s spermatogonia are generally considered the SSCs in rodents (de Rooij, 1998; de Rooij and Grootegoed, 1998). It has recently been suggested that the A_s cells are 'actual' stem cells that can self-renew, whereas the A_{pr} and A_{al} cells are 'potential' stem cells that do not self-renew under normal physiological condition, but they could switch to become 'actual' stem cells in response to loss of 'actual' stem cells or an emptied stem cell niche, the microenvironment for SSCs (Nakagawa et al., 2007). SSCs can divide into either two new A_s cells or two type A_{paired} spermatogonium (A_{pr}) that then produces the aligned spermatogonia (A_{al}) (de Rooij and Grootegoed, 1998). The A_{al} spermatogonia, in turn, give rise to several generations of spermatogonia, including type A_1 - A_4 , intermediate, and type B spermatogonia (de Rooij and Grootegoed, 1998). Type B cells

produce spermatocytes, spermatids, and mature sperm, successively. In humans, Clermont first distinguished the A_{dark} and A_{pale} spermatogonia and speculated that the A_{dark} cells were reserve stem cells while the A_{pale} cells were renewing stem cells (Clermont, 1963; 1966b; a; 1972). Most researchers have adopted this classification for human as well as for monkey spermatogonia. Other classifications have been proposed for human testis (Ehmcke and Schlatt, 2006), but so far the functional identity of the true human SSCs remains an enigma. It is likely that the human SSCs are a subset of either the A_{dark} or the A_{pale} spermatogonia based upon our observations that the number of GPR125 cells (a potential marker for SSCs in the human) in each tubule cross section is lower than the number of either A_{dark} or A_{pale} spermatogonia (Dym et al., 2009).

SSCs can be identified by morphological features, phenotypic characteristics, and the functional SSC transplantation assay. As shown in Figure 1, the morphological features of mouse spermatogonia isolated from mouse testis using the STAPUT technique (Bellvé et al., 1977; Dym et al., 1995) include large spherical nuclei, a high nuclear/cytoplasm ratio, and few organelles in the perinuclear cytoplasm. Our group has identified three subtypes of type A spermatogonia, namely dark, transition, and pale spermatogonia, in the testis of immature mice. Dark spermatogonia, putative stem cells in the seminiferous tubules, display a homogeneously distributed chromatin, one or two nucleoli, and a characteristic round centrally located nuclear vacuole, as shown by light and electron microscopy (Figures 2A and B) (Dettin et al., 2003).

A great deal of information is available on the phenotypic identity of SSCs/progenitors in rodents (Dym, 1994; Beumer et al., 2000; Oatley and Brinster, 2006; Brinster, 2007). Markers for mouse SSCs and differentiating spermatogonia have been summarized in Table 1. It has been shown that $\alpha 6$ -integrin (CD49f), $\beta 1$ -integrin (CD29), and Thy1 (CD90) are surface markers for mouse SSCs (Shinohara et al., 1999; Kubota et al., 2003) and CD9 is a surface marker for mouse and rat SSCs (Kanatsu-Shinohara et al., 2004b). GFRA1 and RET are co-receptors for GDNF and markers for spermatogonial stem/progenitor cells (Buaas et al., 2004; Costoya et al., 2004; Buaas et al., 2005; Hofmann et al., 2005; Naughton et al., 2006). PLZF is characterized as a hallmark for spermatogonial stem/progenitor cells (Buaas et al., 2004; Costoya et al., 2004), whereas KIT is regarded as a marker for differentiating spermatogonia (Yoshinaga et al., 1991; Schrans-Stassen et al., 1999; Dolci et al., 2001). PCNA indicates proliferating spermatogonia (Schlatt and Weinbauer, 1994) and POU5F1 (Oct-4) is used as a marker for spermatogonial stem/progenitor cells (Ohbo et al., 2003; Ohmura et al., 2004; Hofmann et al., 2005). Recently, GPR125 (G protein-coupled receptor 125) (Seandel et al., 2007) and neurogenin3 (Yoshida et al., 2004; Yoshida et al., 2007) were demonstrated to be expressed in spermatogonia and their progenitors. STRA8 is a marker for spermatogonia including SSCs and differentiating spermatogonia (Giuli et al., 2002), while TNAP (tissue-nonspecific alkaline phosphatase) is expressed at a low level in SSCs but not in differentiating spermatogonia (Matzuk, 2004). However, even though there is a great deal of information regarding spermatogonial markers, there is still no unique biochemical marker available to distinguish SSCs from other progenitor spermatogonia. A combination of phenotypic markers (2 or more) may be used to identify the SSCs. We did a double staining, using antibodies to GFRA1 and POU5F1 (also known as Oct-4), showing that mouse SSCs co-express GFRA1 and POU5F1 (Figure 3) (He et al., 2007). Work on the expression of markers on primates including human spermatogonia and SSCs might help to identify the stem cell pool in human testis.

