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Prospermatogonia

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Prospermatogonia

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Abstract

Prospermatogonia (ProSG)—also called gonocytes or pre-spermatogonia—are a transient population of male germ cells derived from primordial germ cells (PGCs) in the fetus. ProSG undergo successive developmental stages that alternate between quiescence and proliferation. ProSG re-establish the DNA methylation marks erased at the PGC stage. They differentiate into spermatogonial stem cells (SSCs), an event that, in rodents, is accompanied by migration to the SSC niche in the periphery of the seminiferous tubule. In this chapter, we summarize what is known about ProSG, including findings from recent studies that have identified regulatory factors driving key events in ProSG.

Key Points

- Prospermatogonia are understudied transient germ cells that undergo dynamic cellular and molecular changes.
- Prospermatogonia are critical for the genesis of SSCs.
- Epigenetic events occur in prospermatogonia that are essential for the next generation.

Introduction

Prospermatogonia (ProSG) are immature male germ cells derived during embryogenesis from primordial germ cells (PGCs). First characterized by Clermont in the 1950s (Clermont and Perey, 1957), ProSG (originally called gonocytes) are large cells with a prominent spherical euchromatic nuclei that develop into spermatogonial stem cells (SSCs), the stem cells that support long-term spermatogenesis (Mccarrey, 2013; Tan and Wilkinson, 2020; Kluin and de Rooij, 1981). In rodents, a small proportion of ProSG give rise to SSCs, while the rest give rise to differentiating SG that go on to generate the "first wave" of spermatogenesis to permit rapid fertility (Yoshida *et al.*, 2006; Yoshida *et al.*, 2004). While ProSG are a "transient" cell type, they are not optional. These cells undergo dynamic changes critical for laying the foundation for spermatogenesis (Tan and Wilkinson, 2020). They "reset" the DNA methylation pattern erased at the PGC stage and disturbance of ProSG development results in complete sterility (Kaneda *et al.*, 2004). In addition, the failure of ProSG to differentiate appropriately in the gonad can result in the formation of germ cell tumors (Clark, 2007). ProSG have been studied in a wide variety of mammalian species, including mice, rats, yaks, humans, and non-human primates (Loebenstein *et al.*, 2020; Wang *et al.*, 2019; Sharma *et al.*, 2017), but most of what we know about ProSG comes from work in mice. In this review, we focus on studies from mice and humans.

ProSG Development

ProSG are not a uniform cell population. As they develop, they undergo asynchronous changes that involve shifts between cell proliferation and quiescence, as well as activation of a migration event that occurs during the same time frame as reactivation of proliferation (Mccarrey, 2013; Tan and Wilkinson, 2020).

In mice, ProSG first develop from PGCs between E12.5–13.5. This PGC-to-ProSG transition involves morphological changes (Hamer and De Rooij, 2018; Clermont and Perey, 1957) and occurs when PGCs complete their migration to the genital ridge. Once ProSG form at the genital ridge, they are enclosed by differentiating Sertoli cells, a process that generates seminiferous cords.

The first mouse ProSG to form are mitotically-active cells called mitotic or multiplying (M)-ProSG. Many papers refer to these post-migratory germ cells as "PGCs" rather than "M-ProSG," perhaps because both are actively dividing cells. This M-ProSG stage is particularly subject to apoptosis. Lineage tracing analysis showed that apoptosis occurs independent of intercellular bridges, suggesting that death is triggered by intrinsic properties of individual ProSG (Nguyen *et al.*, 2020). It is not known why many M-ProSG die, but it is tempting to speculate that a checkpoint exists at the M-ProSG stage to eliminate poor-quality developing germ cells. In support, apoptosis-susceptible M-ProSG have elevated DNA methylation levels, raising the possibility that errors in epigenetic programming are recognized by a checkpoint mechanism that kills such cells (Nguyen *et al.*, 2020).

Between E14.5 and 16.5, those M-ProSG that survive convert into transition (T) 1-ProSG, whose hallmark trait is quiescence. This transition to a non-proliferative state depends on Sertoli cells (Yan *et al.*, 2020), as well as many protein factors, as described below. While T1-ProSG do not proliferate, they are molecularly active, as they re-establish the DNA methylation marks erased—genome-wide—in PGCs. The proteins that mediate the re-introduction of DNA methylation marks in ProSG and the consequences of this genome-wide epigenetic reprogramming are discussed below.

