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Self-renewal and differentiation of spermatogonial stem cells

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1. ABSTRACT

Mammalian spermatogenesis is a complex but well-coordinated process in which spermatogonial stem cells (SSC) of the testis develop to form spermatozoa. During testicular homeostasis, the spermatogonial stem cells self-renew to maintain the stem cell pool or differentiate to form a progeny of germ cells which sequentially transform to spermatozoa. Accumulating evidence from clinical data and diverse model organisms suggest that the fate of spermatogonial stem cells towards self-renewal or differentiation is governed by intrinsic signals within the cells and by extracellular signals from the SSC niche. Here, we review the past and the most recent developments in understanding the nature of spermatogonial stem cells and the regulation of their homeostasis in mice. We also review the potential clinical applications of spermatogonial stem cells in male infertility as well as in germline modification, by virtue of gene correction and conversion of somatic cells to biologically competent male germline cells.

2. INTRODUCTION

Men in most cases continue to be sexually competent until they are sixty years old, and if that limit be overpassed then until seventy years; and men have been actually known to procreate children at seventy years of age.

— Aristotle

These words by Aristotle signified that the continuity of fertility throughout life in men was noticed as early as in the 350 BC. Each day, approx. 100 million sperms are made in each human testicle, and each ejaculation releases 200 million sperms. During his lifetime, a human male can produce 10^{12} to 10^{13} sperms (1). Decades of research has led to the appreciation of the continuous nature of spermatogenesis as the reason for extended fertility in males compared to females. Mammalian spermatogenesis is a well-coordinated and a highly regulated process involving the sequential development of haploid spermatozoa from the diploid precursor germ cells in the testis. The testis is comprised of somatic cells, and a subset of undifferentiated spermatogonial cells (SSC), which can self-renew continuously or give rise to a progeny of germ cells at different stages of development until they mature to spermatocytes. Consequently, the high productivity and longevity of spermatogenesis relies primarily on the proliferation of SSCs.

The self-renewal and differentiation of SSCs during the initial steps of spermatogenesis produce heterogeneous SSC subpopulations under the regulation of multiple intrinsic and extrinsic factors, with each subpopulation differing in their stem cell properties. The extremely low number of SSCs and lack of SSC-specific markers had made the identification, isolation and study of these cells challenging. However, over the years, the development of spermatogonial transplantation techniques, efficient *in vitro* culturing, fluorescence-activated cell sorting (FACS), lineage tracing studies, single cell RNA sequencing (scRNA-seq) and mathematical modeling have made it possible to

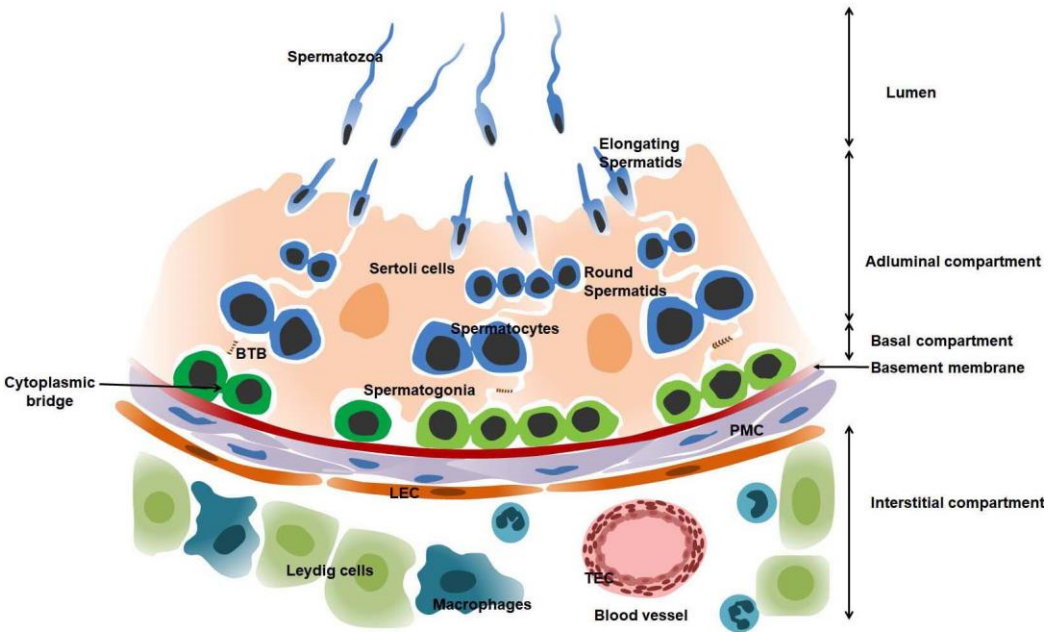

Figure 1. Schematic overview of tissue organization in mouse testis. Organization of the seminiferous epithelium shows hierarchy of germ cells supported by Sertoli cells and basement membrane. The primitive spermatogonia are localized next to the basement membrane in the basal compartment of seminiferous tubule. The basal compartment is followed by adluminal compartment, separated by the blood-testis barrier (BTB), wherein the spermatocytes derived from spermatogonia, round spermatids and elongating spermatids reside. The lumen shows presence of mature spermatozoa. The accessory somatic cells such as Leydig cells, peritubular myoid cells (PTMs), lymphatic endothelial cells (LECs), macrophages and testicular endothelial cells (TECs) of the vasculature reside in the interstitial compartment. The color key depicting different cell types is used throughout this article. Figure adapted with permission from Gauthier-Fisher *et al. (225).*

decode the secrets of SSCs. The ease of handling and short reproductive lifespan makes the mouse a preferred animal model for reproductive biology studies. As a result, mouse testis is one of the well-studied and well-understood systems for spermatogenesis. This chapter summarizes our understanding on the self-renewal and differentiation of mouse SSCs and the possible clinical implications emerging from this knowledge base.

3. OVERVIEW OF MOUSE SPERMATOGENESIS

3.1. Site of spermatogenesis

Spermatogenesis takes place in the seminiferous tubules of the testis (2), which form long convoluted loops that pass into the mediastinum and join a network of tubules called the rete testis. The seminiferous tubules harbor the seminiferous epithelium which contains the somatic Sertoli cells supporting the male germ cells at various stages of development. Surrounding the seminiferous epithelium is a layer of basement membrane (basal lamina). Between the tubules is the interstitial space that contains blood and lymphatic vessels, immune cells including macrophages and lymphocytes and Leydig cells (Figure 1). The spermatozoa exit the testis via the rete testis and enter the efferent ductules prior to their passage through the epididymis where they undergo maturation. From the epididymis, the spermatozoa enter the vas deferens for ultimate ejaculation.

The undifferentiated spermatogonial cells lie along the basal lamina at the periphery of the tubule interspersed between Sertoli cells. Adjacent Sertoli cells form specialized tight junctions that divide the seminiferous tubule into the basal compartment, in which spermatogonia reside and the adluminal compartment that is occupied by

differentiating germ cells. The tight junctions also constitute the blood-testis barrier which is a semi-permeable barrier that prevents immune system cells from infiltrating the lumen of seminiferous epithelium; making the testis an immune-privileged site. In the adluminal compartment, preleptotene spermatocytes, derived from spermatogonia in the basal compartment, undergo meiosis and subsequently go through successive stages of primary (leptotene, zygotene, pachytene and diplotene) and secondary spermatocytes. The end-products of meiosis are round spermatids that undergo morphological changes by the process of spermiogenesis and give rise to elongating spermatozoa occupying positions closer to the lumen. The mature spermatozoa are ultimately released into the lumen of the tubule by the process of spermiation (Figure 1).

3.2. Organization and timing of spermatogenesis

Spermatogenesis in mammals is organized and timed in a manner that maximizes sperm production. The central aspect of this organization and timing is the 'wave' and the 'cycle' of seminiferous epithelium. The seminiferous epithelium is characterized by asynchronous repeating series of germ cell associations. As these cells progressively differentiate, the initial associations are observed again after a fixed interval (8.6 days in mice and 16 days in humans), as the individual cells have shifted to the next layer. This periodic change in the seminiferous epithelium is called the 'seminiferous epithelial cycle' and was first discovered in rat testes (3). The seminiferous epithelial cycle is divided into stages I through XII in mice. The repetitive patterning of the epithelial stages along the length of a seminiferous tubule is called the spermatogenic wave. The 'cycle' and the 'wave' of seminiferous epithelium represent the key for asynchronous germ cell differentiation, allowing the constant production of spermatozoa (4). Following the first wave of spermatogenesis, which proceeds at a quicker rate than the adult cycles, regular cycles of asynchronous sperm production begin, each lasting approx. 35-36 days in mice. The spatiotemporal coordination of the cycle relies on intrinsic signals from the germ cells and extrinsic signals from the somatic support cells. It is established that retinoic acid (RA) is one of the major signaling molecules responsible for regulating the distinctive cycle and wave formation that induce spermatogonial cell differentiation (5).

3.3. Types of spermatogonia

The spermatogonial cell (SPG) population is enormously heterogeneous with respect to morphology, phenotype and function. SPGs can be broadly classified as undifferentiated cells that display the stemness or the progenitor properties to varying extents, and differentiating cells that have characteristics of being committed to enter meiosis. The undifferentiated SPGs include A- type spermatogonia (A_{undiff}) which in the mouse are found as single cells (A-single, A_s) or as syncytia of typically 2, 4, 8 and 16 cells interconnected by cytoplasmic bridges (A-paired, A_{pr} and A-aligned, $A_{al4\text{-}16}$). While a minority among the population of A_{undiff} has stem cell activity and functions as SSCs, a subset of A_{undiff} cells (A_{al} cells) has transit-amplifying roles and functions as progenitors. The differentiating SPGs are cells that are committed to meiosis and include A_1 SPG which undergoes sequential mitotic divisions to produce A_2, A_3, A_4, Intermediate (In) and type B spermatogonia. The A_{undiff} can be distinguished morphologically from the differentiating SPG by the absence of heterochromatin in the nuclei. In-type spermatogonia contain a moderate amount of heterochromatin, whereas, B-type spermatogonia display clumps of heterochromatin around the periphery of the nuclei (6). Thus, due to numerous transit amplifying divisions, one mouse SSC has the potential to produce up to 4096 sperm cells in a single spermatogenic cycle (7), although this has been shown to be a highly overestimated calculation which has not considered the significant level of apoptosis occurring at the A_{undiff} stage (8–10).

4. SPERMATOGONIAL STEM CELLS

4.1. Origin of SSCs

In metazoans, primordial germ cells (PGC) are the progenitors for both male and female gametes, giving rise to spermatozoa and oocytes, respectively. In mice, the precursors of PGCs arise

at about embryonic day 6 (E6) from the equipotent epiblast cells in response to bone morphogenetic protein (BMP) signals emanating from the extraembryonic ectoderm (BMP4 and BMP8b) and visceral endoderm (BMP2) (11–14). B lymphocyte-induced maturation protein-1 (BLIMP1), PR-domain containing protein 14 (PRDM14) and transcription factor AP-2 gamma (TFAP2C) form a tripartite transcription factor network that facilitates mouse PGC specification by suppressing somatic gene expression of homeobox A1 (*Hoxa1)*, homeobox B1 (*Hoxb1)*, LIM homeobox 1 (*Lim1*), even-skipped homeobox 1 (*Evx1)*, fibroblast growth factor 8 (*Fgf8)* and snail family transcriptional repressor 1 (*Snai1*1*)* genes, while initiating the germ cell transcriptional program and triggering genome-wide epigenetic reprogramming. From E7 onwards, the specified PGCs express the PGC-specific markers, viz., tissue non-specific alkaline phosphatase (TNAP), stage-specific embryonic antigen 1 (SSEA1) and developmental pluripotency associated 3 (DPPA3 or STELLA) (11, 15–17). However, PGC specification in the mouse and human exhibits some differences. The origin of human PGCs from mesodermal precursors, the requirement of the Wingless/integrase 1 (WNT) pathway along with BMP signaling for development of PGCs and lack of PRDM14 and SRY (sex determining region Y)-box 2 (SOX2) expression in human PGCs are the major contrasting differences (18, 19). PGCs proliferate while migrating through the hindgut and colonize the genital ridges (the future gonads) between E7.5 and E11. The proliferation and directional migration of PGCs are facilitated by two germ cell-soma signaling pathways: cKIT-STEEL (20) and stromal cell-derived factor 1 (SDF1)- C-X-C chemokine receptor type 4 (CXCR4) (21). Once in the genital ridges, PGCs undergo approximately five additional mitotic divisions from E10.5 to E14.5 with incomplete cytokinesis to form germline cysts. Around this time (E11.5), the testis development will be initiated by the somatic cells expressing FGF9/SRY/SOX9 proteins marking the Sertoli cell population. On the basis of the cues from these somatic cells, the germ cells also undergo sex differentiation and become developmentally restricted (22). The germ cells in the differentiating testis are now referred to as gonocytes or prospermatogonia.