One study on monkey spermatogonia demonstrates the presence of a number of markers on spermatogonia that were similar to those present in rodents; these markers included PLZF and GFRA1 (Hermann et al., 2007). The frequency of PLZF in adult monkey testis was about 1.86 per tubule cross section (Hermann et al., 2007). The same group suggested that

the spermatogonial stem cell population in the monkey is a subset of either the A_{dark} and/or the A_{pale} spermatogonia. A recent study in the human identified the following four spermatogonial markers [CD49f ($\alpha 6$ -integrin), CD90 (Thy-1), CD133, GFRA1], that may also be SSC markers (Conrad et al., 2008).

The transplantation assay was first developed in 1994 by Brinster and colleagues who demonstrated that mouse germ cells (including SSCs) could be transplanted to infertile testes of nude mice and they are able to colonize the seminiferous tubules and generate spermatogenesis (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994; Ogawa et al., 2000). Rodent recipient models can be genetically infertile (e.g., W/W^v mutant mice) or become depleted of endogenous germ cells by using chemotoxic drugs such as busulfan. Regeneration of spermatogenesis can be achieved by xenotransplantation within and among rodent species (Clouthier et al., 1996) and in larger animal models (e.g., pig, goat) (Ogawa et al., 2000; Honaramooz et al., 2002a; Honaramooz et al., 2002b). In humans, Nagano and colleagues demonstrated the colonizing ability of baboon SSCs and human spermatogonia in the nude mouse (Nagano et al., 2001; Nagano et al., 2002). Recently this procedure was also expanded to the primate testis in monkey to nude mouse xenotransplantation (Hermann et al., 2007). Both in monkey and human, the transplanted cells resulted in colonization of the mouse testis confirming the function of SSCs. However, in primates full spermatogenesis was not achieved but the reasons for this are not clear.

SSCs were previously considered as unipotent stem cells since they were believed to only differentiate to produce sperm. However, this concept has now been challenged. Recent convincing evidence indicates that SSCs and/or spermatogonial progenitors from both mouse and human testis when detached from the niche and cultured in a defined medium can convert in vitro to become ES-like cells that are able to differentiate into the derivatives of three embryonic germ layers [reviewed in (de Rooij and Mizrak, 2008)] (Kanatsu-Shinohara et al., 2004a; Guan et al., 2006; Seandel et al., 2007; Conrad et al., 2008; Kanatsu-Shinohara et al., 2008; Kossack et al., 2008). It is worth noting that SSCs share certain phenotypic characteristics with ES cells, e.g. the cultured SSCs also express SRY-box-containing gene 2 (Sox2) (Oatley et al., 2006), POU5F1 (Oct-4) and alkaline phosphatase (Kubota et al., 2004a; b), markers for pluripotent ES cells, reflecting that SSCs may have pluripotent potential. The unlimited potential of SSCs has recently been confirmed by the findings that human spermatogonia and/or progenitors can acquire pluripotency to become ES-like cells that can differentiate into various lineages of the three germ layers (Conrad et al., 2008; Kossack et al., 2008). These observations suggest that SSCs have great potential for cell-based and autologous organ regeneration therapy for various human diseases without involving ethical issues and immunorejection.

2. Key signaling molecules in fate determination of SSCs

Self-renewal and differentiation of SSCs are tightly controlled by intrinsic and extrinsic factors. The known key signaling molecules and their physiological roles in regulating cell fate decisions of SSCs or spermatogonia are outlined in Table 2.