Around birth, T1-ProSG convert to T2-ProSG, which re-initiate proliferation. This re-initiation of proliferation occurs approximately co-incident with their migration from the center of seminiferous tubules to their periphery: the "SSC niche." While it is possible that these 2 events are triggered by a common mechanism, they are not always synchronous (in terms of timing) (Nagano *et al.*, 2000; Kluin and de Rooij, 1981) and these two events can be genetically separated (Zou *et al.*, 2024), and thus they must also be controlled by independent mechanisms.

ProSG have two fates: (1) conversion into progenitors/differentiating spermatogonia, which proceed through a first round of spermatogenesis to allow rapid fertility, and (2) conversion into SSCs for long-term spermatogenesis (Mccarrey, 2013; Tan and Wilkinson, 2020; Kluin and de Rooij, 1981). Given that SSCs are responsible for maintaining the germline throughout adulthood, there is great interest in defining the mechanism(s) dictating which particular ProSG become SSCs. In principal, ProSG that become SSCs could be (i) chosen randomly (the "stochastic hypothesis"), (ii) chosen through a selection mechanism operating in T2-ProSG during the SSC establishment phase (the "selection hypothesis") or (iii) chosen by a mechanism operating at an earlier stage of germ cell development; i.e., at the PGC, M-ProSG, or T1-ProSG stage (the "pre-determination hypothesis") (McCarrey, 2023). Both the selection and predetermination hypotheses revolve around the idea that a quality control mechanism is in place to select high quality cells to maintain the germline. In favor of the predetermination hypothesis, studies have identified markers specifically labeling T1-ProSG destined to become SSCs (McCarrey, 2023; Law *et al.*, 2019). Evidence that mechanisms may also be in place to maintain the fidelity of the germline comes from analysis of mutational frequencies (McCarrey, 2023).

Human ProSG are poorly characterized, in part, due to the challenge of obtaining these cells for analysis. As with mouse ProSG, human ProSG are derived from PGCs; this conversion event is characterized by well-defined cell morphological changes (Levina and Velikanova, 1967). Human ProSG begin forming at about 7 weeks of gestation when these germ cells (along with remaining PGCs) become progressively enclosed within the testis cords by pre-Sertoli cells (De Felici, 2016). This PGC-to-ProSG conversion occurs in the center of the testes cords (by analogy with what occurs in mice) and is thought to continue up to the end of the first trimester of pregnancy (De Felici, 2016). Human ProSG have been characterized in different ways. For example, Wartenberg *et al*. distinguished three types of human ProSG based on morphology: (i) primitive phase l-gonocytes, (ii) phase 2-gonocytes, and (iii) fetal spermatogonia, the latter of which are more differentiated (Wartenberg *et al.*, 1971). Gondos and Hobel (1971) categorized ProSG based on their localization within the tubules: (i) migrating gonocytes that are centrally situated, and (ii) fetal spermatogonia clustered near the basal lamina. A later study—Fukuda *et al.* (1975)—defined three types of fetal male germ cells: (i) gonocytes, which have several unique morphological features, including a high nucleus-to-cytoplasm ratio, (ii) intermediate cells, which are connected by intercellular bridges and have altered cytoplasmic features, and (iii) fetal spermatogonia, which have the most condensed chromatin and other unique features. These 3 morphologically-distinct fetal germ cells are considered to be ProSG at progressive stages of differentiation, with gonocytes predominant up to 10 weeks of gestation, the intermediate cells predominant at 15 weeks of gestation, and fetal spermatogonia as the predominant cell type after 22 weeks of gestation. Interestingly, all three of these putative ProSG subsets were found to contain cytoplasmic bodies, a stage-specific feature of germ cell subsets across the phylogenetic scale; these bodies (also called "nuage") contain high concentrations of ribonucleoprotein granules that are thought to mediate RNA processing and turnover events (Tauber *et al.*, 2020). It is not clear when human ProSG convert into SSCs, but it is worth noting that single-cell RNA sequencing (scRNAseq) analysis has indicated that human male newborns have a subset of germ cells that have a transcriptome very similar to that of adult human spermatogonia with the characteristics of SSCs (Sohni *et al.*, 2019), as described below.