In the female mice, the germ cells begin to enter meiosis at E13.5 in response to RA signaling from the mesonephros. In contrast, the gonocytes fail to enter meiosis in the developing testis due to expression of CYP26B1 in the Sertoli cells, which catalyzes the oxidation of RA into inactive metabolites. Instead, the gonocytes exit the cell cycle, get arrested at G0, and remain quiescent from approximately E14.5 until postnatal day 1-2 (P1-2). They re-enter the cell cycle on P3 and migrate to the basement membrane of the seminiferous epithelium. The subset of gonocytes that express neurogenin 3 (NGN3) transforms to form the founding SSC population in mice between P3-6. Those gonocytes which lack NGN3 expression directly differentiate into progenitor spermatogonia that undergo further differentiation, initiating the first wave of spermatogenesis at approximately P3 (23, 24). Thus, the first wave of spermatogenesis occurs without the contribution of SSC activity. Intriguingly, a recent study using scRNA-seq analysis revealed the presence of cell populations with characteristics of PGCs (referred to as PGC-like cells, PGCLCs) and SSCs (referred to as prespermatogonia, PreSPG) in human neonatal (day 2 and Day 7) testis (25). The authors hypothesized a model in which human fetal PGCs differentiate into PGCLCs and, subsequently, PreSPGs, both of which populate the human testes at birth. These neonatal germ cells are replaced by SSCs during the first year of the male child.

4.2. Kinetics of SSC cell division

It is unanimously accepted that SSCs are contained within the A_{undiff} pool. However, which cells among the A_{undiff} contribute to the SSC pool is an area of debate till date. A_s cells were traditionally regarded as the actual stem cells, whereas A_{pr} and A_{al} were thought to represent transit-amplifying progenitors (26). However, with the advent of improved experimental tools and molecular markers, it became apparent that stem cell potential may not be limited to A_s cells alone and may extend to A_{pr} and A_{al} cells also and that the developmental hierarchy of A_{undiff} cells is more complex than originally anticipated. This resulted in the proposal of three different models to explain the true identity of SSCs, viz., the A_s model, the revised A_s model and the fragmentation model, which are described in the following paragraphs.

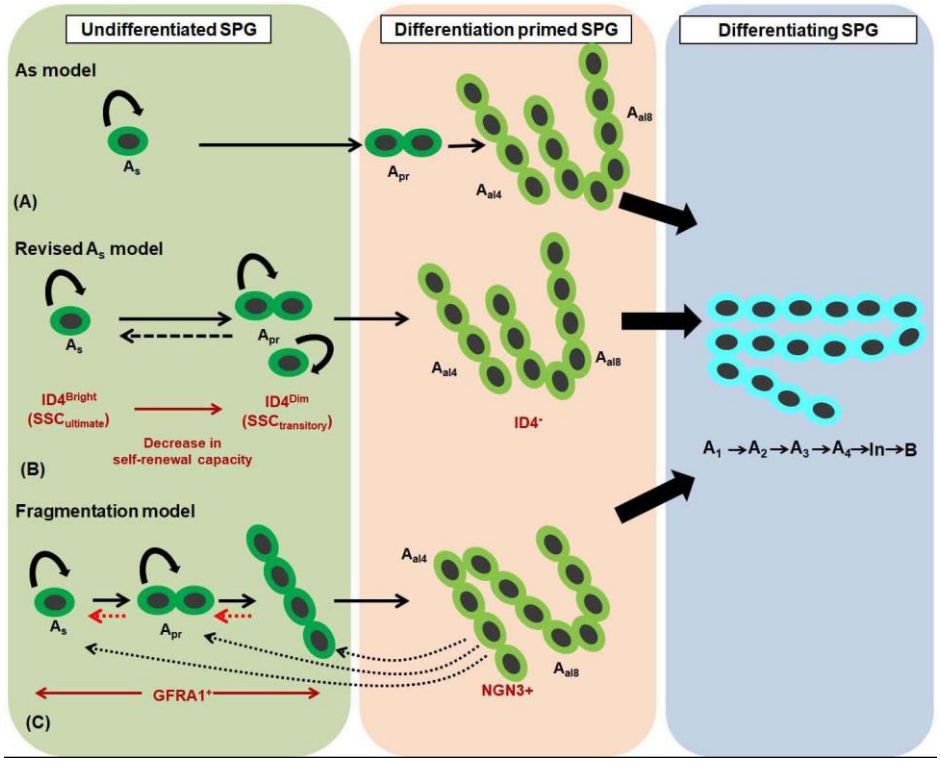

Figure 2. Kinetics of SSC division in adult mouse testis. A) According to A_{single} (A_s) model, the self-renewal (curved arrow) capacity is restricted only to A_s cells of the undifferentiated A type (A_{undiff}) spermatogonial cell (SPG) population that divide (solid arrows) into two daughter cells interconnected by cytoplasmic bridges called A_{paired} (A_{pr}) cells which subsequently divide to form longer syncytia of 4, 8 and 16 cells termed as $A_{aligned\ 4,\ 8,\ 16}$ ($A_{al4,\ 8,\ 16}$). The A_{al} cells finally differentiate (solid block arrow) into differentiating SPGs including A_1, A_2, A_3, A_4, Intermediate (In) and B type SPGs. Thus, the A_s cells constitute the SSC pool. B) In the revised A_s model the population of A_s SPGs is heterogeneous. The SSC activity resides in a subpopulation of A_s cells that express high levels of ID4 (termed as $ID4^{Bright}$ cells). In A_{undiff} cells (A_s and A_{pr}) that are produced subsequently, ID4 levels drop ($ID4^{Dim}$) and these cells exhibit a decreasing chance of self-renewal and subsequently form clones of A_{al}. The $ID4^{Bright}$ cells with the highest chance of self-renewal have been called $SSC_{ultimate}$ and $ID4^{Dim}$ cells with limited self-renewal capacity are called $SSC_{transitory}$ (with respect to the transit to progenitor state). The $SSC_{transitory}$ cells divide and give rise to $ID4^-$ differentiation-primed progenitor SPGs. Reversion of cell fate (dashed arrow) from $SSC_{transitory}$ to $SSC_{ultimate}$ state is possible but from a progenitor to stem state is strictly not possible under any conditions. C) The fragmentation or dynamic SSC model proposes that $GFRA1^+$ A_{undiff} cells continuously interconvert between equipotent single cell and short syncytial states via fragmentation (dashed red arrow). $GFRA1^+$ A_{undiff} also give rise to $NGN3^+$ progenitor cells that undergo differentiation priming. Under steady-state conditions $NGN3^+$ A_{undiff} do not typically revert back to the self-renewing state but in regenerative conditions $NGN3^+$ progenitor cells may revert to the SSC state (dashed curved arrows). The color key depicting different cell types is used throughout this article.

4.2.1. A_s model

In 1971, Huckins and Oakberg proposed the A_s model of spermatogonia multiplication, which was endorsed by most researchers in the field and was held for over 40 years (8, 9, 27, 28). This proliferation scheme was developed by studying whole-mounts of seminiferous tubules instead of sections, which enables one to observe the topographical arrangement of the spermatogonia on the tubule basal lamina. According to this model, only the A_s spermatogonia are the SSCs. SSCs divide and their daughter cells either migrate away from each other and become two new SSCs or they stay together (A_{pr}), constituting the first step along the differentiation pathway (differentiation-committed progenitors). Subsequently, the pairs can proliferate further to form A_{al} cells (Figure 2A). Thus, according to this model, there are two types of SSCs: reserve SSCs that function only in response to injury and active SSCs that divide slowly on a regular basis to maintain homeostasis.

4.2.2. Revised A_s model

The number of A_s cells in the adult mouse testis is estimated to be approximately 35,000 (29). However, following the transplantation of an unselected total donor testis cell population in recipient testes, the number of regenerated spermatogenic colonies was only 3000. (30). As this was less than 10% of the expected value, it implied that not all A_s are stem cells. Studies by Oatley *et al*. and others, using transplantation and lineage tracing experiments, concluded that SSC activity is almost exclusively contained within a fraction of A_s cells marked by expression of transcription factor known as inhibitor of DNA binding 4 (ID4), supporting a "revised" A_s model in which stem cell activity is said to be limited to a subset of A_s (termed as $SSC_{ultimate}$) while remaining A_s, A_{pr} and A_{al} cells are transiting into a differentiation-committed state (31–36). This model is also termed the 'hierarchical A_s model', as it suggests the existence of SSC hierarchy. Furthermore, this model proposes that the cells expressing high levels of ID4 (termed as $ID4^{Bright}$ population) has the greatest capacity of self-renewal and that the capacity for self-renewal decreases as ID4 expression among A_s cells regresses from bright via intermediate to dim ($SSC_{transitory}$) (35). This model also supports that some plasticity may exist for A_{undiff} at the early phase of transition from $SSC_{ultimate}$ to $SSC_{transitory}$ population which is at the progenitor state (Figure 2B). However, recent reports analyzing *Id4* expression by scRNA-seq, immunostaining and reporter assays have shown that *Id4* expression is substantially more widespread within the A_{undiff} cells than previously described, indicating that *Id4* expression may not be limited to SSCs (37–40). *Id4* expression in A_{undiff} cell fractions with the highest SSC capacity has also been disputed (40), further questioning the validity of this model.

4.2.3. Fragmentation model

Yoshida *et al* performed a series of lineage tracing and live imaging experiments to monitor SPGs in transgenic reporter mice models (41–43). They analyzed the expression of glial cell line derived neurotrophic factor (GDNF) receptor alpha 1 (GFRA1), which marks early A_s and A_{pr} SPGs that do not yet express a differentiation marker and the expression of the differentiation marker NGN3, which marks A_s, A_{pr} and A_{al} SPGs that may have taken a first step towards differentiation. Under the steady state, the SSC pool comprising of all GFRA1 expressing A_{undiff} transition into $GFRA1^-/NGN3^+$ cells, which are assumed to be the progenitor cells with increased differentiation propensity (Figure 2C). Notably, in a direct contradiction to traditional schools of thought that depicted the progenitors were irreversibly committed to a differentiating fate, it was reported that, under regenerative conditions in the testis (during restoration of spermatogenesis after transplantation into an infertile recipient testis or during regeneration after tissue injury), the $NGN3^+$ progenitor population can also experience fragmentation, with single progenitor spermatogonia breaking off from chains and reverting to a $GFRA1^+$ state to re-join the self-renewing pool (42, 44). Thus, the fragmentation model proposes that A_s cells almost always divide into A_{pr} and that the A_{undiff} syncytia (A_{pr} and A_{al}) can fragment into singles and pairs to replenish the self-renewing SSC pool (43). Due to the dynamic nature of A_{undiff} proposed here, this model is also referred to as the 'dynamic SSC model' (45). Currently, evidences for these phenomena are based primarily on observations of fragmentation using live imaging of testes in mice that are maintained in a stress condition of long-term anesthesia and computer generated biophysical models. Further, qualms also exist regarding the specificity of GFRA1 and NGN3 as markers for the SSC and progenitor populations respectively. However, the lack of functional evidence for self-renewal capacity in the fragmented cells and for the mechanisms regulating the fragmentation of A_{undiff} syncytia compels further investigation into this proposed model.

