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INVITED REVIEW

Germ Cell Biology

Extrinsic and intrinsic factors controlling spermatogonial stem cell self-renewal and differentiation

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Spermatogonial stem cells (SSCs), the stem cells responsible for male fertility, are one of a small number of cells with the abilities of both self-renewal and generation of large numbers of haploid cells. Technology improvements, most importantly, transplantation assays and *in vitro* culture systems have greatly expanded our understanding of SSC self-renewal and differentiation. Many important molecules crucial for the balance between self-renewal and differentiation have been recently identified although the exact mechanism(s) remain largely undefined. In this review, we give a brief introduction to SSCs, and then focus on extrinsic and intrinsic factors controlling SSCs self-renewal and differentiation.

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INTRODUCTION

Germline stem cells play crucial roles in transmitting genetic information to subsequent generations.[1–4] In rodents, spermatogonial stem cells (SSCs) comprise a subpopulation of undifferentiated spermatogonia derived from gonocytes approximately 6 days after birth, which in turn are derived from primordial germ cells during embryonic development.[5,6] The existence of SSCs has long been proposed; however, lacking of appropriate technology to define SSCs greatly retards its progress. Traditional study of spermatogonia is morphology dependent, making it tough to study *in vitro*.[5] In 1994, Brinster and Avarbock[7] developed transplantation technique that firstly allows us to identify SSCs functionally, and this technique has still been the golden standard to confirm SSCs. Briefly, dissociated donor-derived testicular cells are transplanted into the efferent duct of the sterile recipient mice, and offspring is produced with the donor haplotype. This technique is of fundamental importance, for it firstly allows us to identify SSCs and even to count the number of SSCs in the seminiferous tubule.[8] Another progress is the development of long-term culture system for mouse SSCs (DBA/2 background) in 2003 by Kanatsu-Shinohara *et al.*[9] In the presence of glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2), leukemia inhibitory factor (LIF) and epidermal growth factor (EGF), germ cells from neonatal mouse termed germline stem (GS) cells are able to proliferate in clusters *in vitro* for a long time. And transplantation assay confirms greatly increased GS cells number in this system. *In vitro* culture system allows us to obtain large numbers of cells for basic study, such as signal transducing and regulation mechanism.[10] These two technologies combined with other methods, such as gene-modified model, have greatly facilitated SSCs study.

SPERMATOGONIAL STEM CELL IDENTIFICATION AND CHARACTERIZATION

As noted above, SSCs comprise a sub-fraction of undifferentiated spermatogonial cells.[5] In rodents, there are three types of undifferentiated type A spermatogonial cells, classified according to cell morphological arrangement: A_s (single), A_{pr} (two paired cells), and A_{al} (aligned cells of 4, 8, 16 and sometimes even 32 cells). The traditional model considers A_s cells as SSCs while A_{pr} and A_{al} cells are progenitors committed to proliferate and eventually produce haploid cells. A_s cells will either undergo division, producing two daughter A_s cells, or division, producing A_{pr} cells connected by a cytoplasmic bridge that with further division produce connected A_{al} cells of 4, 8, 16 and then eventually haploid cells.[6] However, this classical model has been challenged in recent years.[11] Firstly, not all A_s cells are actual SSCs and transplantation assays show that only 10% of total A_s cells are able to populate the recipient testis, indicating that only a small number of A_s are actual stem cells.[10] Additionally, A_{pr} and A_{al} cells are not always transit-amplifying cells. In fact, they may act as colony-forming cells during tissue regeneration or even in normal situations, and have been proposed as potential stem cells when compared with actual stem cells. Through a tamoxifen-inducible pulse-label system, Nakagawa *et al.* proved that the number of colonogenic cells are more abundant than the actual stem cells after transplantation or regeneration and they determined that the transit-amplifying is the main contributor to the extra colony-forming cells.[12] Furthermore, cells expressing *c-Kit*, the

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major signal marking spermatogonial differentiation, which can be detected in A_{pr}, are also able to form colonies after transplantation, albeit with much lower abilities, demonstrating the strong regenerative ability among the undifferentiated spermatogonial cells.[13] In 2010, Nakagawa *et al.*[14] proposed an extended model, whereby "stemness" is present at all stages of undifferentiated spermatogonia, but the potential decreases with progression to later stages. Overall, these experiments largely demonstrate stemness among the undifferentiated type A spermatogonia, not the previous thought A_s. Moreover, lineage tracing and topology feature experiments have revealed heterogeneity among SSCs.[15,16] Fragmentation of A_{al} and A_{pr} cells occurs infrequently, but can reverse molecular expression changes during normal rounds of spermatogenesis and further increase study complexity.[17,18]