scRNAseq Analysis Has Deepened Our Understanding of ProSG

Recently, a plethora of studies have leveraged scRNAseq analysis to dissect the cell types in the testis, including ProSG (Tan and Wilkinson, 2020; Dong *et al.*, 2023; Suzuki, 2023). Two scRNAseq studies—Law *et al.* (2019) and Tan *et al.* (2020)—have provided insights into mouse ProSG development. Law *et al.* (2019) performed scRNAseq analysis on perinatal germ cells

purified from multi-lineage reporter mice. They define several ProSG cell clusters, including 4 clusters likely enriched for T1-ProSG (cluster 1–4 from E16.5 mice) and 2 clusters likely enriched for T2-ProSG (clusters 5 and 6 from P0 mice). Thus, this study suggested that, unlike the previous view based on morphology alone, that both T1- and T2-ProSG are heterogenous. It remains to be determined whether these different scRNAseq-defined cell clusters are distinct developmental stages of ProSG and/or have distinct functions. This study also identified DPPA5A and VPS8 as potentially useful ProSG protein markers, as they selectively label E18.5 and P0 Id4-eGFP+ germ cells, respectively.

Tan *et al*. performed scRNAseq analysis on dissociated cells from whole testes instead of purified germ cells (Tan *et al*., 2020). Their analysis of E18.5 and P2 testes identified two discrete ProSG cell clusters that largely correspond to the T1- and T2-ProSG subsets, respectively, previously defined by morphology and proliferative status. This analysis allowed them to define ProSG stage-specific marker genes and proteins, including DNMT3L and ETV4, which label T1- and T2-ProSG, respectively. This study also defined testicular somatic cell subsets and candidate signaling pathways operating between these somatic cells and ProSG (as well as other germ cell subsets) during the perinatal period. Tan *et al*. also defined a novel cell cluster—which they named "intermediate (I)-ProSG"—that lie developmentally between the T1- and T2-ProSG stages. The timing of the appearance of I-ProSG cells and their location in the seminiferous tubule raise the possibility that I-ProSG are the stage that migrate from the center of the seminiferous tubule to its periphery, an obligate event that places ProSG at the site of the "SSC niche" (Kluin and de Rooij, 1981; McGuinness and Orth, 1992). In the future, it will be important to define the mechanisms responsible for the formation of I-ProSG and their migration. In this regard, Tan *et al*. defined I-ProSG-enriched genes encoding migration-associated proteins that are candidates to be involved in migration of ProSG to the SSC niche.

To investigate human ProSG development, Sohni *et al*. (2019) used scRNAseq analysis to elucidate the nature of testicular cells in human newborns. They identified 2 germ cell clusters, as well as several somatic cell clusters. One germ cell cluster had an expression profile highly reminiscent of PGCs (e.g., expression of pluripotency genes such as *POU5F1* and *NANOG*) and thus these cells were named "PGC-like (PGCL)." Immunofluorescence analysis showed that this highly undifferentiated cell subset selectively expresses the POU5F1, PIM2, and ETV5 proteins. The second cell cluster is dominated by cells that largely lack the expression of PGC genes and instead express many ProSG genes. Pseudotime analysis indicated that this second cluster partially overlaps with the most primitive adult undifferentiated spermatogonia cluster, raising the possibility that emergent SSCs have already formed at birth in humans. Whether any of the germ cells present at birth are *functional* SSCs remains to be determined.

Another study—Guo *et al*. (2018)—analyzed two infant (~1-yr old) testes and found these contain a ProSG/primitive undifferentiated spermatogonia cell cluster similar or identical to that observed by Sohni *et al*. (2019) in newborn testes. However, these infant testes lacked the PGCL cluster observed in newborn testes, consistent with classical studies showing that fetal germ cells are absent in infants (Paniagua and Nistal, 1984). This suggests that the PGCL stage differentiates or dies between birth and 1-yr of age in humans. A later study from the same group—Guo *et al*. (2020)—examined subsequent time points and found that the ProSG/primitive undifferentiated spermatogonia cell cluster remained the only germ cell cluster in human testes until at least age 7. As expected, by puberty, later stage germ cells emerge, including differentiating spermatogonia and spermatocytes.

A likely critical aspect of ProSG generation, development, and maintenance is signals from the adjacent somatic cells. Using scRNAseq analysis, Sohni *et al*. (2019) defined signaling pathways that are candidates to operate between ProSG and testicular somatic cells in testes from newborn humans. To define signaling pathways important for the initial generation of ProSG from PGCs, Overeem *et al*. (2021) analyzed previously published scRNAseq datasets from human male gonads derived from human embryos between 6 and 28 wks of gestation. This scRNAseq analysis revealed an enrichment for genes involved in the WNT, NOTCH, TGFβ/BMP pathways, as well as signaling pathways involving receptor tyrosine kinases. Thus, somatic cells may use these signaling pathways to influence both the initial generation and the subsequent development of ProSG.