It can be argued that the differences between the revised A_s model and the fragmentation model are rather insignificant under steady state conditions as they primarily differ in the proposed mechanism for maintenance of the A_s population, i.e., by complete cytokinesis vs syncytial fragmentation respectively. Both models claim that the SSC capacity is restricted to A_s and A_{pr} cells in contrast to only A_s cells as proposed by the A_s model. In conclusion, the A_{undiff} population displays in-built heterogeneity and has their propensity for alteration or reversion of gene expression profiles in response to different requirements within the niche.

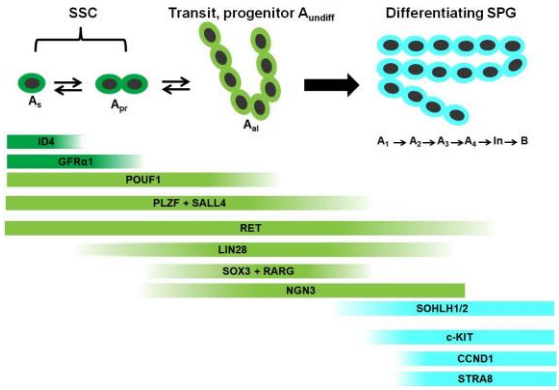

Figure 3. A schematic showing the expression pattern of markers that define different subsets of spermatogonial cells (SPGs). The transcription factor ID4 is an A_{single} (A_s) specific marker. The membrane receptors GFRA1 and RET bind the ligand GDNF, which is crucial for stem cell self-renewal. GFRA1 marks undifferentiated A type SPGs (A_{undiff}) including A_s and A_{paired} (A_{pr}) which exhibit SSC activity (marked in dark green) whereas RET expression is found across the A_{undiff} cells population (A_s, A_{pr}, A_{al}). PLZF and POU5F1 are transcription factors expressed by SSCs and A_{undiff}. The progenitor (marked in light green) A_{undiff} cells (A_{pr} and A_{al}) which arise on retinoic acid (RA) stimulation are marked by the expression of LIN28, RARG, SOX3 and NGN3. The progenitor cells under the influence of RA differentiate into differentiating SPGs (A_1-A_2-A_3-A_4-Intermediate (In)-B). The differentiating SPGs (marked in light blue) are characterized by the expression of SOHLH1/2, c-KIT, CCND1 and STRA8, which will induce them to enter meiosis and give rise to primary spermatocytes. The color key depicting different cell types is used throughout this article.

4.3. Markers of SSCs

Mice have 2-3 × 10^4 SSCs in the testis, which comprise only 0.02%-0.03% of the total germ cell population (29). Therefore, the identification of the phenotype of SSCs is critical for the functional investigation of SSCs at the single-cell level. Several methods to enrich SSCs from testes have been developed, including differential plating, density-gradient centrifugation, experimental surgical cryptorchidism, and antibody-based selection methods such as FACS and magnetic-activated cell sorting (MACS). An approach using FACS/ MACS together with a functional transplantation assay was widely used to isolate SSCs (46, 47). Currently, with the advent of fluorescence tagged proteins, cell lineage-tracing experiments are being used to study SSCs.

Flow cytometry-based transplantation experiments showed that the stem cell activity was concentrated in fractions of mouse SPGs with surface antigenic profile as follows - alpha 6-integrin $(ITGA6)^+$, beta 1 integrin $(ITGB1)^+$, thymus cell antigen 1 $(THY1)^+$, $CD9^+$, $GFRA1^+$, epithelial cell adhesion molecule $(EPCAM)^+$, $CD24^+$, E-cadherin $(CDH1)^+$, melanoma cell adhesion molecule $(MCAM)^+$, KIT^- and major histocompatibility complex class I $(MHC\text{-}I)^-$ (46, 48–51). However, considering the dynamic nature of SSC hierarchy and the SPG heterogeneity, it is ambiguous to delineate a universal array of markers for SSCs.

Hence, it is important to note that the gene expression profiles of A_s, A_{pr}, and A_{al} spermatogonia are different (39, 42), as represented in Figure 3. The expression of promyelocytic leukemia zinc finger (PLZF or ZBTB16) and CDH1 is relatively constant in the A_s, A_{pr}, and A_{al} spermatogonia, and has been used to identify all A_{undiff} (49, 52). *Gfra1*, *Id4*, *Bmi1*, *Pax7*, *Nanos2*, *Lhx1*, *Bcl6b*, *Etv5*, *T (Brachyury)*, *Sall4* are shown to be preferentially expressed in A_s cells whereas *Pou5f1 (Oct4)*, *Ngn3*, *Lin28a*, *Sohlh1*, *Sox3* and *Rarg* are expressed by A_{al} progenitor cells (34, 39, 43, 53). Later studies have shown that SSC activity is evident in progenitor A_{al} cells (43, 54). Subsequently, it was also found that the state and function of A_{undiff} is context-dependent. Thus, there are different interconvertible subsets of A_{undiff} cells that contribute to SSC activity during steady state adult spermatogenesis, postnatal testicular development and under tissue regenerative conditions (discussed in detail in section 5.5). Hence, it is important to consider the expression profiles of these functionally distinct subsets of A_{undiff} when delineating SSC activity.

5. SPERMATOGONIAL STEM CELL NICHE

The microenvironment surrounding the stem cells is called the stem cell niche, which provides juxtacrine and paracrine factors that maintain stem cell competence and decide the fate of the stem cell towards self-renewal or differentiation. The interaction of testicular “niche” cells with SSCs occurs via both cellular contact and soluble signaling. Cellular components of the niche include Sertoli cells and germ cells of the tubules, peritubular cells (peritubular myoid cells and peritubular macrophages) and interstitial cells (Leydig cells, interstitial macrophages and vasculature).

In the basal compartment, A_{undiff} cells localize preferentially to the basement membrane in areas adjacent to the vasculature network of arterioles and venules that accompany the interstitial cells, including Leydig cells (43, 55, 56). Kitadate *et al*. recently demonstrated that the self-renewal and proliferation of SSCs are favored at areas of high fibroblast growth factor (FGF) concentration, lying in close proximity to the vasculature and interstitium. However, A_{undiff} cells do not cluster to a restricted domain, but intermingle and migrate between differentiating spermatogonia and immotile Sertoli cells (43). Since the SSC localization is not restricted to any specialized area in the niche, it is designated as an 'open' or 'facultative' niche, contrary to the canonical 'closed' or 'definitive' niche observed in other stem cell systems (57–59). Although research is still ongoing to decode the complex mechanism of the coexistence of a heterogeneous SSC pool and an open niche, it is well established that germ cell-niche interaction determines the density and the fate of SSCs.

5.1. Cellular components of SSC niche

5.1.1. Sertoli cells

Sertoli cells are arguably the most important component of the SSC niche. In addition to producing a number of factors essential for SSC maintenance such as GDNF, they also physically support, nurture and protect the SSCs (60). Intriguingly, Sertoli cells that have already terminated their cell cycle before puberty expand their plasma membrane to an extreme degree and simultaneously 'hold' germ cells of all four stages (spermatogonia, spermatocytes, round spermatids and elongating spermatids) at different areas of their plasma membrane. Lack of a report describing a germ-cell-only tubular phenotype implies that SSCs and more advanced germ cells cannot exist without Sertoli cells *in vivo*.

5.1.2. Peritubular myoid cells

Seminiferous tubules are encased by contractile smooth muscle cells called peritubular myoid cells (PMCs). Besides providing structural support and propelling the flow of luminal fluid towards the rete testis, PMCs also secrete paracrine factors important for SSCs, including GDNF, leukemia inhibitory factor (LIF) and CC-chemokine monocyte chemoattractant protein-1 (MCP-1) (61–63).

5.1.3. Testicular macrophages

The role of testicular macrophages (peritubular and interstitial macrophages) within the SSC niche is not well-understood, although some reports have led to the speculation that they potentially influence SSCs proliferation and differentiation either directly via colony stimulating factor 1 (CSF1) and RA synthesis or indirectly by influencing testosterone synthesis in Leydig cells through the production of 25- hydroxycholesterol, an intermediate compound within the testosterone biosynthetic pathway (64, 65). However, these claims have not been functionally validated.

5.1.4. Leydig Cells

Leydig cells, upon luteinizing hormone (LH) stimulation via LH receptors (LHR), regulate the expression levels of steroidogenic enzymes, such as 17-beta hydroxysteroid dehydrogenase, in order to increase the production of testosterone. While testosterone is strictly indispensable for spermatogenesis, it also regulates the expression of thousands of genes in different somatic cell populations in the testis under normal conditions. One of the targets of testosterone is the Sertoli cell-controlled attachment mechanisms (66, 67). Besides testosterone, Leydig cells also produce factors that directly target SSCs such as insulin-like growth factor 1 (IGF1) and CSF1.

5.1.5. Vasculature

Vasculature cells (testicular endothelial cells or TECs) are rich sources of several cytokines and growth factors such as vascular endothelial growth factor A (VEGFA) that are required for SSC maintenance and localization. It has been proposed that $ID4^+$ SPGs (A_s cells) are mainly localized at the avascular sections of the tubule whereas the $NGN3^+$ progenitor SPG cells, derived from A_s cells, relocate to vascular areas to fulfill their new requirements for different levels of oxygen, metabolites, and various growth factors (33, 56, 64).

Vasculature associated lymphatic endothelial cells (LECs) are found at the border of

seminiferous tubules and testicular interstitium, and cover the surface of the lymphatic space. LECs located in proximity to vasculature express a number of FGFs (FGF4, 5, and 8), that are shown to regulate the density of GFRA1$^+$ A_{undiff} (40).

5.2. Signaling pathways of SSC niche

The vital soluble niche factors include GDNF, FGF, RA, follicle stimulating hormone (FSH), testosterone, CSF1, WNT and NOTCH. These somatic cell-derived factors govern multiple signaling pathways in SSCs and the resulting germ cell-soma communications are the paramount forces governing SSC self-renewal and differentiation.

5.2.1. GDNF signaling

While Sertoli cells have been considered as the primary source of GDNF during steady state spermatogenesis, TECs and PMCs are also found to be GDNF producers. GDNF is a well-defined prime factor that is required for promoting SSC renewal and maintenance, both *in vitro* and *in vivo* (68, 69). GDNF belongs to the transforming growth factor beta superfamily molecules and binds to glycosylphosphatidylinositol (GPI)-anchored GFRA1, triggering signaling via the transmembrane receptor tyrosine kinase called REarranged during Transfection (RET), which does not directly bind to GDNF.

The loss of GDNF signals from Sertoli cells or peritubular myoid cells *in vivo* results in the loss of undifferentiated germ cells, whereas overexpression leads to an expansion of the undifferentiated SSCs and the development of tumors (61, 68). Similarly, the absence of GDNF receptors (GFRA1 and RET) triggers rapid depletion of SSCs resulting in a Sertoli-cell-only phenotype (68, 70). Moreover, culturing of mouse SSCs *in vitro* requires GDNF (71, 72). These results suggested that GDNF is a *bona fide* self-renewal factor for SSCs. Furthermore, the expression of GFRA1 within the A_{undiff} cells is reduced as the syncytial length is increased. While approximately 90% of A_s spermatogonia express GFRA1, approx. 75% of A_{pr}, approx. 40% of A_{al4}, and approx. 15% of A_{al8} spermatogonia are positive for GFRA1. A_{al16} spermatogonia lack GFRA1 expression altogether (41, 45). In addition, the expression level of GFRA1 per cell is typically lower in aligned syncytia than single cells or pairs (73).