Another surprising characteristic of SSCs is the ability to convert into pluripotent cells without gene manipulation under certain culture conditions. This was first reported by Kanatsu-Shinohara *et al.*[19] who found that cultured SSCs from neonatal mice occasionally convert into embryonic stem cell-like cells (ES-LCs) with similar phenotypes and the ability to form teratomas in all three embryonic germ layers after subcutaneous injection, thereby confirming functional pluripotency. However, the ES-LCs were only able to be produced from young mice, raising the possibility that SSCs lose their ability to convert into pluripotent cells as they mature. Nevertheless, soon after, several groups successfully generated ES-LCs from cultured SSCs of both young and adult mice using different models and conditions, further confirming that SSC can convert into pluripotent cells in certain culture conditions.[20–22] Although the pluripotent cells of different sources show different behavior after transplantation, some generate teratomas while others contribute to germline chimeras following blastocyst injection.[21] This is possibly due to culture of different SSC subpopulations using different isolation methods. The exact mechanism that SSC can convert into pluripotent cells remains unknown, but it is likely to involve the expression of *Oct4*, *Sox 2*, *c-Myc*, and *Klf4*, core transcription factors for induction of iPSs.[23] *Nanog*, another core pluripotent transcription factor, is not detected in SSCs and may be the last barrier to pluripotency. It is feasible that *Nanog* is relatively easily activated under certain conditions (e.g., the expression of *Sox2* and *Oct4*, since it's well known that *Nanog* expression can be activated by *Oct4* and *Sox2*), and combined with other pluripotent factors, pluripotent cell behavior is induced.[24] Intriguingly, the ability to convert into pluripotent cells was also seemed possible in human SSCs.[25–27]

Spermatogonial stem cells are difficult to distinguish morphologically among undifferentiated spermatogonial cells, and until now, no specific marker molecules have been identified.[28] SSC rarity (0.002% of total testis cells),[29] determined by transplantation assay, hinders progress of SSC mechanistic studies.[10] Encouragingly, a large number of genes related to SSC regulation have recently been identified. Surface markers, such as β1-integrin, α6-integrin, EPCAM, GFRA1, RET, and GPR125, are of relevance to SSCs and many are chosen as antigens to enrich SSCs by fluorescence-activated cell sorting or magnetic-activated cell sorting.[22,30–34] Many transcription factors crucial for SSC self-renewal, maintenance, and differentiation have also been found (**Table 1**).

REGULATION OF SPERMATOGONIAL STEM CELL SELF-RENEWAL AND DIFFERENTIATION

In male mammals, self-renewal and differentiation properties of SSCs

Table 1: Important transcription factors for SSCs

Molecule	Experimental proof	KO henotype	Possible function on SSCs	Expression			References
				A_s	A_{pr}	A_{al}	
PLZF	Mu, KO, WHI, CI	Infertility Progressive germ cell loss	Maintenance	++	++	++	55,56
Etv5	KO, CI	Infertility Progressive germ cell loss	Self-renewal	++	++	++	41,48
Bcl6b	KO, CI	Progressive Germ cell loss	Self-renewal	++	++	++	41,47
Lhx1	CI	Lethal	Self-renewal	++	++	++	36,47
ID4	KO, TG, WHI, CI	Young fertile Progressive germ cell loss	Self-renewal	++	––	––	53
Nanos2	KO, TG, WHI, LT, OE	Infertile Early PGC loss	Self-renewal	++	++	+	54,100,101
Nanos3	KO, WHI, TG, WHI	Infertile Late PGC loss	Maintenance	+	++	++	100–102
Ngn3	LT, TR, WHI	Not tested	Differentiation	+	++	++	15,49
FOXO1	CKO	Infertile Progressive germ cell loss	Self-renewal Differentiation	++	++	++	62
DMRT1	CKO, CC	Young fertile Premature initiation of meiosis	Differentiation	++	++	++	93
Taf4b	KO, TR	Young fertile Progressive germ cell loss	Self-renewal	++	++	++	61
OCT6	KO, CI	Lethal	Maintenance	++	++	++	103
Oct4	CI	Lethal	Pluripotent Maintenance	++	++	++	65,104,105
Sohlh1/2	KO, CC, WHI	Infertile Accumulated spermatogonial with few spermatocytes	Differentiation	+	++	++	89–91
Stra8	WHI, TG, TR, IHC	Not tested	Differentiation	++	++	++	106,107
SOX3	KO, IHC	Infertile Spermatogenesis defect	Differentiation	+	++	++	108
STAT3	CI	Not tested	Differentiation	+	++	++	95

SSCs: spermatogonial stem cells; Mu: mutant mice; KO: knockout mice; WHI: whole mount immunostaining; TR: transplantation assay; CI: culture interference; OE: overexpression; LT: lineage tracing; TG: transgene; CC: chip-chip; CKO: conditional knockout mice; IHC: immunohistochemistry; PGC: primordial germ cell; "+" and "++" mean relative expression change of each particular gene

lay the foundations for continual genesis of mature spermatozoa, and this process requires coordinated and balanced gene expression of both extrinsic and intrinsic factors. In this section, we discuss recent findings regarding the intrinsic and extrinsic factors regulating the SSC fates of self-renewal and differentiation (major in the mouse model).