Glial cell line-derived neurotrophic factor (GDNF), produced by Sertoli cells, was the first identified molecule that regulates the self-renewal and differentiation of mouse SSCs in a dose-dependent manner via a paracrine pathway. In vivo, overexpression of GDNF leads to an accumulation of undifferentiated spermatogonia including SSCs, and conversely, ablation of GDNF by gene targeting results in the depletion of spermatogonia (Meng et al., 2000), suggesting that GDNF is essential for the self-renewal and maintenance of SSCs. In vitro, the addition of GDNF to cultured SSCs promotes their proliferation significantly over a short and long-period of time (over 2 years), further demonstrating that GDNF is a crucial

factor for self-renewal and maintenance of mouse SSCs (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004b; Hofmann et al., 2005).

GDNF signals via a receptor complex containing GFRA1 and RET, both of which are expressed in SSCs and probably in some A_{pr} cells (Hofmann et al., 2005; Naughton et al., 2006; He et al., 2007) and A_{al} spermatogonia (Tokuda et al., 2007). Loss of GDNF/GFRA1 signaling triggers differentiation of SSCs. Recently, we have demonstrated that the silencing of GFRA1 by RNA interference results in a switch from proliferation of mouse SSCs to differentiation into A₁-A₄ spermatogonia (He et al., 2007), an initial stage of spermatogenesis. Furthermore, both GFRA1 and RET receptors have been shown to be important for GDNF-mediated proliferation of SSCs (Kubota et al., 2004b; Naughton et al., 2006). Together, these studies suggest that both GDNF ligand and its receptors are required for self-renewal and maintenance of SSCs.

Promyelocyte leukemia zinc-finger factor (PLZF), co-expressed with POU5F1 in undifferentiated spermatogonia, is the first transcription factor shown to be required for the self-renewal of SSCs. Mutations in PLZF results in an intrinsic defect in SSC self-renewal (Buaas et al., 2004; Costoya et al., 2004). Intriguingly, PLZF can suppress the transcription of KIT (Filipponi et al., 2007), a marker for differentiating spermatogonia. However, it seems likely that GDNF doesn't act via PLZF (Oatley et al., 2006), and thus the mechanisms for the role of PLZF in SSC self-renewal need to be explored further. Another transcription factor Neurogenin3 (Ngn3) is expressed type A spermatogonia and their progenitors and may play a role in the differentiation of SSCs (Yoshida et al., 2004; Yoshida et al., 2007); however, the upstream and downstream events of Ngn3 signaling in SSCs are still unknown.

Ets-related molecule (ERM), a transcription factor produced by Sertoli cells but not male germ cells, is required for self-renewal of SSCs. Disruption of ERM results in spermatogonial depletion due to the failure of stem cell renewal (Chen et al., 2005). However, loss of ERM expression doesn't change the expression of GDNF, indicating that ERM doesn't signal through the GDNF pathway. Instead, GDNF has been demonstrated to regulate the expression of ERM in vitro (Oatley et al., 2007), suggesting that GDNF is an upstream molecule for ERM. Fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF), secreted by Sertoli cells, are important for proliferation of SSCs in vitro. (Kubota et al., 2004b). Other members of the FGF family including FGF2, FGF1, and FGF9, but not FGF7 or FGF10, induce the transcription of ERM (Chen et al., 2005). These findings suggest that certain FGF ligands may signal via ERM to regulate the fate decisions of SSCs in vitro, although this function needs to be confirmed in vivo. GDNF has been demonstrated to regulate the expression of transcription factors LIM homeobox 1 (Lhx1) and B cell CLL/lymphoma 6 member B (Bcl6b) in vitro (Oatley et al., 2006). The transplantation assay further reveals that Lhx1 and Bcl6b are essential for self-renewal of SSCs (Oatley et al., 2006). These observations suggest that GDNF signals via the activation of ERM, Lhx1, and Bcl6b to stimulate SSC renewal and indicate regulatory networks that control the fate determination of SSCs. While GDNF doesn't signal via PLZF (Oatley et al., 2006), it is clear that GDNF acts through Bcl6b, a member of the POZ (poxvirus and zinc finger) family of transcription factors that include PLZF (Oatley et al., 2006; Payne and Braun, 2006). TATA-binding protein-associated factor 4b (TAF4b) is also suggested to regulate the proliferation and specification of SSCs (Falender et al., 2005); however, GDNF doesn't regulate TAF4b expression (Oatley et al., 2006).