Sharma and Braun reported that GDNF is expressed cyclically in Sertoli cells and its level is at its highest during the stages when SSC self-renew (X-IV). They proposed that GDNF acts to promote self-renewal not by regulating SSC proliferation, but by inhibiting SSCs from differentiating into transit amplifying A_{undiff} SPGs by using LIN28-null A_s cells in their study (74). Additionally, the stage specific cyclic nature of GDNF availability is also associated with chemotactic migration of undifferentiated SPGs (75). Target genes of GDNF in A_{undiff} spermnatogonia include *Nanos2*, *Etv5*, *Lhx1*, *T(Brachyury)*, *Mycn*, *Bcl6b*, *Id4* and *Ccna* (76–81). Other paracrine factors involved in SSC maintenance in synergy with GDNF include FGF2, different forms of VEGFA and C-X-C motif chemokine 12 (CXCL12) (72, 81, 82).

5.2.2. FGF signaling

Fibroblast growth factors (FGF) belong to a large family of over 15 FGF members that activate receptor complexes including FGFR1, FGFR2, FGFR3, and FGFR4. FGF2 together with GDNF is crucial for proliferation of prospermatogonia and SSC *in vitro* (39, 69, 83). GDNF-independent action of FGF2 on SSC self-renewal has also been reported using transplantation assay and *in vitro* culturing (84). Intriguingly, SPGs cultured in presence of FGF2 have morphology, doubling time, and SSC activity distinct from those of SPGs cultured in presence of GDNF. FGF2 promotes survival and proliferation of SSCs through signaling pathways which are distinct from those involving GDNF. Nevertheless, studies in mice have confirmed that both GDNF levels and SSC numbers increase in FGF2-depleted testis, thereby, implying that a balance between FGF2 and GDNF influences SSC self-renewal (84).Though FGF2 has been considered as a self-renewal promoting factor, it has also been reported to induce expression of RA receptor gamma (RARG) in SPGs marking them as differentiation-primed or differentiating SPGs (85). Moreover, FGF2 also regulates the availability of RA by suppressing the expression of RA-degrading enzyme *Cyp26b1* (86). Further research is needed to better understand the role of GDNF/FGF2 ratio or FGF2 alone in SSC fate determination. It has been reported that FGF5, FGF4, and FGF8 are expressed

in LECs covering the outer surface of the tubules near the interstitium. Although the expression of FGF5 persists throughout the seminiferous epithelial cycle, its spatial availability is heterogeneous with more proximity to the interstitium. Interestingly, the distribution of A_{undiff} spermatozoa shows spatial correlation with FGF5 expression (40). It is proposed that the fate of SSCs may be determined by the competition among the SSC population for a limited supply of FGFs whose availability on the basement membrane is inversely proportional to the distance from the source and the number of A_{undiff} spermatogonia (40). However, further investigation is required to decipher the roles of different FGF ligands in determining the fate of SSCs.

5.2.3. FSH Signaling

Follicle Stimulating hormone (FSH) is a gonadotropin hormone synthesized by the anterior pituitary that acts via its cognate G-protein coupled receptor, FSH receptor (FSHR). During the perinatal period, FSH induces Sertoli cell proliferation and establishes the final Sertoli cell number. Later in development, FSH stimulates the transcriptional and metabolic activities of the Sertoli cell, which contributes to the hormonal and nutritional environment necessary for germ cell survival and development (87–89). FSH has been shown to stimulate GDNF expression in Sertoli cells and to increase the proliferation of undifferentiated SPGs *in vivo* (90). FSHR knockout male mice are fertile but display small testes and partial spermatogenic failure, with defects in sperm viability and motility (91). These data suggest that FSH plays a role in maintaining quantitatively normal spermatogenesis, but may not be absolutely required for fertility in male rodents. Interestingly, men with FSH deficiency or inactivating mutation in FSHR are infertile signifying a species specific prominence of FSH in spermatogenesis (92, 93).

5.2.4. WNT signaling

Wnt genes encode WNT ligands, which are cysteine-rich, glycosylated and lipid-modified secreted proteins that engage Frizzled (*Fzd*) receptor family members to transduce signals into target cells. In many cases, the “canonical” WNT pathway, mediated by beta-catenin, acts to maintain the stem cell pool by inhibiting their differentiation (94). On the contrary, in mouse spermatogenesis, both *in vitro* culture and *in vivo* transplantation based studies suggest that Wnt/beta-catenin signaling (activated by WNT3a) stimulates the proliferation of differentiating progenitors (95, 96). Tokue *et al*. further demonstrated that transition from stem ($GFRA1^+$) to progenitor ($NGN3^+$) state is driven by WNT6 which is prominently expressed by the Sertoli cells (97). Moreover, they identified SHISA6, a cell-autonomous WNT inhibitor, expressed by a subset of $GFRA1^+$ A_{undiff} spermatogonia. It is proposed that SHISA6 might play a role in the maintenance of the $GFRA1^+$ pool by reducing the Wnt/beta-catenin signaling strength in the $SHISA6^+$ A_{undiff} cells and preventing premature entry into the differentiation-primed state.

Interestingly, the availability of GDNF and WNT6 (a WNT family member that is abundantly expressed by Sertoli cells) during the seminiferous epithelial cycle differs, suggesting that they have distinct windows of action (96, 97). Androgen-regulated Sertoli cell gene WNT5A (an activator of beta-catenin-independent pathway) has also been implicated in control of SSC self-renewal, but the available data indicates that WNT5A is an A_{undiff} mitogen (98). Whether it supports adoption of either the stem or progenitor state is unclear.

5.2.5. Retinoic acid signaling

Genetic and molecular studies have elegantly proven that RA signaling is important for SPG differentiation, meiotic initiation, spermatid elongation, and sperm release (99, 100). Vitamin A (retinol) undergoes two oxidation steps to form RA which activates the RA receptors (RARA and/or RARG) and is then quickly (RA half-life in mouse testis is 1.3 hr) oxidized to inactive metabolites by two P450 enzymes (CYP26A1 and CYP26B1). During the first wave of spermatogenesis, RA is produced by Sertoli cells and is required for spermatogonia differentiation (101). In subsequent spermatogenic cycles, meiotic and post-meiotic germ cells become the major source of RA (101, 102). The lack of RA or vitamin A resulted in an accumulation of A_{undiff} spermatogonia, resulting from an inhibition of differentiation of A_{undiff} spermatogonia to A_1 spermatogonia. On the other hand, administration of exogenous vitamin A released this inhibition in vitamin A-deficient mice (103). Similarly, RARG is

predominantly expressed by differentiation primed NGN3$^+$ SPGs (85) and inactivation of *Rarg* in spermatogonia impairs the A_{al} to A_1 transition in the course of some of the seminiferous epithelium cycles (104). Additionally, RA has also been found to downregulate GDNF expression in Sertoli cells (resulting in the expression of differentiation-supporting factors, such as *Bmp4* and stem cell factor (*Scf*)) and antagonize the effect of GDNF in A_{undiff} (105–107). The periodic, pulsatile and stage-specific nature of RA synthesis is the prime regulator of asynchronous seminiferous epithelial cycle (108). Despite the extensive research on the role of RA in spermatogenesis, information on the molecular targets and interacting partners of retinoic acid receptors at various stages of germ cell development is still scarce.

5.2.6. NOTCH signaling

The NOTCH proteins (NOTCH 1-4) are large cell-surface receptors that are activated by membrane bound ligands on neighboring cells such as JAGGED (JAG1 and JAG2) and DELTA-like (DLL1, DLL3 and DLL4). Upon activation of the canonical pathway, the NOTCH intracellular domain (NICD) is cleaved and translocated to the nucleus, where it associates with and consequently activates a DNA-binding protein called recombining binding protein suppressor of hairless (RBPJ). The Hes/Hey family of transcriptional repressors are targets of RBPJ (109). NOTCH receptors (NOTCH 1-4) and NOTCH ligands (JAG1, JAG2 and DLL1) are reported to be expressed by spermatogonia whereas, NOTCH2, JAG1 and DLL1 are expressed by Sertoli cells as well (110). Gain-of-function mouse model that constitutively activates NOTCH1 signaling only in Sertoli cells led to a complete loss of germ cells around birth due to premature differentiation of gonocytes in fetal testis (111). Further investigations described a downregulation of *Gdnf* and *Cyp26b1* which are niche factors required for maintaining undifferentiated state of germ cells. A contrasting phenotype was observed in *Rbpj*-conditional knockout mice where NOTCH signaling was disrupted with significant increase in SSCs and overall germ cell numbers (112). The data so far is suggestive of a role of NOTCH signaling as a negative regulator of germ cell proliferation and promoter of differentiation. However, other studies reported that NOTCH blockage in germ and Sertoli cells had no effect on spermatogenesis and that NOTCH signaling is dispensable for mouse spermatogenesis (113).

5.2.7. Chemokine signaling

CXCL12, also known as SDF-1, is one of the chemokines produced by the Sertoli cells. It acts via its cognate receptor, known as C-X-C motif chemokine receptor 4 (CXCR4), a seven-transmembrane protein which signals via G-proteins, leading to MAPK activation. CXCR4 is expressed by PGCs, gonocytes and A_{undiff} SPGs. In the fetal testis, CXCL12/CXCR4 signaling facilitates the later stages of PGC migration into the genital ridge and is required for gonocyte survival (114). In the adult testis, CXCL12/CXCR4 signaling is crucial for proper homing of SSCs to their cognate niche. Evidence also suggests that CXCR4 expression is stimulated by GDNF in A_{undiff} and that CXCL12/CXCR4 signaling may promote the self-renewing state and prevent transition from A_{undiff} to progenitor state *in vitro* (81).

6. REGULATION OF SSC FATE IN MOUSE SSC NICHE

For healthy spermatogenesis to occur, it is important to maintain the number and function of SSCs during steady state and also in response to environmental and genetic insults. Frequent self-renewal of SSCs can lead to the over-accumulation, leading to defects in spermatogenesis. Conversely, SSCs get “exhausted” if there is insufficient SSC self-renewal, resulting in progressive germ cell loss. Hence, it is critical to achieve an appropriate balance of self-renewal and differentiation in the life cycle of SSCs by the niche factors. The life cycle of SSCs in the mouse testis can be described in three stages: 1) maintenance of self-renewing state 2) differentiation priming and 3) differentiation commitment. Each stage is governed by a specific network of niche factors (Table 1).

6.1. Maintenance of self-renewing state

The property of self-renewal encompasses cell proliferation, cell survival and the proportion of self-renewing cell divisions relative to differentiating cell divisions. GDNF is the key factor for maintenance

Table 1. Summary of SSC niche derived factors required for mouse SSC maintenance or differentiation.