Self-renewal and maintenance

Spermatogonial stem cell self-renewal provides the basis for spermatogenesis. SSCs with self-renewal problems lead to defects in their maintenance, with few advanced germ cells and occasional fertility in early days, but definite infertility later coupled with severe testicular atrophy. Genetically modified or mutant mice provide a powerful tool for identifying the molecules underlying regulation of SSC status. Here, we introduce in detail recent findings regarding SSC self-renewal.

Extrinsic factors

Of the extrinsic factors, GDNF, a molecule secreted by Sertoli cells in seminiferous tubules, was first identified with a crucial role in both *in vivo* development and *in vitro* culture. Meng *et al.* were the first to find that GDNF regulates undifferentiated spermatogonia in a dosage-dependent manner, specifically, gene-targeted mice with decreased expression of GDNF show age-dependent germ cell loss while mice overexpressing GDNF exhibit accumulation of undifferentiated spermatogonia.[35] Based on these extraordinary findings, a long-term SSC culture system with the addition of GDNF, FGF2, EGF and LIF was successfully developed from neonatal mice.[9] In this system, cultured cells termed GS cells proliferate for more than 2 years as confirmed by transplantation assay, implicating a critical role for GDNF signaling in SSC self-renewal. GDNF acts through a multicomponent receptor complex consisting of GFRA1 and RET, and depletion of both receptors results in the same phenotype as GDNF ablation, with SSCs self-renewal defect and germ cell depletion.[36] Using combined *in vitro* culture and transplantation assays, several groups have presented evidence for activation of phosphoinositide 3-kinase (PI3K)-AKT by GDNF.[37–39] Significant phosphorylation in ser-476 is detected 20 min after adding GDNF to culture medium following overnight starvation, while preincubation with a PI3K chemical inhibitor prevents this activation. Moreover, when AKT inhibitor IV is added to the medium, single cells, and rarely clusters are observed, indicating that AKT plays a central role in both self-renewal and survival.[38] GDNF also activates Src family kinase (SFK) signaling, promoting self-renewal partly through AKT signaling. Incorporation of the SFK chemical inhibitor, SU6656, into culture medium results in slower cluster formation compared with controls. AKT ser476 phosphorylation is also downregulated. Compared with the SFK pathway, AKT appears to be dominant molecular for proliferation as AKT inhibitor completely prevents SSCs from proliferating while SFK inhibitor partly impedes proliferation. Intriguingly, He *et al.* found that the RAS/ERK1/2 pathway is also involved in GDNF-induced self-renewal and proliferation, via upregulation of CREB/activating transcription factor 1 family member phosphorylation and c-FOS transcription factor.[40] Overall, GDNF promotes SSC self-renewal and proliferation through multiple signaling pathways, making the process complex.

Fibroblast growth factor 2 is another indispensable extrinsic factor in GS cell culture. Both GDNF and FGF2 activate MAP2K1, but FGF2 is a stronger stimulator, and an activated form of MAP2K1 can substitute for FGF2, confirming that FGF2 promotes proliferation through MAP2K1 pathways. Three transcription factors (*Bcl6b*, *Etv5*, and *Lhx 1*) act downstream of FGF2 pathways as they are downregulated following MAP2K1 inactivation.[41] However, in a previous study, these three factors were shown to act downstream of GDNF through SFK signaling, suggesting that GDNF and FGF2 signaling may be partially redundant.[38] In another study, they found that MEK (mitogen-activated protein kinase/ERK1 kinase), whose activation is a key signal for cell cycle progression, seems to be the downstream of FGF2. Inhibitor of MEK markedly suppresses GS cells proliferation, while GS cells with activated MEK successfully proliferate only with GDNF, indicating that MEK can be a substitution for FGF2.[41] Intriguingly, Shinohara lab found that GS cells with activated H-RAS proliferate without extra cytokine additions, while GS cells with transduction of K-RAS, another activated form of RAS, which activates AKT pathway more strongly compared to H-RAS, fail to proliferate, indicating the importance of balanced pathway between AKT and MEK.[42]