Stem cell factor (SCF, KIT ligand, steel factor) seems to play a distinct role in regulating the fate of mouse spermatogonia, specifically the type A₁ to A₄ (Yoshinaga et al., 1991). Other studies by Rossi et al. indicate that SCF stimulates proliferation of primary spermatogonia in culture (Rossi et al., 1993). More recently, our group demonstrated that SCF induces a

spermatogonial cell line to differentiate into meiotic spermatocytes and haploid round spermatids in the absence of Sertoli cells, as evidenced by the formation of synaptonemal complex and acrosome-like structure, respectively (Feng et al., 2002). This suggests that SCF is a key factor for the full differentiation of SSCs and gametogenesis in vitro. The transplant assay further confirms that SCF is required for the differentiation but not proliferation of SSCs. Transplantation of undifferentiated germ cells (KIT-negative spermatogonia) into seminiferous tubules of *steel factor (Sl)/Sld* mutant testes results in proliferation and colony formation that are unable to further differentiate. In contrast, re-transplantation of these undifferentiated KIT-negative spermatogonia into *Sl*-positive seminiferous tubules resumes their differentiation (Ohta et al., 2000). In vitro culture studies also reveal that SCF is necessary for proliferation of mouse KIT-positive type A spermatogonia, i.e., A₁-A₄ spermatogonia, the differentiating spermatogonia, whereas SCF is not required for the self-renewal and differentiation of KIT-negative undifferentiated type A spermatogonia, the potential SSCs (Tajima et al., 1994). Considered together, these studies indicate that SCF is essential for differentiation but not for renewal of SSCs.

Bone morphogenetic proteins (BMP) are members of the transforming growth factor beta (TGF β) superfamily. In *Drosophila*, Kawase et al. (Kawase et al., 2004) show that SSCs uses BMP signaling pathway to promote self-renewal and maintenance partly by repressing the expression of bag of marbles (bam), a gene that is necessary for stimulating differentiation of SSCs (Suzuki et al., 1998; Chen and McKearin, 2005; Szakmary et al., 2005). In mouse, one key member of the BMP family identified thus far to have an important role in regulating fate decisions of SSCs is BMP4. In vitro exposure of undifferentiated spermatogonia to BMP4 induces both their proliferation and differentiation, as shown by the increase of [³H] thymidine incorporation and KIT expression, respectively. Therefore, BMP4 appears to be one of the signaling molecules that are important for both proliferation and differentiation of SSCs, which may be due to the fact that SSCs can self-renew and progress to differentiate at the same time. However, BMP4 is not required for the survival of undifferentiated spermatogonia (Pellegrini et al., 2003).

3. Crucial signaling pathways in controlling self-renewal and differentiation of SSCs and differentiating spermatogonia

Signaling ligands use various signaling pathways to regulate self-renewal and differentiation of SSCs. The signaling pathways known to be activated by a number of ligands and the regulatory networks have been illustrated in Figure 4 and discussed in detail below.

3.1 Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway—

The JAK/STAT signaling pathway was first identified to stimulate self-renewal and maintenance of SSCs activated by the ligand Unpaired (Upd) in *Drosophila* testis. In the Upd-deficiency mutants, SSCs are depleted rapidly, whereas overexpression of Upd results in an accumulation of stem cell-like cells and block their differentiation (Kiger et al., 2001; Tulina and Matunis, 2001). Moreover, the blockage in JAK/STAT signaling by the mutation of JAK (the Hopscotch) or Stat92E leads to SSC loss, and conversely, the activation of JAK/STAT signaling by Upd results in self-renewal of SSCs (Kiger et al., 2001; Tulina and Matunis, 2001). Removal of JAK-STAT signaling causes germ line stem cells to differentiate into clusters of interconnected spermatogonial cysts (Brawley and Matunis, 2004). However, JAK2-dependent pathway appears not to be activated by SCF in differentiating spermatogonia (Dolci et al., 2001). In contrast, JAK/STAT signaling pathway may be activated by BMP signaling, but the machinery is still unknown.