Factor	Testicular expression	Function	Reference
GDNF	Sertoli cells, TEC, PTM	Self-renewal of SSC	(68, 69, 206)
FGF2	Sertoli cells, Leydig cells, germ cells	Expansion and induction of differentiation in A_{undiff}	(39, 69, 83, 86)
FGF4/5/8	LECs	Regulates SSCs and their niche	(40)
WNT6	Sertoli cells and Interstitial cells	WNT ligand, promotes entry of SSC into differentiation-primed progenitor state	(96, 97)
RA	Sertoli cells, spermatocytes	Differentiation of SSCs into differentiating spermatogonia and spermatocytes	(5, 99, 101)
GFRA1	Self-renewing A_{undiff} (A_s, A_{pr})	Forms GDNF receptor, SSC self-renewal.	(68, 70)
SHISA6	A subset of GFRA1$^+$ A_{undiff}	WNT inhibitor prevents premature entry of SSCs into the differentiation-primed state.	(97)
RARG	Differentiation-primed progenitor A_{undiff} (majorly A_{al})	Promotes differentiation of A_{undiff} cells into differentiating spermatogonia	(85, 104)
PLZF	A_{undiff} (A_s, A_{pr}, A_{al}), early differentiating spermatogonia	Promotes SSC self-renewal	(106, 118, 120, 121)
POU5F1	A_{undiff} (A_s, A_{pr}, A_{al})	Proliferation and maintenance of SSCs	(125, 126)
NANOS2	GFRA1$^+$ A_{undiff}	Prevents premature entry of SSCs into the differentiation state	(53, 80, 128)
ID4	GFRA1$^+$ A_{undiff}, differentiating spermatogonia	Promotes SSC self-renewal	(35)
NGN3	Differentiation-primed progenitor A_{undiff} (majorly A_{al} and few A_s and A_{pr})	Sensitizes progenitor A_{undiff} to retinoic acid signaling and mark their entry into differentiation state.	(42, 43, 85)
STRA8	Differentiating spermatogonia	Induces the entry of differentiating spermatogonia into meiosis	(5, 149, 151)
KIT	Differentiating spermatogonia	Initiates entry of differentiating spermatogonia into meiosis	(85, 150)

of self-renewal of GFRA1$^+$ SSCs. GDNF acts through two different signaling pathways to induce target genes that promote SSC self-renewal - the phosphatidylinositol 3-kinase (PI3K)/Ak strain thymoma (AKT)-dependent pathway (115) and the Src family kinase (SFK) pathway (78). The well-studied GDNF-inducible self-renewal genes include *Ets-variant gene-5* (*Etv5*), *Bcl6b, and Lhx1, Pou3f1* (Oct6), *Brachyury (T)* and *Id4*, as reviewed by Song and Wilkinson (116). *Id4* promotes the undifferentiated cell state by its ability to inhibit basic helix-loop-helix transcription factors, most of which promote differentiation. Moreover, ID4 is unique in being the only protein known to be expressed in A_s and not A_{pr} or A_{al} SPGs (31). There are many GDNF-independent and SSC-derived factors such as PLZF, FOXO1, GILZ and TAF4B that also contribute to regulate the self-renewal state of SSC (Figure 4).

Promyelocyctic leukemia zinc finger (PLZF), also known as ZBTB16 or ZFP145, is a transcriptional repressor that binds to DNA via its Kruppel-type zinc finger domains and recruits histone deacetylases (HDACs) via its POZ domain. It is expressed throughout the A_{undiff} population and therefore is widely used as a marker for A_{undiff} SPGs (117). Accordingly, loss of functional PLZF results in progressive loss of germ cells and infertility (118). In mouse SSCs, PLZF has been suggested to work in at least three different ways to ensure SSC maintenance - firstly, by modulating the activity of Sal-like protein 4 (SALL4), whose action is associated with spermatogonia differentiation; secondly, by directly and indirectly (via *Foxo1 and Etv5*) repressing differentiation genes (including *c-Kit*) and stimulating stemness genes of the spermatogonia (many of which are also GDNF targets) and thirdly, by indirectly opposing the

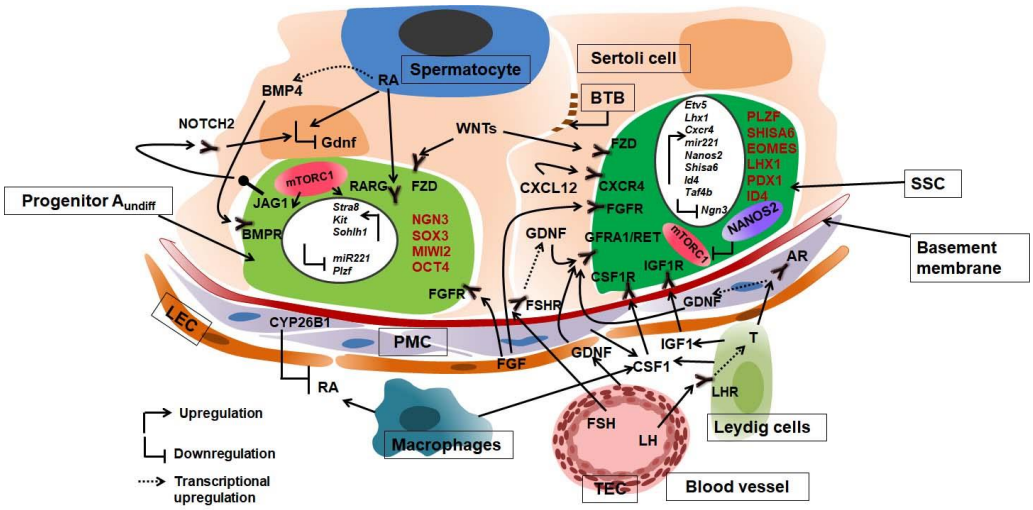

Figure 4. Molecular mechanism regulating SSC fate in adult mouse testis. The SSC niche is contributed by different juxtacrine and paracrine factors secreted by the somatic cells and germ cells respectively. Paracrine factors such as GDNF, FGF, CSF1, IGF1 and CXCL12 derived from Sertoli cells, Leydig cells, testicular endothelial cells (TECs) of the vasculature and lymphatic endothelial cells (LECs) maintain the self-renewal state of SSC via their cognate receptors by upregulating the expression of genes including *Etv5*, *Lhx1*, *Cxcr4*, *Nanos2*, *Shisa6* and *Id4*. These SSCs are marked by the expression of PLZF, SHISA6, EOMES, LHX1, PDX1, and ID4. Certain self-renewal factors function by inhibiting differentiation pathway, for example, SHISA6 is a WNT inhibitor and Nanos2 sequesters and inhibits activity of mTORC1 pathway. Follicle stimulating hormone (FSH), derived from vasculature, and testosterone (T), derived from peritubular myoid cells (PMCs), act indirectly by upregulating GDNF expression. Differentiation primed progenitor cells of A type undifferentiated (A_{undiff}) spermatogonia pool express an exclusive set of genes compared to SSCs. The progenitor cells are marked by the expression of NGN3, SOX3, MIWI2, and RARG. The major characteristic of progenitor cells is the responsiveness to retinoic acid (RA), synthesized by pre-meiotic and post-meiotic germ cells such as spermatocytes, through RARG receptor resulting in upregulation of *Stra8*, *Kit*, *Sohlh1* and downregulation of *Plzf* and *kit*-degrading miR221 making the cells vulnerable to differentiation into differentiating spermatogonia. The progenitor cells also ensure the shutting down of self-renewal pathway by RA and NOTCH signaling mediated inhibition of GDNF expression. RA degrading enzyme, CYP26B1, secreted by PMC ensures removal of RA from extratubular sources like peritubular macrophages. The color key depicting different cell types is used throughout this article. Figure adapted with permission from Mäkelä and Hobbs (45).

differentiation-promoting mTORC1 pathway through the upregulation of mTORC1 inhibitor DDIT4 (119–122). The activation of DDIT4 transcription by PLZF is likely to be important for SSC maintenance since the repression of mTORC1 signaling by DDIT4 is also necessary for maximal expression of both components of the GDNF receptor, viz., GFRA1 and c-RET in SSCs. Together, these data support a model in which PLZF operates in a molecular circuit that amplifies the responsiveness to GDNF signals as a means to maintain SSCs.

Forkhead box protein O1 (FOXO1) is a member of the forkhead transcription factor family that has a variety of functions, including regulation of glucose metabolism, insulin signaling, control of cellular growth and stem cell homeostasis. FOXO1 is highly expressed in SSCs and germ cell specific knock-out of FOXO1 results in spermatogonia arrest. FOXO1 plays a role in SSC maintenance by directly or indirectly upregulating SSC self-renewal genes including *Lhx1, c-Ret, Egr2 and Tex19*. In addition, FOXO1 regulates stem cell marker genes *Gata2* and *Dppa4* (123). In contrast to its role in GDNF induced response, PI3K/AKT pathway has a "pro-differentiation" role in gonocytes, wherein it prevents cytoplasmic FOXO1 from entering the nuclei of gonocytes and activates a cell proliferation program precociously. Hence, the precise role of FOXO1 activity in SSCs needs further investigations.

TATA-box binding protein associated factor 4b (TAF4B) is a gonad-specific subunit of transcription initiation factor TFIID, which is a component of RNA polymerase II pre-initiation complex. Mice null for *Taf4b* exhibit a unique testicular phenotype that includes normal fertility at early ages followed by a complete loss of fertility by

P84, characterized by spermiogenesis defects, loss of germ cells and testicular degeneration. This phenotype is attributed to defective perinatal germ cell development (gonocytes to spermatogonia transition) and SPG proliferation (124). Thus, TAF4B appears to be active in maintaining proliferation of gonocytes and SSCs.

POU Class 5 Homeobox 1 (POU5F1 or OCT4) is a POU-subclass homeobox transcription factor that is essential for the establishment and maintenance of stem cell activity. The major cell types that express POU5F1 in mice are the embryonic primordial germ cells, gonocytes, the precursors of SSCs that are most abundant at birth and A_{undiff} SPGs that are present after birth (125). Knock-down of POU5F1 in cultured SSC caused decrease in the proliferation rate, survival levels and SSC activity as assessed by transplantation assay (126). However, Wu *et al*. found that transient knockdown of POU5F1 did not significantly reduce SSC numbers in $Thy1^+$ SPG cultures. This discrepancy in the data can be attributed to the difference in the two studies with respect to origin of the cells, the culture conditions, and the genetic background of mice (127). Hence, further investigations are required to determine the function of POU5F1 in SSCs.

Similar to ID4 and PLZF, NANOS2 is a SSC self-renewal factor that functions by preventing premature differentiation of SSCs. NANOS2 is an RNA-binding protein that acts by sequestering and consequently inhibiting the activity of components of differentiation promoting mTORC1 pathway in a ribonucleoprotein complex (128). It was recently shown that NEDD4 (neural precursor cell expressed developmentally downregulated protein 4), an E3 ubiquitin ligase, targets NANOS2 for degradation and thus promotes differentiation (129). NANOS2 also associates with DND1 (Dead end protein homolog 1) in A_s and A_{pr}, and deletion of either results in gradual depletion of SSCs. Conditional disruption of postnatal *Nanos2* in mouse testis depleted SPG reserves, whereas overexpression of NANOS2 in mouse testis resulted in accumulation of A_{undiff} SPGs implicating the importance of NANOS2 in SSC self-renewal (53).

The stem cell property of SSCs is also governed by post-transcriptional and epigenetic mechanisms. The post-transcriptional mechanism of gene regulation include an array of regulatory noncoding RNAs (ncRNAs), such as microRNA (miRNA), long ncRNA (lncRNA), piwi-interacting RNA (piRNA) and circular RNA (circRNA), that have been observed to be involved in regulating the SSC self-renewal through forming an intricate regulatory network together with protein-coding genes (130). Dozens of miRNAs have been identified that are specifically or preferentially expressed in SSCs and have been found to modulate expression of known SSC self-renewal genes. For example, miR-21 is regulated by ETV5 (131), miR-20 and miR-106a upregulates the expression of the self-renewal factor *Plzf*; whereas, miR-221 and miR-146a suppresses the expression of the differentiation factor c-Kit (132, 133). LncRNAs are arbitrarily defined as transcripts of greater than 200 nucleotides in length that lack functional ORFs and can be localized to both the nucleus and cytoplasm. Accumulating evidence suggests that lncRNA also has substantial contributions in SSC maintenance (130). Two spermatogonia-specific lncRNA candidates, known as SPGA-lncRNA1 and 2, have exhibited a significant inhibitory effect on differentiation in an *in vitro* model (134). PiRNAs are a distinct class of small non-coding RNAs primarily expressed in the germline cells (135). These 21-31 nucleotide-long non-coding RNAs produced by a Dicer-independent mechanism are loaded into specific PIWI orthologs to form the piRNAs-PIWI complex and this ribonucleoprotein complex along with other protein components perform their function (136). PIWI proteins are composed of three proteins in mice namely, MIWI, MIWI2 and MILI. Although piRNAs-PIWI have been delineated to be involved during the meiosis of spermatocytes and spermiogenesis stages of spermatogenesis, some *in vitro* and *in vivo* (137, 138) studies are suggestive of their involvement in SSC maintenance and self-renewal. However, further research is required to explore the functions of PiRNAs in early stages of spermatogenesis. CircRNAs are an emerging class of single-stranded RNA molecules with a covalently closed loop structure generated through a special type of alternative splicing termed backsplicing, derived mostly from exons, but also from antisense,

intergenic, intragenic, or intronic regions. CircRNAs can modulate gene expression via multiple actions, including sponging miRNAs and proteins as well as regulating transcription and splicing. 5,573 circRNAs are identified so far in SSC and the average levels of circRNAs exhibited dynamic changes during male germ cell development, indicating that these circRNAs are probably involved in SSC self-renewal and differentiation (139). Nevertheless, the biological function of these circRNAs in the SSCs remains elusive.