In addition to these essential factors, others such as CSF1 and WNT5A, produced by Leydig and Sertoli cells, respectively, are essential constituents of the SSC niche and may act as enhancers for SSC self-renewal. Microarray transcript profiling, performed by Oatley *et al.* found approximately 200 genes expressing 10-fold or higher in THY1^{+} cells compared with THY1^{-} depleted testis cells, including colony stimulating factor 1 receptor (*Csf1r*). *Csf1r* expression is highly enriched in a subpopulation of cultured THY1^{+} germ cells, implying a potential role for CSF1 in undifferentiated spermatogonial cells. Adding recombinant soluble CSF1 to chemically defined and serum-free culture medium caused a significant increase in SSC number *in vitro* without total THY1^{+} germ cell expansion, confirmed by functional transplantation assay.[43] Thus, it is reasonable to speculate that CSF1 promotes SSC propagation only, and not the entire population of undifferentiated spermatogonial cells, although the mechanism remains unknown. *Wnt5a* expression is confined to Sertoli cells in mouse testis with all WNT5A receptors present on the surface of undifferentiated spermatogonia, and since β-catenin-dependent Wnt signaling promotes self-renewal of various stem cell types, it's reasonable that this signal is involved in SSCs self-renewal. Although transgenic reporter mice showed that β-catenin-dependent signaling was not active in SSCs *in vitro* and most spermatogonia *in vivo*, WNT5A antagonist significantly reduced SSC colonies *in vitro*, while exogenous WNT5A enhanced SSC colony formation in a β-catenin-independent manner, indicating a potential role for WNT5A in enhancing SSCs self-renewal.[44] Our study showed that short-type PB-cadherin promoted self-renewal of SSCs via activating Janus kinase/signal transducer and activator of transcription (STAT) and PI3K/AKT, and blocking transforming growth factor-beta1 signaling.[45] Morimoto *et al.*[46] reported that reactive oxygen species are required for mouse SSC self-renewal.

Intrinsic factors

According to the response to GDNF, there are two categories of transcription factors: GDNF-inducible and-independent. Microarray analysis identified six transcripts (*Bcl6b*, *Etv5*, *Lhx 1*, *Egr2*/3, and *Tspan8*) that were first downregulated with GDNF removal from culture medium, and then significantly up-regulated with addition to the medium. Culture intervention by siRNA and knockout experiments implicated *Bcl6b* as an important maintenance factor.[47] *Etv5* ablation in mice causes infertility, although the first wave of spermatogenesis is not impaired, the following waves are severely impaired.[48] The first wave of spermatogenesis is a distinct process independent of SSC self-renewal while subsequent spermatogenesis relies on SSC amplification.[49] SiRNA-mediated ablation of *Etv5* in THY1^{+} cultured spermatogonial cells results in reduced SSC numbers, as determined by functional

assay.[38] Microarray analysis shows that RNAi depletion of *Etv5* leads to downregulation of *Bcl6b*, *Lhx 1*, and Brachyury, all important molecules for SSC proliferation.[50] Interestingly, Chen *et al*. found that *Etv5* in Sertoli cells promotes many adhesion molecules (e.g. CXCL-12, CXCR4, and CCL9), some are known for regulating the SSCs niche and indicative of a role for *Etv5* in niche formation.[48,51,52] ID4, an inhibitor of DNA binding protein 4, is another important transcription factor induced by GDNF. ID4 is unique in that it is exclusively detected in A_s spermatogonia. ID4 knockout mice exhibit age-dependent germ cell depletion, suggesting a role in SSC maintenance.[53] *Nanos2*, a zinc-finger RNA-binding protein, plays an important role in SSC maintenance. *Nanos2* knockout mice display progressive germ cell loss, while *Nanos2* overexpression in mice causes accumulation of promyelocytic leukemia zinc finger ($PLZF^+$) spermatogonia.[54] Although many downstream factors of GDNF signaling have emerged, the underlying gene networks remain largely undefined and require further investigation.