3.2 Src signaling pathway—In neural cells, GDNF activates the Src family kinases in a RET-independent manner (Trupp et al., 1999). Our group has recently shown that the Src family kinases, including Src, Yes, Lyn, and Fyn, play important roles in GDNF-mediated proliferation of SSCs. GDNF activates Src family kinases, which further stimulates the phosphoinositide 3-kinase (PI3K)/Akt pathway and eventually up-regulates N-Myc expression and promotes proliferation of SSCs (Braydich-Stolle et al., 2007). Notably, in a subsequent study by Brinster's group using transplantation assay, the Src family pathway was confirmed to be essential for GDNF-regulated self-renewal of mouse SSCs (Oatley et al., 2007).

3.3 Phosphoinositide 3-kinase (PI3K)/Akt pathway—Phosphoinositide 3-kinase (PI3K) acts as an immediate downstream molecule of ligands and receptors to drive cells to proliferate (Klippel et al., 1998). Recently, it has been demonstrated that the PI3K/Akt pathway is related to GDNF-induced self-renewal of SSCs (Lee et al., 2007). Inhibition of PI3K by its specific inhibitor LY294002 blocks proliferation of SSCs in culture, and conversely, conditioned activation of Akt in SSCs by transfecting myr-Akt-Mer plasmid and in the presence of 4-hydroxy-tamoxifen stimulates their self-renewal and rescues apoptosis of SSCs due to the absence of GDNF. These observations implicate that the PI3K/Akt pathway is essential for self-renewal and survival of SSCs. Notably, the transplantation assay demonstrates that SSCs expressing myr-Akt-Mer have spermatogonial stem cell activity and undergo spermatogenesis to produce round spermatids, although complete spermiogenesis was not observed (Lee et al., 2007).

Interestingly, the PI3K/Akt pathway has also been implicated in regulating differentiating spermatogonia. In vitro studies by our group and others demonstrate that SCF promotes the proliferation of A₁-A₄ spermatogonia expressing KIT protein (Feng et al., 2000; Dolci et al., 2001). Furthermore, in vivo research confirms that the failure of binding of PI3K to KIT receptor diminishes activation of Akt, which leads to a decrease of proliferation and an increase of apoptosis of SSCs and eventually results in an arrest of spermatogenesis (Blume-Jensen et al., 2000; Kissel et al., 2000). Collectively, these studies reflect that different signaling molecules can act through the common PI3K/Akt pathway to mediate self-renewal, survival, and proliferation of SSCs and differentiating spermatogonia.

3.4 Ras/Erk1/2 signaling pathway—The small guanosine triphosphatase protein Ras, a key mediator for cell proliferation and differentiation (Wittinghofer, 1998; Perez-Sala and Rebollo, 1999; Yamamoto et al., 1999) and the extracellular signal-regulated kinases (ERK), an important member of the mitogen-activated protein kinases (MAPK), are involved in modulating a variety of cellular functions, including cell proliferation, differentiation, and cell cycle progression (Dolci et al., 2001; Yoon and Seger, 2006). Blocking the MAPK/ERK pathway by the MEK-specific inhibitor PD098059 results in a slight decrease in the proliferation of male germline stem cells from neonatal mice. We have recently demonstrated that GDNF signaling results in the activation of the Ras/ERK1/2 pathway in the SSCs. GDNF stimulates the phosphorylation of Ret tyrosine kinase and the docking protein Shc, the recruitment of adaptor protein Grb2, the rapid activation of the Ras/ERK1/2 pathway, the phosphorylation of transcription factors CREB/ATF family, the induction of the immediately early gene c-Fos transcription, and the enhancement of cyclin A and CDK2 expression. This leads to accelerate S-phase entry in the SSCs and promotes DNA synthesis and SSC proliferation (He et al., 2008). Interestingly, the Erk1/2 pathway can also be activated by SCF in KIT-expressing spermatogonia to stimulate their proliferation (Dolci et al., 2001). Thus, Erk1/2 pathway can be activated by different ligands to regulate the proliferation of SSCs and their progeny.