Interestingly, the epigenome (DNA methylation at CpG sites plus histone modifications) of male germ cells undergoes profound changes during fetal development, whereas in postnatal germ cells the epigenetic marks are more stable. It has been shown that the epigenetic landscape of SSCs is plastic and is similar to that of pluripotent cell types, characterized by bivalent (both activating H3K4me3 and repressing H3K27me3) histone modifications placing promoters in a poised state capable of dynamic activation (140).

Emerging evidences have also identified many potential players of SSC maintenance. Reactive oxygen species (ROS), which were considered to be inhibitory for stem cell function, have striking self-renewal promoting effects in SSCs (141, 142). Cyclin M1 (CNNM1) protein that belongs to the Ancient Conserved Domain Protein family appears to act as a cytosolic copper chaperone. Using *in vitro* cultured mouse SSCs and spermatogonial cell lines, CNNM1 was found to be associated with SPG self-renewal (143). Recently, preferentially expressed antigen of melanoma 12 (PRAME12) protein, expressed in A_{undiff} and early differentiating SPGs, was found to be contributing to SSC maintenance. Knock-out of *Pramef12* impaired SSC self-renewal and early differentiation, resulting in a Sertoli cell-only syndrome in adult mice (144).

6.2. Differentiation priming

This stage is marked by the exit of SSCs from the self-renewing state to a differentiation primed progenitor state, wherein the A_{undiff} cells become sensitive to retinoic acid signals. The most significant molecular event triggering differentiation priming is the activation of mTORC1 pathway in SSCs (38, 121, 129, 145). In addition to mTORC1 pathway, the WNT/beta-catenin signaling also promotes transition from self-renewing to RA-responsive progenitor state of SPGs (95–97, 146). These pathways result in the downregulation of self-renewal genes including *Gfra1, Ret, Lhx1, Eomes* and *Pdx1* and upregulation of genes including *Ngn3, Sox3, Lin28* and *Rarg* (39) as shown in Figure 4. Hence, these upregulated genes are used as markers to identify progenitor SPGs.

6.3. Differentiation commitment

The $NGN3^+$ progenitor SPGs are shown to express RARG which increases their differentiation competence by making them responsive to differentiation inducing RA signaling (85, 147). Progenitor SPGs have the capacity to transition into a self-renewing state or to enter differentiation state. Accordingly, the timely onset of differentiation is regulated by managing the availability of RA and RARG expression within the seminiferous tubule. Since the meiotic and post-meiotic germ cells are the primary source of RA during spermatogenesis, the extratubular supply of RA is kept blocked by its degradation by the CYP26B1 enzyme expressed in PMCs (146, 148). An alternate mechanism of sequestration of RA precursors by round spermatids at stages II-VI has also been proposed as a mechanism to prevent pre-mature entry of progenitors into differentiation states (103). It is also believed that the somatic cell derived niche factors determine cyclic expression of RA specifically during the VII-VIII stages which dictate the spermatogenic wave (101). As a result of rise in RA levels at stages VII-VIII, the $RARG^+$ progenitor cells transit into type A_1 differentiating SPGs and expresses early markers of spermatogonial differentiation including c-KIT and stimulated by retinoic acid 8 (STRA8) as shown in Figure 4 (85, 105, 149–151).

6.4. Stage specific regulation of niche factors

The three stages of SSC life cycle mentioned above (Section 6.3) can be correlated with stages of the seminiferous epithelial cycle (Figure 5). GDNF levels are high during the stages XII-IV which

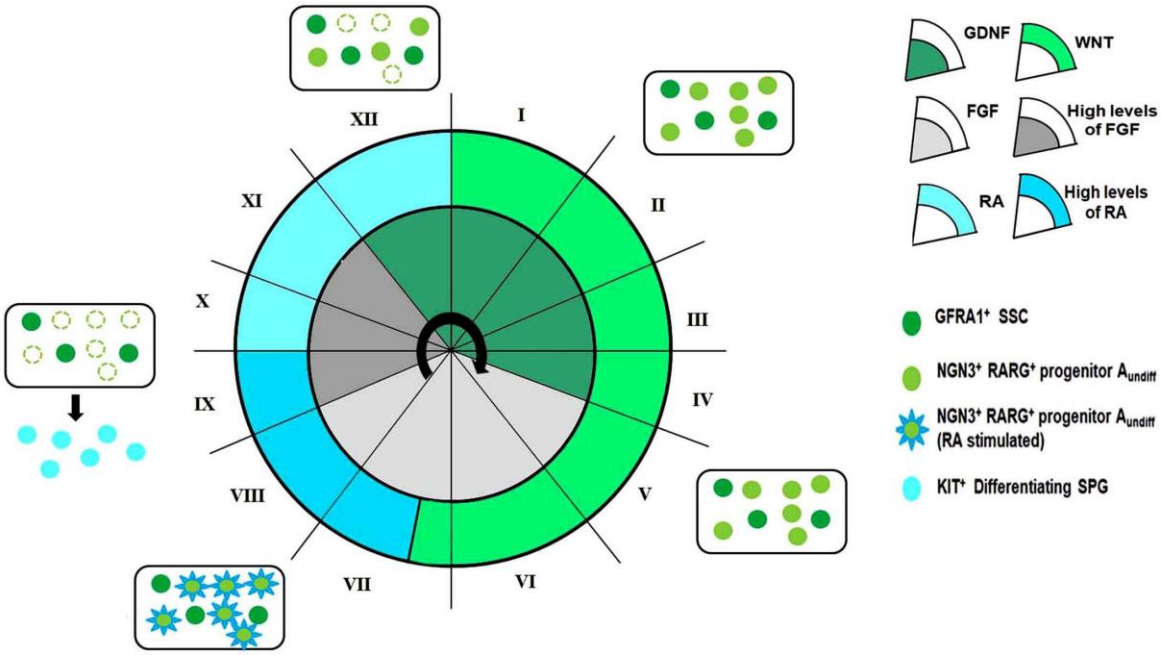

Figure 5. Regulation of SSC niche across the seminiferous epithelial cycle in adult mouse. During the steady state spermatogenesis, the high level of GDNF present at stages XII-IV is conducive for maintenance of self-renewing subset of undifferentiated A type (A_{undiff}) spermatogonial cells (SPGs) which constitute the SSC population. The presence of WNT ligands in stages II-VII ensures existence of progenitor A_{undiff}. The peak in the concentration of retinoic acid (RA) which is observed during stages VIII-IX results in the stimulation of the progenitor cells to give rise to differentiating SPGs. The reduction in the concentration of RA levels during stage X-XII and the concomitant increase in the level of FGF mark the onset of the next SSC self-renewal and differentiation cycle. The color key depicting different cell types is used throughout this article. Figure adapted with permission from Mäkelä and Hobbs (45).

are marked by proliferation of A_{undiff} and self-renewal of SSCs (29). Wnt6 signaling is strongly active in stages I-VIII (73, 97). Moreover, RA pulses start at late stages of VII. Consecutive to the action of WNT and RA signaling on SSCs, $RARG^+$ progenitor cells are observed during the stages of II-VII (85). The highest levels of RA are recorded at stages VII-IX which coincide with the appearance of KIT^+ differentiating SPGs. Mäkelä and Hobbs proposed a model, wherein the reducing levels of RA and a sharp decline in the number of FGF-consuming cells (due to A_{undiff}-to-A_1 transition) at later stages (X-II) of the cycle allow GDNF and FGF levels to rise, resulting in the next wave of proliferation of A_{undiff} (45).

6.5. SSC maintenance during homeostasis and regeneration

SSC niche is dynamic in nature and varies with the state of the biological system, as is observed during postnatal and pre-pubertal testis development, homeostasis and regeneration after testicular tissue injury. The dynamic SSC niche subsequently results in the dynamic interconversion of undifferentiated SPGs into different states. In developing testis, the SSC niche produces abundant growth factors and less inhibitory factors resulting in an environment that supports self-renewing proliferation. On the other hand, the SSC niche in homeostatic adult testis produces a moderate amount of mitogenic factors to maintain a stable SSC number. Accordingly, the majority of SSCs in developing testis are in mitotic state, while the SSCs in homeostatic condition are likely to be quiescent or in a slow cycling state. In regenerating testis, the SSC niche again stimulates growth factor production for SSC expansion.

It has been reported that during regenerative conditions, progenitor A_{undiff} cells (*Gfra1*$^-$ *Ngn3*$^+$) re-express the self-renewal genes and acquire SSC activity. Furthermore, during

transplantation assay which represents a regenerative condition, *Ngn3*$^{+}$ *Miwi2 (Piwil4)*$^{+}$ *Gfra1*$^{-}$ *Kit*$^{-}$ $A_{undifff}$ cells identified as differentiation primed progenitor cells also display reconstitution of stem cell activity (42). Recently, a minor subset (0.2% of testicular cells) of GFRA1^{+} cells were identified as co-expressing *Pdx1*, *Brachyury*, *Eomes* and *Lhx1* (39). Interestingly, it was observed that this subset of SPGs adopts a different expression profile signifying different cellular states in response to the niche conditions. The self-renewal state marked by *Pdx1*$^{+}$, *Eomes*$^{+}$ and *Lhx1*$^{+}$ prevails during homeostasis. However, during postnatal development and under regenerative conditions, when the niche provides excess of self-renewal signal, *Eomes* and *Lhx1* expression are upregulated and *Pdx1* is down-regulated (39). Hence, *Pdx1*, *Eomes* and *Lhx1* expression might be required for long term maintenance of SSCs under steady state spermatogenesis. Thus, replenishment of cells in the differentiation-primed state and restoration of self-renewing fractions after genotoxic damage are possible via dynamic interconversion of these A_{undiff} states

7. *IN VITRO* MANIPULATION OF MOUSE SPERMATOGONIAL STEM CELLS

SSCs are the only cells in the adult body which can transmit genetic information to subsequent generations and increase in number following birth. Thus, SSCs provide an accessible and renewable source of genetic code which can have enormous valuable applications for germline modifications in the field of medicine and molecular breeding. The development of spermatogonial transplantation techniques paved the way for *in vitro* manipulation experiments on SSCs (152). The transplantation assay for SSCs was primarily developed by Brinster and Zimmermann. They injected testis cell suspension containing SSCs into seminiferous tubules of busulfan-treated infertile mouse and congenitally infertile Kit^{W}/Kit^{W-v} mouse. The transplanted SSCs colonized the recipient seminiferous tubule and started spermatogenesis demonstrating the self-renewal and reconstitution properties of the injected cell suspension. The generated spermatozoa were able to produce offspring (153). Moreover, it was reported that one colony generated by spermatogonial transplantation is derived from a single SSC (154, 155), implying that the spermatogonial transplantation technique can be used as a biological assay for SSC identification and quantitation. The first transgenic animals using SSCs were created by transduction of mouse SSCs using a retrovirus vector containing the beta-galactosidase gene (156). Subsequent development of long-term culture systems has allowed a variety of techniques to be used for genetic modification of SSCs such as homologous recombination and gene-editing using the TALEN and CRISPR/Cas9 system (155, 157).