Spermatogonial stem cell maintenance can be regulated independent of GDNF, and in this regards, several nuclear factors have been identified. PLZF was first described as an important maintenance factor specifically expressed in undifferentiated spermatogonia. A nonsense mutation in *Zbtb16*, the gene encoding PLZF, causes infertility and progressive germ cell loss in mice,[55] and appears to promote SSC proliferation, not survival, as no significant apoptosis is detected. Additionally, SSCs from PLZF-null mice cannot repopulate the testis when transplanted into germ cell deficient mice,[56] but can be cultured in medium with excessive GDNF, indicating PLZF and GDNF work independently. However, the exact mechanism by which PLZF maintains the SSC pool is not clear. Filipponi *et al*. found that PLZF binds to the *c-Kit* promoter region and thus directly represses the expression of *c-Kit*, a marker for spermatogonia differentiation.[57] Moreover, Hobbs *et al*. showed that SALL4 antagonizes PLZF, and increasing SALL4 expression leads to *c-Kit* transcription.[58] Relative levels and mutual effects of SALL4 and PLZF determine SSC status. Another transcription factor directly affected by PLZF is Redd1, an inhibitor of mammalian target of rapamycin complex 1 (mTORC1). In stem cells, mTORC1 hyperactivity leads to an abnormal translation of downstream targets and eventually results in cell exhaustion.[59,60] Additionally, activated mTORC1 in SSCs represses expression of the GDNF receptor, RET. Another important GDNF-independent transcription factor, *Taf4b*, although not exclusively expressed in testis, is detected in both spermatogonia and Sertoli cells, and shows an essential role in SSC maintenance, with *Taf4b* loss in mice inducing age-dependent germ cell loss.[61] Transplantation of normal SSCs into *Taf4b*-depleted testis induces normal and sustained spermatogenesis, suggesting that *Taf4b* behaves in a cell-autonomous manner. FOXO1 has also been shown to play an indispensable role in SSC maintenance, and may be at the upper levels of GDNF-independent transcription regulation. FOXO1 knockout mice exhibit similar defects of SSC maintenance to the previous maintenance factors (e.g., PLZF, *Taf4b*, *Etv5*) with progressive age-dependent decline in spermatogenesis, however microarray analysis revealed decreasing expression of SSC maintenance genes (e.g., *Lhx 1*, *c-Ret*) and increasing expression of SSC differentiating genes (e.g., *Egr2*, *Tex 19*).[62] Furthermore, in gonocytes, FOXO1 is located within the cytoplasm but resides in nuclei of spermatogonia, indicating a change in subcellular location during gonocyte transition to SSCs. Previous studies using transgenic models show that this shift is controlled by AKT signaling.[63,64] Conditional knockout of *Pten* or *Pdk1* (repressor or stimulator of AKT activity, respectively) both result in germ cell deficiency in mice. Conditional *Pten* ablation causes AKT hyperactivity, FOXO1 phosphorylation, and subsequent cytoplasmic localization of FOXO1 in spermatogonia, while *Pdk1* conditional knockout shows FOXO1 nuclear preference in gonocytes rather than spermatogonia. This indicates that AKT activity is important to normal FOXO1 function.[62] Other GDNF-independent transcription factors, *Oct4* for instance, though much less well understood, they also play an important role in SSC self-renewal.[65]

Differentiation

In this section, we discuss the extrinsic and intrinsic factors contributing to SSC differentiation.[5] Two types of differentiation are involved here. One is SSC conversion to progenitors or transit amplifying cells, and the other is generation of differentiated spermatogonia from undifferentiated spermatogonia. It is a challenge to identify factors in the former process since progenitors share the same phenotype as SSCs, and can reverse to SSCs in certain cases. The second differentiation process is retinoic acid (RA) dependent and largely marked by expression of *c-Kit*, the receptor for KITL known as stem cell factor (SCF).

Extrinsic factors

It has been a while since the finding that mice receiving a long-term vitamin A deficient diet develop spermatogenesis defects, with only undifferentiated spermatogonial and Sertoli cells found in the seminiferous epithelium. Replacement of vitamin A in the diet counters this spermatogenic arrest.[66] Vitamin A is normally stored and transported as retinol, and RA biosynthesis undergoes two sequential oxidative steps in both Sertoli and spermatogenetic cells via alcohol dehydrogenases and retinaldehyde dehydrogenases (RALDH).[67] Bis-[dichloroacetyl]-diamines, WIN 18,446, inhibits spermatogenesis and acts as an inhibitor of RALDH2, an essential enzyme for RA biosynthesis. Adding WIN 18,446 to organ culture medium causes a germ cell developmental defect compared with controls.[68] RA receptor (RAR) is the receptor for RA, and when formed as a complex they can bind to RA response elements and induce transcriptional changes, including expression of the meiotic initiator gene, *Stra8*. In organ culture, the RAR antagonists, BMS-204493 and AGN193109, inhibit expression of the meiotic marker genes, *Stra8*, *Scp3*, and *Dmc1*, while RA agonists do not.[69,70] In males, RA is degraded by CYP26B1, and RA degradation may protect from premeiosis during embryonic development. Although *Cyp26b1* knockout mice are embryonic lethal, high *Stra8* and *Scp3* expression is detected, and even more surprisingly, the pachytene stage of meiosis I germ cells are present at 16.5 dpc.[70,71] Besides, *in vitro* study proved that RA induces differentiation of GS cells and down-regulation the expression of *Oct4* and PLZF.[65] Taking all these studies together, RA is an indispensable extrinsic factor for normal differentiation of spermatogonia, and *Stra8* is the main target for the meiosis initiation.