3.5 Smad signaling pathways—The Smad signaling pathway has been implicated in the maintenance and pluripotency of human ES cells (Besser, 2004; James et al., 2005). However, mouse and likely human ES cells are distinct from tissue stem cells including SSCs in their proliferation and differentiation pattern, and the self-renewing and differentiating mechanisms between these two cell types may also be different (Oatley et al., 2006).

In vitro studies show that BMP4 stimulation of mouse spermatogonia induces a rapid nuclear translocation of Smad4 and Smad5 to form a Smad4/5 complex (Pellegrini et al., 2003). One downstream event of the Smad pathway by BMP4 is that the transcriptional coactivators CBP and p300 form an active complex with Smad4/5 to promote the differentiation of spermatogonia. In a co-culture system of immature mouse spermatogonia and Sertoli cells, BMP2 and BMP4 stimulate the expression of Smad1, Smad5, and Smad8 in the nucleus of spermatogonia (Itman and Loveland, 2008). Recently, we have demonstrated that Nodal regulates self-renewal of mouse SSCs through Smad2/3 activation via an autocrine signaling pathway. We found that both Nodal and its receptors are present in SSCs, but not in Sertoli cells or differentiated germ cells. Nodal promotes self-renewal of SSCs and activates Smad2/3 phosphorylation, Pou5f1 transcription, cyclin D1, and cyclin E, but not cyclin A expression (unpublished data). Considered together, these studies indicate that different ligands use distinct Smad pathways to regulate self-renewal and differentiation of SSCs. Table 3 outlines the crucial signaling pathways known to regulate cell fate decisions of SSCs and differentiating spermatogonia.

4. The association of aberrant expression of signaling molecules and cascades with abnormal spermatogenesis and testicular cancer

Spermatogenesis is mainly regulated by extrinsic and intrinsic signaling factors. Abnormal expression of signaling molecules and the disruption of signaling cascades have been demonstrated to result in disrupted spermatogenesis. Genetic studies show that abnormality of GDNF expression blocks spermatogenic development and results in successive germ cell depletion (Meng et al., 2000). BMP4 deficient male C57BL/6 mice have compromised fertility owing to degeneration of germ cells, reduced sperm counts, and decreased sperm motility (Hu et al., 2004). FGFR1 signaling cascade has also been implicated in normal spermiogenesis and male fertility, and disrupted FGFR1 signaling results in a remarkable reduction in daily production of sperm with functionally compromised capacitation (Cotton et al., 2006). Studies with the PI3K pathway indicate that KIT-induced activation of the PI3K pathway is essential for male fertility since mutant males with disruption of PI3K binding to KIT are sterile due to a blockage of both the proliferation of spermatogonia and early stages of spermatogenesis (Blume-Jensen et al., 2000; Kissel et al., 2000). Together, these results illustrate the close association of signaling molecules and cascades with male infertility.

The incidence of malignant tumors including testicular cancer is on the rise across the world (Huyghe et al., 2003; Walschaerts et al., 2008). Aberrant expression or disruption of the signaling molecules and intracellular signaling pathways may result in an enhanced risk of testicular cancer and other tumors. For example, mice overexpressing GDNF develop malignant testicular cancer that resembles classic seminoma in humans (Meng et al., 2001). Transcription factor Bcl6b is a target gene of GDNF and it is important for the self-renewal of SSCs (Oatley et al., 2006). Bcl6b is the mouse orthologue of human BCL6, which is involved in chromosomal translocations associated with non-Hodgkin's lymphoma (Baron et al., 1993). The PI3K/Akt signaling pathway is also involved in testicular germ cell tumors (TGCT) (McIntyre et al., 2008), and importantly, SCF has recently been suggested to be a valuable diagnostic marker for TGCT (Stoop et al., 2008). The development of new

inhibitors, such as antibodies or small molecules (e.g. small interference RNAs), to inhibit these signaling pathways could be of great practical value in male contraception and against testicular cancer.

5. Potential future directions to pursue in signaling pathway research of SSCs

In summary, there are a number of signaling molecules and multiple signaling pathways identified to determine cell fate decisions of SSCs. Knowledge of these signaling molecules and pathways is crucial not only for a better understanding of how SSC functions are controlled but also for the application of SSCs in regenerative medicine and cancer therapy in the future. However, the research on the signaling pathways controlling the fate decisions of SSCs needs to be explored further.