7.1. Establishment of germline stem cell (GS) culture

Two-dimensional (2D) culture of isolated SSCs has become a popular approach to study the influence of niche factors involved in the regulation of their proliferation and the differentiation of their progeny. The first report of culture and maintenance of mouse SSCs *in vitro* was published in 1998 (158). In this study, unfractionated testicular cells from neonatal and adult transgenic mice expressing beta-galactosidase were cultured for approximately 4 months on SIM mouse embryo-derived thioguanine and ouabain resistant (STO) feeder cells, which have been routinely used for mouse embryonic stem (ES) cell cultures, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). In transplantation assay, the cultured cells derived from neonatal testis formed spermatogenic colonies in the recipient testis demonstrating the stem cell potency of the cultured cell. However, no expansion of SSCs was observed, and the number of surviving SSCs was very low. Since the proliferation of stem cells is regulated intrinsically and extrinsically by the stem cell niche, several modifications in the culture condition were performed to identify the soluble factors which would support the maintenance and expansion of SSCs in culture. A beneficial effect of GDNF, minimal essential medium (MEM) and OP9 bone marrow stroma or fibroblast cell lines as feeder layers was observed on SSC maintenance in this short-term culture experiment (30). However, the expansion of SSC and consequent long-term

culture was not obtained using these culture conditions.

The first report of successful *in vitro* expansion of mouse SSCs was in 2003 by Kanatsu-Shnohara *et al*. The SSCs in this study were enriched by differential plating and cultured on mouse embryonic fibroblasts (MEFs) feeders in a serum-supplemented proprietary StemPro-34 (Gibco)-based medium, which contained the original StemPro-34 supplement plus 16 individual compounds and FBS with a cytokine mixture of GDNF, FGF2, LIF, and epidermal growth factor (EGF). Using the enriched culture media, quiescent SSCs resumed proliferation and formed grape-like clusters that expressed spermatogonia markers ITGA6, ITGB1 and EPCAM. The cultured cells proliferated for approx. 5 months in a logarithmic manner without losing colonization activity in transplantation assays. Moreover, the haploid male germ cells could produce offspring, proving that the cultured cells possessed the proper SSC activity (71). Although the cultured cells exhibited stem cell activity, these cells appeared (grape-like aggregates) different from the isolated SSCs in seminiferous tubules. Hence, these cultured SSCs were termed as germline stem cells. Subsequently, some studies reported comparable results regarding GS cell derivation from other mouse strains under similar conditions (69, 159). These results suggested that the combination of mouse strain and age, feeder cells used, and medium composition affected the *in vitro* expansion of SSCs.

7.2. Genome editing of GS cells

GS cells are considered to be more suitable than embryonic stem cells (ESC) for genome editing of germline lineages as GS cells have the following advantage over ES cells: 1) stable epigenetic/ genetic properties, 2) normal karyotype, 3) normal genomic imprinting status, 4) susceptible for drug selection and 5) can be maintained *in vitro* for as long as 2 years (71, 160). Transgenes can be introduced and established in GS cells through conventional gene transfer techniques such as lipofection, electroporation, and retroviral vector infection, lentivirus-, adenovirus-, and adeno-associated virus-mediated gene transductions (160–162). However, the genetic modification of GS cells has proved to be more difficult than that of ESCs, mainly due to low gene transfer and genome targeting efficiency in GS cells. The targeting efficiency of genome editing using homologous recombination has been increased by several fold using site specific double strand break producing nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases effector nucleases (TALENs) (157, 163). Successful genome editing using clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 technology for base pair substitutions and transgene knock-in is also reported in mouse GS cells (164).

7.3. *In vitro* spermatogenesis

Although SSCs can be maintained and expanded for several months in 2D culture (section 7.1), it is difficult to induce meiosis or later stages of spermatogenesis in these conditions (71, 165). A few studies have demonstrated the ability of immortalized spermatogonia cells to differentiate into spermatocytes and round spermatids (166, 167). However, this strategy has its own limitations such as manipulation of the genome for immortalization of germ cells which is not feasible for reproduction of animals, l*et al*one for humans. The passage of SPGs into meiosis (and hence, migration through the seminiferous epithelium) depends upon the structural support of the seminiferous epithelium, interaction with the extracellular matrix (ECM) and the availability of SSC niche factors

The importance of Sertoli-germ cell interaction was also revealed in Sertoli-spermatogenic cell co-cultures established from 13- to 18-day-old mice that were able to convert pachytene spermatocytes to round spermatids capable of developing normal and fertile offspring when injected into mature oocytes (168). The requirement of spatial and temporal testicular microenvironment was understood in *ex vivo* organ culture. The first description on *in vitro* spermatogenesis was reported by Martinovitch, who used newborn mouse testis tissue cultured on a clot composed of equal parts of fowl plasma and fowl embryo extract and demonstrated the development of pachytene spermatocytes from presumably immature spermatogonia in the culture (169). Organ

culture experiments in the later years also achieved limited success till the pachytene spermatocyte stage. In 2003, Suzuki and Sato isolated seminiferous tubules from 5-day old mice and cultured it on an agarose gel block at the liquid-air interface (170). The round spermatids obtained were injected into the oocytes resulting in embryos that developed up to the 8-cell stage but no offspring was produced. This technique is also applicable to adult and cryopreserved tissues (157, 171).

Reproducing the testicular third dimension *in vitro* (three-dimensional or 3D culture) has been achieved by embedding various (dissociated) cell types of the seminiferous tubule in a collagen gel matrix. In this way, a suitable support is provided for isolated germ cells to interact with Sertoli cells and other structural and hormone-producing elements. Stukenborg *et al*. established soft agar culture system (SACS), wherein enriched SSCs from 10-day-old mice were mixed with the gel-agar medium (0.35%) and incubated on a solid-agar base (0.5%). The agar was mixed with a high glucose DMEM solution. This approach yielded enhanced viability, germ cell meiosis, and differentiation up to post-meiotic stage (172). SACS technique has reported morphologically normal spermatozoa from pre-meiotic germ cells (173, 174). Although the study of *in vitro* spermatogenesis progressed significantly over the last century, mouse tissues have been more feasible for spermatogenesis under culture conditions (175).

7.4. Germ cell induction from pluripotent stem cells

There have been attempts to generate gametes or PGCs *in vitro* from ESCs both in mice and humans by isolating cells that express a germ cell marker(s) in spontaneously differentiated embryoid bodies (176). However, these attempts were inefficient in obtaining the induced cells (less than 1.0%) and in generating induced gamete-like cell derived healthy offspring, thus unsuitable for analyzing the events that take place before the emergence of germ cell-like cells. While ESCs are pluripotent stem cells (PSCs) derived from the inner cell mass (ICM) of preimplantation blastocysts at E3.5-E4.5 *in vitro*, epiblast stem cells (EPiSCs) are PGCs derived from epiblast (which are the precursors of PGCs *in vivo*) of post-implantation embryos at E5.5-E6.5 *in vitro* (177). EpiSCs exhibit a primed pluripotency and retain attributes of the original epiblasts making them a superior source for the generation of germ cell-like cells compared to ESCs *in vitro* (178). Hence, recent studies have focused on inducing PGC like cell (PGCLCs) from epiblast-like stem cells (EpiLCs) generated from ESCs and induced pluripotent stem cells (iPSCs). *Fragilis*, *stella* (*Pgc7/Dppa3*) and *Blimp1* (also known as *Prdm1*) genes in the epiblast and BMP4 signaling from the extraembryonic ectoderm were found to be required for the specification of germ cell fate in mice (11). Subsequently, a transgenic mouse stain and ES cell line were established by Ohinata *et al*., which showed germ cell commitment by dual fluorescence reporter genes (*Blimp1-Venus*:: *Stella-Cfp* reporter mouse/ES cells) (179). Thereafter, Hayashi *et al*. in 2011 succeeded in inducing EpiLCs from ES and iPS cell lines using activin A and FGF2, and then PGCLCs were derived from aggregated EpiLCs in suspension culture by stimulation with BMP4, BMP8b, SCF, LIF, and EGF. The resulting PGCLCs were then transplanted into infertile mouse testes to produce haploid male germ cells (180).

8. CLINICAL APPLICATIONS OF SPERMATOGONIAL STEM CELLS

Infertility is a worldwide problem affecting 15-20% of couples globally with male factor involvement estimated to be present in about 50% of cases, with sole responsibility in 30% of cases and with a co-contributing female factor in 20% of cases (181, 182). Severe male infertility, including azoospermia and oligoasthenozoospermia, as well as testicular dysfunction can result from genetic or medical conditions such as Klinefelter syndrome, environmental insults such as infections, inflammation/autoimmunity or gonadotoxic medical treatments such as oncotherapies (183–185). Fertility preservation is proposed for all these health conditions, especially in pediatric cancer patients from the perspective of future interventions allowing parenthood. For adult men or adolescents, cryopreservation of ejaculated or surgically retrieved sperm is routinely proposed before gonadotoxic therapies, while for prepubertal boys,

cryopreservation of a testicular biopsy of immature testicular tissue (ITT) containing SSCs is now ethically accepted as the only way to offer a fertility preservation strategy (186).

There are at least four potential ways to theoretically use cryopreserved pre-pubertal or adult testicular tissue biopsies or germ cell preparations to obtain functional sperm. These includes the following: 1) autologous transplantation (auto-transplantation) of testicular tissue termed as testis tissue transplantation (TTT), 2) xenografting of testicular tissue under the back skin or scrotal skin of mice, 3) isolation of spermatogonial stem cells, with and without expansion, for auto-transplantation and 4) isolation, expansion, and maturation of germ cells *ex vivo* via 2D, 3D, and organoid tissue cultures.

8.1. Testicular tissue transplantation

TTT has the advantage that it retains SSCs within their niche and ensures germ cell and supporting cell interactions, providing an optimal microenvironment for cell proliferation, maturation and differentiation. However, since TTT carries a potential risk of reintroducing cancerous cells back to the patient and causing malignant relapse (187), it should only be considered for patients diagnosed with non-systemic cancer and/or non-malignant hematopoietic disorders (188). Complete spermatogenesis following auto-transplantation of ITT was first demonstrated in mice (189). Successful autologous/allogeneic TTT has been reported in rhesus monkeys (190), but not in other species including humans. In recent years, ectopic grafting of immature testicular tissues from various mammalian species under the back skin of immunodeficient mice (xenotransplantation) has been developed as a strategy for preserving testicular function and generating mature spermatozoa (189). However, complete spermatogenesis was not achieved in human xenotransplantation cases. Pachytene spermatocytes and spermatid-like cells were reported in human ITT xenotransplants placed into the scrotum of castrated immunodeficient mice (191, 192), while early spermatocytes were detected in xenotransplants under the dorsal skin (193). Intratesticular xenotransplantation also led to differentiation only up to pachytene spermatocytes stage (194). The reason for inefficient spermatogenesis in human xenotransplantation studies is believed to be the long duration of prepubertal development (8-10 years) observed in humans which may not be achieved in transplant recipient mouse systems (193).

8.2. SSC transplantation

Brinster's group was the first to demonstrate successful transplantation of testicular cell suspension containing SSCs with development of mature sperm in mice using freshly isolated and cryopreserved prepubertal or adult mouse testicular cell suspension (152, 195). Many studies thereafter have reported live offspring generation in different species including mice, rats, goats, chickens, and sheep and embryo development in non-human primates following auto-transplantation of cultured SSCs (196–200). So far, only one report has described autotransplantation of cryopreserved human testicular cell suspension in patients cured of non-Hodgkin's lymphoma, but no follow-up was published (201, 202). The small size of human testicular biopsy samples makes it difficult to isolate SSCs for preservation and transplantation, making *in vitro* expansion and maturation of human SSC critical. Other important concerns are the risk of neoplastic contamination of cryopreserved tissue with subsequent possibility of re-inducing the disease in a cured patient and the need for standardization of an efficient cell injection technique. The availability of an undamaged recipient niche which would support migration, proliferation and differentiation of the transplanted SSC is also an important factor for the success of SSC transplantation compared to TTT.