Stem cell factor is also known as KIT ligand or the steel factor and is encoded at the Steel locus (Sl). Mutation at this site results in disruption of hematopoiesis, melanogenesis, and gametogenesis. Similarly, *c-Kit* mutation results in the same phenotype. It has been shown that SCF is a ligand for *c-Kit*, a tyrosine kinase receptor. The SCF/*c-Kit* interaction is critical for spermatogonia survival and proliferation, differentiation, and subsequent meiosis.[72,73] Here, we briefly introduce its role in proliferation and differentiation of spermatogonia. In the testis, SCF is secreted by Sertoli cells while *c-Kit* is expressed in differentiated type A and B spermatogonia.[74] In Sl17H/Sl17H mice, with SCF splicing defects, A_s, A_{pr} and A_{al} undifferentiated spermatogonia are noted according to their morphology and arrangement. However, no advancing germ cells exist, not even A_{al} spermatogonia expressing *c-Kit*, suggesting

arrest of spermatogonia differentiation due to SCF/*c-Kit* signaling defect.[75] Intraperitoneal injection of anti-*c-Kit* antibody in adult mice leads to reduced proliferation of differentiating spermatogonia, whereas undifferentiated spermatogonia are unaffected.[76] Sl/Sld and Sl17H/Sl17H mice are both SCF mutant, while W/Wv mice are *c-Kit* mutant, and all of them are infertile. Transplantation of green fluorescent protein-labeled undifferentiated spermatogonia into the testis of Sl/Sld or Sl17H/Sl17H mice results in proliferation of undifferentiated spermatogonia.[75] W mutant mice receiving the same transplantation have spermatogenesis restored, demonstrating the essential role of SCF/*c-Kit* in spermatogonia differentiation and proliferation.[77] The PI3K-AKT pathway may be involved in SCF/*c-Kit* induced proliferation and differentiation, as shown by both *in vitro* and *in vivo* experiments.[78] Blume-Jensen *et al*. took advantage of the Cre-loxP system to mutate the codon for Tyr719 within the SCF/*c-Kit* binding site for PI3k. This mutant shows significantly decreased (90%) PI3K – dependent activation of AKT. Moreover, homozygous mutant mice are infertile owing to a spermatogenesis defect, with an initial reduced proliferation rate and then widespread apoptosis occurring in undifferentiated spermatogonia.[79] Further experiments have found that SCF/*c-Kit* promotes rapamycin-sensitive proliferation, through activation of p70s6k by PI3K-AKT and then the cell progression transcription factors, cyclin D3 and RB.[80] Co-transfection of constitutively active v-AKT or dominant negative AKT-K179M (using the p70s6k plasmid) in spermatogonia cultured in serum-free medium, remarkably promotes or inhibits, respectively, p70s6k phosphorylation and can be blocked by rapamycin, suggesting that AKT activates p70s6k via FRAP/mTOR kinase. Dolci *et al*. reported that SCF administration transiently activates ERK1/2 kinase and sustained activation of PI3K-dependent AKT kinase, indicating that MEK signaling also plays a role in spermatogonia proliferation. Inhibition of either signaling pathway in spermatogonia completely blocks SCF-induced proliferation, showing both signaling pathways are indispensable. Additionally, both pathways converge when cyclinD3 translocates from the cytoplasm to the nucleus.[81] As opposed to the proliferation, it is still not clear how the differentiation marker *c-Kit* is induced.

Other factors secreted by Sertoli cells, such as BMP4 and Activin A, may also be involved in extrinsic regulation of differentiation.[82] In a 7-day spermatogonia primary culture system, BMP4 or activin A decrease colony forming ability after transplantation, while total cell number remains unchanged, suggesting these two factors promote differentiation.[83] Accordingly, Carlomagno *et al*. found that in rat SSC lines, BMP4 upregulated expression of *c-Kit*. Furthermore, A time-course DNA micro-array analysis highlights the pivotal role for adhesion molecules in BMP4-induced differentiation, with many adhesion molecules upregulated, thereby facilitating migration out of the niche.[84] The BMP4 receptor is a serine/threonine kinase, exclusively expressed in spermatogonia. Adding BMP4 to cultured spermatogonia induces phosphorylation, nuclear translocation of the SMAD4/5 heteromeric complex, and the formation of a DNA-binding complex with the various transcription co-activator p300/CBP.[85]