There are many interesting and important aspects of signaling pathway studies in SSCs that warrant further investigation. The possible future directions of particular importance include: 1) further definition of novel signaling molecules and pathways that are important for the self-renewal and differentiation of mouse SSCs. This may eventually lead to the development of new methods for in vitro generation of functional sperm. There are a number of interesting molecules whose roles need to be elucidated, e.g., notch signaling pathway, other members of the TGF β superfamily signaling pathways, and Wnt signaling pathways. Notch ligand Delta 1 seems to be an autocrine molecule because it is expressed in spermatogonia rather than in Sertoli cells, while Notch receptors 1, 2, and 3 are also expressed in spermatogonia (Dirami et al., 2001). However, the role of Delta 1 in regulating the fate of SSCs and Delta 1/Notch signaling pathways are still unclear; 2) the cross-talks between multiple signal transduction pathways and how these signaling pathways integrate to control SSC self-renewal and differentiation; 3) it is imperative to unravel the endogenous signaling molecules within SSCs that signal via autocrine pathways. This would be helpful to modulate spermatogenesis in vitro when SSCs are cultured in the absence of Sertoli cells and makes it possible for the endogenous stem cells in the testis to be therapeutic targets for male infertility and testis cancer; 4) the signaling pathways regulating human SSC self-renewal and differentiation are still unknown due to ethical issue and logistical difficulties in obtaining sufficient human testis for research purposes. There may be certain differences in SSC regulation machinery between humans and rodents since the number of spermatogonial divisions, the spermatogonial subtypes, and the time required for a type A to form a fully differentiated spermatid are distinct between rodents and man. Studies on SSCs in rodents and *Drosophila* have provided and will continue to offer guiding principles for understanding the machinery governing cell fate decisions of human SSCs; however, it is necessary to explore whether these signaling pathways are applicable to human SSCs; 5) the signaling pathways underlying the conversion of SSCs into pluripotent ES-like cells and the differentiation of ES-like cells derived from SSCs into the committed cell lineages of three embryonic germ layers need to be elucidated. Overall, such studies mentioned above would provide a more thorough understanding of stem cell regulation in the testis and spermatogenesis.

CONCLUSION

From the morphological features, phenotypic characteristics, and signaling molecules and pathways regulating SSC fate decisions, we have obtained greater insights into the SSC biology. The advances made in the signaling molecules and pathways of SSCs would be helpful to a better understanding of the complex process of spermatogenesis, the etiology of male infertility and testicular cancer. However there are still many more new research directions to pursue in signaling pathways regulating the fate determination of SSCs. In particular, all the observations on the function of signaling molecules and signal transduction pathways have been obtained from mice or *Drosophila*, and thus it is necessary

to confirm whether these results are applicable to other mammalian species, especially humans. A major impediment to research on the SSC in rodents is that we still do not have a specific marker that can be used to isolate pure populations of SSCs. In addition almost no information is available on the identity of markers that may be used to isolate SSCs in man and then examine their signaling mechanisms. It is no doubt that the studies on signaling pathways of SSCs from both rodents and humans would eventually lead to cell-based and gene therapy for male infertility, testicular cancer, and various human diseases.

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Figure 1. Morphological features of spermatogonia isolated from immature mice
Phase-contrast microscopy shows the morphology of isolated spermatogonia following the STAPUT procedure.

Figure 2. Morphological features of the A_{dark} spermatogonia

(A) Light microscopy reveals the morphology of A_{dark} spermatogonia (asterisks) from immature mice. The arrows and arrowheads delineate other spermatogonia. Sertoli cell (SC). (B) Electron microscopy shows the ultrastructure of the characteristic vacuole (V) in the nucleolus (n) of an A_{dark} spermatogonia with mitochondria (m) in the cytoplasm and few other organelles. Heterochromatin clump (h). Scale bar in A = 10 μm and bar in B = 1 μm . Figure 2 is adapted from Dettin et al. (2003).

Figure 3. Phenotypic characteristics of SSCs isolated from immature mice

Immunocytochemistry shows that a subpopulation of type A spermatogonia co-express GFRA1 and POU5F1 (arrowheads). Some type A cells were negative for both GFRA1 and POU5F1 (arrows). Scale bar = 10 μ m. Figure 3 is adapted from He et al. (2007).