Long-term *ex vivo* propagation and expansion of pre-pubertal SSC and adult SSC from normozoospermic and infertile men (203, 204) have both been reported and these cells were shown to have stable genetic and epigenetic profiles after culture (205). Recently, Bhang *et al.* discovered that human endothelial cells secreted GDNF, basic fibroblast growth factor (bFGF), stromal cell-derived factor-1 (SDF-1), macrophage inflammatory protein 2 and insulin-like growth factor-binding protein 2 and could support SSC growth for at least 150 days (206).

To eliminate the risk of cancer-cell contamination of testicular cell suspension, attempts have been made to efficiently isolate human SSCs from cancer cells using specific markers and cell-sorting techniques (200, 207). However, these techniques did not allow complete removal of cancer cells. Alternatively, culturing the testicular cells to propagate SSCs led to elimination of all contaminating malignant cells after 26 days of culture (208).

The rete testis ultrasound-guided injection was established as the best approach for SSC transplantation into large testes with 70% of the tubules filled after an average of 30 min in the monkey testis (209). More recently, an infusion pump was used to inject SSCs in human cadaver testes, showing less variability between subjects if compared to the injection under hydrostatic pressure (210). However, leakage in the testicular interstitium was observed and further studies are warranted to improve the injection technique.

Eventually, as SSCs after being re-transplanted have to migrate to colonize the host testicular stem cell niche to gain physical and molecular support for their proliferation and differentiation, an undamaged niche is of paramount importance for a successful SSCs transplantation.

8.3. *In vitro* maturation of SSCs

Although testicular tissue or SSC transplantation are promising fertility preservation strategies, the risks related to the transplantation of residual neoplastic cells limit their application. *In vitro* maturation and differentiation of cryopreserved SSCs into haploid cells for later usage in assisted reproduction techniques (ART) would bring a promising fertility preservation option for childhood cancer survivors. Similarly, it would benefit the infertility treatment of the wide range of non-obstructive azoospermia patients who are not able to produce sperm but still have SSCs. *In vitro* spermatogenesis using human testicular cells was earlier reported in 1967 by organ culture which demonstrated differentiation of spermatocytes from preleptotene to pachytene stage (211). Many studies on organ culture in the following year showed limited success with differentiation of spermatogenic cells, that too achieved only till pre- and post-meiotic spermatocyte stage (175). Recently, development of haploid germ cells from spermatogonia cells using organotypic culture for testicular tissues from pre-pubertal cancer patients was reported (212, 213). Nevertheless, the characterization of non-cultured and cultured human SSC remains challenging/ controversial due to the heterogeneity of spermatogonia, ambiguity of human SSC-specific markers, and the inherent contamination of SSC with other testicular cells during the culture process. In addition, cell culture conditions for *ex vivo* propagation and differentiation of mouse spermatogonia have not been fully translatable to human SSC.

8.4. Pluripotent stem cells

Human ESCs and iPSCs have been considered as valuable sources of pluripotent cells to obtain germ cells *in vitro*. Human ESCs have been utilized to model and improve our understanding of human germ cell development and infertility, and have also been investigated in stem cell-based fertility preservation strategies. Germ cells or gonadal support cells have also been developed from human iPSCs derived from autologous cells such as skin biopsy-derived fibroblasts or blood cells or urine derived cells or hair keratinocytes (214–216). These strategies are encouraging for patients who lack spermatozoa or SSCs. Similar to the case in rodent species, pluripotent stem cell based strategies involve the derivation of PGCLC from a patient's somatic cell (typically dermal fibroblast, keratinocytes, or blood cells) via induction of iPSC, to be used for transplantation into the testis to induce spermatogenesis *in vivo*, or to pursue *in vitro* derivation of gametes (216, 217). These strategies have been employed for Klinefelter and non-obstructive azoospermia patients albeit with limited success (218, 219). However, direct reprogramming of mouse skin fibroblasts into embryonic Sertoli cells and Leydig-like cells has been reported (220, 221). Sertoli cell- and Leydig cell-induced differentiation of human iPSC was also recently reported (222, 223). An important limitation of human ESC and human iPSC-based approaches, apart from the legal and ethical barriers, is the high risk of accumulation of

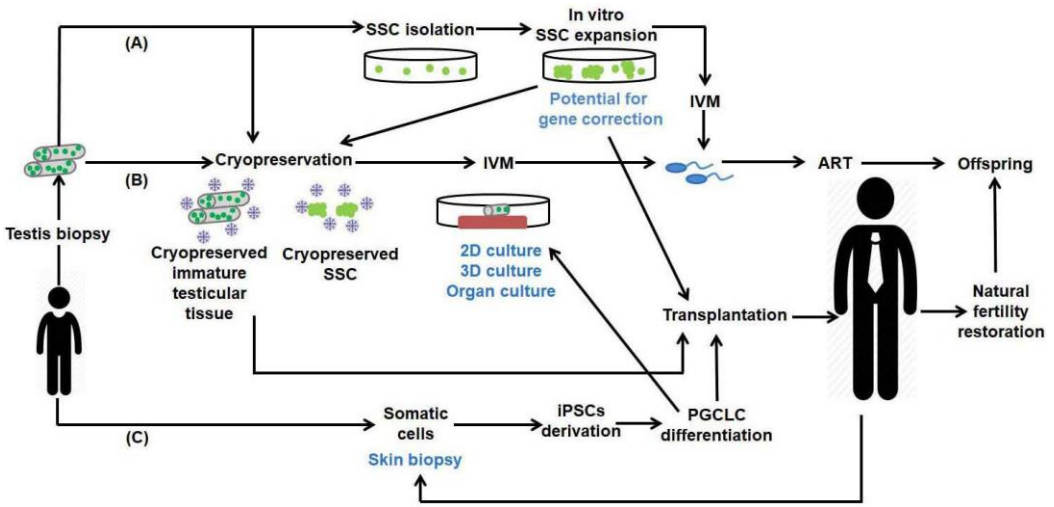

Figure 6. Schematic of potential SSC-based fertility preservation strategies in humans. Three methods for fertility preservation, especially at the prepubertal male stages, have been investigated using animal models and in few instances, using patient samples such as prepubertal cancer patients and Klinefelter patients. A) In the first method, SSCs can be isolated from testis biopsy samples and expanded *in vitro.* The expanded SSC-derived germ-line clusters can be cryopreserved for future application. B) In the second method, the tubules obtained from testis biopsy can be directly cryopreserved for future applications. In the adult stage of the male, the cryopreserved samples can be transplanted back into the testis to restore spermatogenesis and fertility. Alternatively, the cryopreserved samples can be revived by *in vitro* maturation (IVM) in 2D, 3D or organ culture, resulting in the formation of mature sperms that can be applied in assisted reproductive techniques (ART) such as intracytoplasmic sperm injection. Tissue-based approaches have the advantage of preserving the structural integrity of the seminiferous epithelium resulting in efficient restoration. C) In the third method, somatic cells such as fibroblast cells, derived from skin biopsy from prepubertal or adult male, can be reprogrammed *in vitro* to pluripotent cells (induced pluripotent stem cells, iPSCs). These iPSCs can be transdifferentiated *in vitro* into primordial germ cell like cells (PGCLCs) that can be autotransplanted into the adult male to restore natural fertility.

genetic and epigenetic mutations during reprogramming (224). Consequently human ESC or iPSC derivation of germ cells which have the potential to transmit genetic material to the offspring may not be the safest approach for fertility preservation.

9. CONCLUSION

The development of new cellular, molecular and computational technologies such as single-cell transcriptomic analysis has aided the research fraternity to elucidate the heterogeneity of mouse spermatogonial stem cells and somatic cells that contribute to the regulation of SSCs (Figure 6). This should result in a finer characterization of spermatogonia populations and development of a detailed hierarchy of successive cell states in the developmental lineages. Further, a broad-spectrum omics (including transcriptomics, epigenomics and proteomics) based study is envisaged to understand the complexity of SSC function at the molecular and spatiotemporal level. Although the rodent species have been the favored choice of animal model with the ease in handling, housing and breeding, it is important to take into consideration the interspecies differences in spermatogenesis resulting in difficulties in translating rodent data to higher species such as humans. Hence, it is critical to develop an efficient non-human primate model to study the process of spermatogenesis and to improve strategies for fertility preservation and treatment in humans. Availability of efficient manipulation techniques of SSCs would also have non-clinical applications such as improvement of molecular breeding of livestock animals. Significant advances in the area of SSC cryopreservation and *in vitro* maturation point towards the potential of SSC based clinical application to restore fertility in near future. Moreover, evolving germline genome editing research may, in the distant future, allow for the safe use of these approaches for the treatment of genetic factor-induced male infertility. We hope that future research in this line would decode the secrets of

human SSC to the level of our understanding of mouse spermatogenesis, which would enable us to do this task in a safe and efficient manner.

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Abbreviations: SSC, spermatogonial stem cell; FACS, fluorescence-activated cell sorting; RA, retinoic acid; SPG, spermatogonial cell; A_{undiff}: undifferentiated A-type spermatogonia; A_s, A-single; A_{pr}, A-paired; A_{al}, A-aligned; In, intermediate; PGC, primordial germ cell; BMP, bone morphogenetic protein; BLIMP1, B lymphocyte-induced maturation protein-1; PRDM14, PR-domain containing protein 14; FGF, Fibroblast growth factor; WNT, Wingless/integrase 1; SRY, sex determining region Y; CXCR4, SDF1; stromal cell-derived factor 1; CXCR4; C-X-C chemokine receptor type 4; NGN3, neurogenin 3; PGCLC, PGC-like cells; PreSPG, prespermatogonia; ID4, inhibitor

of DNA binding 4; GDNF, glial cell line derived neurotrophic factor; GFRA1, GDNF receptor alpha 1; ITA6, alpha 6-integrin; ITGB1, beta 1 integrin; THY1, thymus cell antigen 1; EPCAM, epithelial cell adhesion molecule; PLZF, promyelocytic leukemia zinc finger; PMC, peritubular myoid cell; CSF1, colony stimulating factor 1; LH, luteinizing hormone; LHR, luteinizing hormone receptor; IGF1, insulin-like growth factor 1; TEC, testicular endothelial cell; VEGFA, vascular endothelial growth factor A; LEC, lymphatic endothelial cells; FSH, follicle stimulating hormone; RET, REarranged during Transfection; CXCL12, C-X-C motif chemokine 12; RARG, retinoic acid receptor gamma; FSHR, follicle stimulating hormone receptor; PI3K, phosphatidylinositol 3-kinase; AKT, Ak strain thymoma; FOXO1, forkhead box protein O1; TAF4B, TATA-box binding protein associated factor 4b; POU5F1, POU Class 5 Homeobox 1; miRNA, microRNA; microRNA, long ncRNA; piRNA, piwi-interacting RNA; circRNA, circular RNA; EGF, epidermal growth factor; STRA8, stimulated by retinoic acid 8; GS, germline stem cell; 2D, two-dimensional; DMEM, Dulbecco's modified Eagle's medium; ESC, embryonic stem cell; 3D, three-dimensional; SACS, soft agar culture system; EPiSc, epiblast stem cells; EpiLCs, epiblast-like stem cells; iPSCs, induced pluripotent stem cells; ITT; immature testicular tissue, TTT; testicular tissue transplantation

Key Words: Spermatogenesis, SSC, Self-renewal, Differentiation, Review

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