Intrinsic factors

Neurogenin 3 (*Ngn3*), a bHLH family transcription factor, is well known for its role in promoting differentiation of the endocrine pancreas, enteroendocrine cells, and other systems. In the testis, *Ngn3* expression is restricted to undifferentiated spermatogonia, indicating a potential role in SSC regulation.[86] *In vitro* culture of THY1$^+$ cells exposed to GDNF suppresses *Ngn3* expression, and transient *Ngn3* knockdown by siRNA enhances colony formation after transplantation whereas the total germ cell number is not changed, indicating a potential role in SSC differentiation to progenitors. Using a shRNA approach that stably knocks down *Ngn3* caused impaired spermatogenesis after transplantation, although patches of A_s, A_{pr}, and A_{al} cells are present, showing that *Ngn3* is indispensable for SSC differentiation.[87] Lineage tracing experiments provided further evidence. A tamoxifen-inducible *Ngn3*-Cre system allows the fate of labeled cells to be traced by pulse-chase analysis. Mating these mice with mice carrying the *LacZ* gene activated by Cre excision, Nakagawa *et al*. reported that predominantly, labeled cells progressed to advanced stages of spermatogenesis and only a few patches remained in the testis 2 months after tamoxifen administration, the period for a spermatogenesis cycle, arguing that most *Ngn3*$^+$ spermatogonia are progenitors and committed to differentiation.[12] It is still not clear what the downstream effector of *Ngn3* signaling is, and how it promotes differentiation, since its expression is parallel to that of *c-Ret*.[86] It is known, however, to be regulated by several established differentiation factors, including STAT and *Sohlh1*/2, which are discussed below.

Sohlh1 is also a bHLH family member, and its expression is limited to premeiotic germ cells in both males and females, indicating a potential role in germ cell development.[88] *Sohlh1* knockout mice are infertile with severely decreased differentiating spermatogonia, whereas PLZF$^+$ spermatogonia are slightly increased, evidence for its important role in differentiation.[89] Interestingly, although there are severely decreased spermatocytes at 10 days postnatal, increasing spermatocytes are detected at 3 weeks, with a few still detected at 7 weeks of age. This transient leaky phenotype is possible owing to compensation by a homologous transcription factor, *Sohlh2*, since a two-fold higher expression of *Sohlh2* is observed in *Sohlh1* knockout mice. Like *Sohlh1*, *Sohlh2* also exhibits germ cell specific expression, limited to a subpopulation of undifferentiated and the majority of differentiating spermatogonia. *Sohlh2*-deficient mice exhibit almost the same phenotype as *Sohlh1* knockout mice, raising the possibility of a related function.[90] Suzuki *et al*. found that *Sohlh1* and *Sohlh2* not only heterodimerize with each other, but also individually homodimerize. Further, they found via chip analysis that these two genes bind to each other's promoter, elaborating their cross-regulation.[91] It is not surprising therefore that the *Sohlh1* and *Sohlh2* double knockout mouse is infertile, and exhibits a similar phenotype as single knockout mice. Gene arrays using double or single knockout mice reveal molecular features of differentiation at both earlier and later steps, suggesting that *Sohlh* induces spermatogonial differentiation. Increasing the expression of *Gfrα1*, *c-Ret*, *Nanos2*, and *Oct4*, traditional maintenance factors, were found in *Sohlh* knockout mice, while expression of the earlier differentiation factors, SOX3 and *Ngn3*, was decreased. Chip analysis confirms these findings, as either *Sohlh1* or *Sohlh2* is able to bind to promoters of these genes. Simultaneously, *c-Kit* (the marker gene for A_{al} to A_1 spermatogonia transition) is significantly decreased in *Sohlh* knockout mice. Chip and electrophoretic mobility shift assay analysis show that both *Sohlh1* and *Sohlh2* are able to bind to the E-box-containing *Kit* promoter region. Transfection of *Sohlh1* and/or *Sohlh2* enhanced endogenous *c-Kit* expression, with these two genes functioning independently and cooperatively.[92] The upstream regulator of *Sohlh* remains largely unknown, and DMRT1 is a possibility, which we will discuss later. *Sohlh* is an important intrinsic factor that regulates both SSC and spermatogonia differentiation. Other intrinsic factors, including DMRT1, SOX3, and STAT3 are also involved in SSC differentiation.

Through restricting spermatogonial RA responsiveness, directly inhibiting *Stra8* expression, and activating *Sohlh1* transcription, DMRT1 guarantees continuous and sufficient sperm production.[93] DMRT1 knockout mice exhibit the premature meiosis initiation and *Stra8* expression in early stages of spermatogonia. ChIP-chip analysis found that *Stra8* and *Sohlh1* were DMRT1 targets, and DMRT1 may perform its role in SSC differentiation through *Stra8* inhibition and *Sohlh1* activation.[94] Transient STAT3 knockdown in cultured THY1+ cells causes increased SSCs, but not total cell number, indicating a possible role for STAT3 in SSC differentiation.[95] Stable shRNA-mediated STAT3 knockout, results in failed spermatogenesis following transplantation into recipient mice. The STAT3 target remains largely unknown, but *Ngn3* is a possible one. STAT3 inactivation in cultured THY1+ cells exposed to GDNF, a key factor for self-renewal, suppressed *Ngn3* gene expression. More direct evidence comes from the finding that STAT3 is able to bind to the distal *Ngn3* promoter/enhancer region *in vitro*.[96] Compared with self-renewal, more unknowns remain regarding the signaling pathway(s) underlying SSC differentiation. Developing an *in-vitro* culture system may greatly facilitate this (**Figures 1** and **2**).