Figure 4. Crucial signaling pathways activated by various ligands and the regulatory networks regulating cell fate decisions of SSCs and differentiating spermatogonia

GDNF and SCF activate both the Ras/Erk and PI3K/Akt pathways, whereas SCF cannot signal via JAK/STAT pathway. GDNF can activate Bcl6b, Lhx1, and ERM, but not PLZF or TAF4b. BMP4 signaling via Smad4/5 pathway and Upd (Unpaired) signaling through JAK/STAT pathway are also shown. Arrows indicated “activation”, whereas the blocked arrows denote “inactivation”.

Table 1

Markers for mouse SSCs and differentiating spermatogonia (Spga)

Marker	SSCs	Differentiating Spga	Representative References
α 6-integrin (CD49f)	+	-	(Shinohara et al., 1999)
β 1-integrin(CD29)	+	-	(Shinohara et al., 1999)
CD9	+	-	(Kanatsu-Shinohara et al., 2004b)
GFRA1	+	-	(Meng et al., 2000)
GPR125	+	+	(Seandel et al., 2007)
KIT	-	+	(Yoshinaga et al., 1991)
Neurogenin3	+	+	(Yoshida et al., 2004)
PCNA	+	+	(Schlatt and Weinbauer, 1994)
PLZF	+	-	(Buaas et al., 2004)
POU5F1	+	-	(Ohbo et al., 2003)
RET	+	-	(Naughton et al., 2006)
STRA8	+	+	(Giuli et al., 2002)
Thy-1 (CD90)	+	-	(Kubota et al., 2003)
TNAP	+	-	(Matzuk, 2004)

Markers known to be expressed (+) or absent (-) in mouse SSCs and differentiating spermatogonia are listed. Differentiating spermatogonia include type A1-A4, Intermediate, and type B spermatogonia. PCNA, proliferating cell nuclear antigen; POU5F1, also known as Oct-4; TNAP, tissue-nonspecific alkaline phosphatase.

Table 2

Signaling molecules regulating spermatogonial cell fate

Molecule	Physiological role	References
Bcl6b	Self renewal of mouse SSCs	(Kubota et al., 2004b)
BMP4	Proliferation/differentiation of mouse Spga	(Pellegrini et al., 2003)
EGF	Proliferation of mouse SSCs	(Kubota et al., 2004b)
ERM	Renewal of mouse Spga	(Chen et al., 2005)
FGF2	Proliferation of mouse SSCs	(Kubota et al., 2004b)
GDNF	Renewal of mouse SSCs	(Meng et al., 2000)
Neurogenin3	Differentiation of mouse SSCs	(Yoshida et al., 2004)
PLZF	Renewal of mouse SSCs	(Buaas et al., 2004) (Costoya et al., 2004)
SCF	Differentiation of mouse SSCs	(Feng et al., 2002)
	Proliferation of differentiating Spga	(Rossi et al., 1993)
TAF4b	Proliferation of mouse SSCs	(Falender et al., 2005)
M-CSF	Proliferation of mouse SSCs	(Kokkinaki et al., 2008)
Unpaired	Renewal of <i>Drosophila</i> SSCs	(Kiger et al., 2001)
Nodal	Renewal of mouse SSCs	(He et al., 2009)
Jagged/Delta	Differentiation of mouse Spga	(Braydich-Stolle et al., 2005) (Dirami et al., 2001)

Table 3

Signaling pathways regulating spermatogonial cell fate

Signaling pathways	Physiological role	References
JAK/STAT	Renewal of <i>Drosophila</i> SSCs	(Kiger et al., 2001)
PI3K/Akt	Renewal of mouse SSCs	(Lee et al., 2007)
	Proliferation of A ₁ to A ₄ Spga	(Feng et al., 2000)
Ras/Erk1/2	Renewal of mouse SSCs	(He et al., 2008)
	Proliferation of A ₁ to A ₄ Spga	(Dolci et al., 2001)
Smad 4/5	Differentiation of mouse Spga	(Pellegrini et al., 2003)
Src	Renewal of mouse SSCs	(Oatley et al., 2007)
Smad 2/3	Renewal of mouse SSCs	(He et al., 2009)