PERSPECTIVES

The last decade has witnessed great progress in reproductive research, with technology improvements such as *in vitro* fertilization and intracytoplasmic sperm injection. However, for those with early stage gametogenesis failure, these technologies cannot help. SSCs are the origin of spermatogenesis, therefore, normal SSC function is of crucial importance and any disruption can result in severe infertility. To help solve this clinical problem, it is important to determine the mechanisms underlying normal functioning SSCs. Many important molecules regulating SSC self-renewal and differentiation have been identified; however, the exact mechanisms remain largely unknown. Difficulties in distinguishing SSCs from progenitors greatly hinder our understanding. It is unclear if there is a specific stage during spermatogonial development that once passed, marks unidirectional differentiation, or if no specific stage exists and a small part of undifferentiated spermatogonia maintain stem potential. If this stage does exist, identification of stage specific markers becomes critical and will aid our understanding.

Proliferation and differentiation of stem cells are regulated intrinsically by the stem cell itself and extrinsically by factors in the stem cell's niche. It is a sophisticated balance ensuring life-long spermatogenesis. Despite the number of extrinsic and intrinsic factors discussed above, SSC fate determination remains uncertain. Currently, the importance of GDNF-dependent signaling pathways is gradually becoming apparent, although GDNF-independent pathways remain to be clarified. The SALL4-PLZF-REDD1-mTORC1 interaction is thought to be an important circuit for maintenance of the undifferentiated state, yet PLZF and SALL4 regulation remains elusive. The maintenance factor, *Taf4b*, is not affected by the known maintenance signaling pathway, raising the possibility that there are more unknown signals and undiscovered core transcription factors. Culture intervention combined with microarray analysis may help investigate this problem.

At present, an increasing number of people are suffering from infertility because of the cryptorchid testis and acquired the infertility after chemotherapy or radiotherapy in cancer treatment. For those with normal SSC function, a prior testis biopsy combined with *in vitro* proliferation and transplantation may solve these problems.[97] Human SSC culture enabling SSC amplification is now possible; however lack of a differentiation culture system hinders its clinical application. Many are trying to develop a system that supports the entire process of spermatogenesis, but attempts have so far failed. Exposure of cultured SSCs to RA increases the proportion of germ cells expressing *c-Kit*, nevertheless, premature meiosis affirming the incomplete spermatogenesis restricts its potential application.[65] In 2012, Sato *et al*. developed an organ culture system that supported the whole spermatogenesis process, and produced functional sperm in about 2 months.[98] However, the demand for organ fragments for those patients with defected spermatogenesis inhibits its potential application. It is presumed that spermatogenesis is highly conserved among rodent animals as demonstrated by spermatogenesis following transplantation of hamster SSCs into mouse testis.[99] Xenotransplantation of human SSCs to primates may solve the problem, but needs further validation. To better improve culture systems supporting differentiation, greater understanding of molecular pathways is of paramount importance while, in reverse, such culture systems will greatly facilitate mechanistic studies.

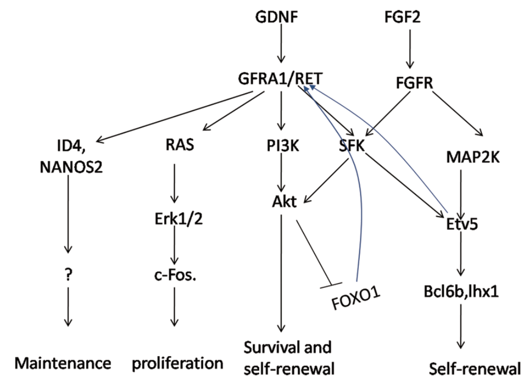

Figure 1: Signaling pathway of the extrinsic factors, glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2). GDNF is the main extrinsic factor for maintaining undifferentiated spermatogonia. FGF2 signaling interacts with GDNF and is more likely to enhance proliferation rate.

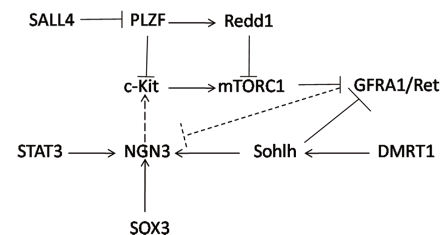

Figure 2: Transcription factor interaction. Solid arrows/lines represent confirmed interactions, and dotted lines/arrows indicate predicted interactions.

AUTHOR CONTRIBUTIONS

XXM, JWa and JW wrote and revised the manuscript. All authors read and approved the final manuscript.

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