Together, the many scRNAseq studies described above tracked the progression of both mouse and human ProSG development, and provide a molecular view of the transitions between ProSG stages.

Epigenetic Regulation in ProSG

ProSG not only undergo dynamic cellular changes, but also critical epigenetic reprograming events, which have been best defined in mice. A key epigenetic event that occurs in ProSG is global DNA re-methylation to replace the DNA methylation marks erased at the PGC stage (Yamanaka *et al*., 2019; Odroniec *et al*., 2023). This "de-methylation-followed-by-re-methylation" process is thought to be critical for resetting the epigenetic signatures for the next generation. The majority of DNA re-methylation occurs in T1-ProSG, with global DNA methylation levels rising from ~10% of loci at E13.5 to ~50% of loci at E16.5 (Tseng *et al*., 2015). While DNA methylation in higher eukaryotes is best known to occur on cytosine residues when 5′ of a guanine (i.e., CpG residues), ProSG exhibit an unusually high percentage of non-CpG methylation (Kubo *et al*., 2015).

DNA methylation is mediated by DNA methyltransferases (DNMTs) and thus the high expression of the *de novo* DNA methyltransferases, DNMT3A and DNMT3B, in ProSG is likely to reflect their key role in DNA methylation in ProSG. In support of a physiologically important role for DNMT3A, male mice with a conditional *Dnmt3a* deficiency specifically in PGCs and ProSG (via breeding floxed-*Dnmt3a* mice with *Tnap-Cre* mice) exhibit defective spermatogenesis and lack germ cells as adults (Kaneda *et al*., 2004). In contrast, analogous conditional-KO (cKO) *Dnmt3b*-mutant mice do not have obvious male germ

developmental defects and are able to generate sperm that fertilize eggs and generate offspring, suggesting that DNMT3B either has little or no role in re-methylation or its role is compensated for by DNMT3A (Kaneda *et al.*, 2004).

A DNMT-related molecule that also plays a key role in ProSG DNA methylation is DNMT3L. While this DNMT family member has no catalytic function due to lack of a key enzymatic domain, DNMT3L is known to strongly facilitate the function of DNMT3A and DNMT3B. Consistent with this, global *Dnmt3l*-KO mice have spermatogenic defects very similar to the *Dnmt3a*-cKO mice described above, but are otherwise viable (Hata *et al.*, 2002). These *Dnmt3l*-KO mice display defective methylation on paternally methylated imprints and transposons, which may be responsible for their germ cell loss and infertility (Hata *et al.*, 2002; Webster *et al.*, 2005). Another protein important for re-methylation of DNA in ProSG is NSD1. This lysine methyl transferase promotes *de novo* DNA methylation in ProSG indirectly via its ability to broadly deposit H3K36me2 in euchromatic regions (Shirane *et al.*, 2020).

Given that methylation of DNA regulatory elements is known to suppress transcription, the burst of genome-wide DNA methylation in ProSG might be expected to inhibit gene expression genome-wide. Surprisingly, the DNA re-methylation that occurs in M- and T1-ProSG (between E13.5 and 16.5) is instead associated with an increase (rather than decrease) in genome-wide expression levels (Ng *et al.*, 2013; Sachs *et al.*, 2013). This suggests that ProSG execute a DNA methylation-independent mechanism of transcriptional induction. Histone modifications are likely to play a role in this. In support, the levels of H3K4me3, H3K9me3, H3K27me3, and H3K79me2/3 are all increased globally just before the M-to-T1 transition, raising the possibility that these histone modifications lead to alterations in gene expression that drive the quiescence program (Abe *et al.*, 2011; Yoshioka *et al.*, 2009). Interestingly, many ProSG genes are marked with both active (e.g., H3K4me3) and repressive (e.g., H3K27me3) modifications at their promoter regions (Ng *et al.*, 2013; Sachs *et al.*, 2013). Such bivalent marks are known to prime genes for subsequent activation in response to developmental cues. Some histone variants may also be critical for transcriptional regulation in ProSG; a good candidate is histone H3.3, as it is known to be highly enriched in ProSG (Tang *et al.*, 2014).

Transposon Defense

One reason it is critical for the genome to be remethylated in ProSG is to protect the germline (and thus future generations) from mutations engendered by transposons. Particularly dangerous are retrotransposons, as these replicate and transpose a new copy to another site by a 'copy and paste' mechanism. This process leads—over evolutionary time—to the accumulation of hundreds of thousands of transposon copies dispersed through the genome (Platt *et al.*, 2018). Transposons are regarded as a double-edged sword. On the positive side, transposons sometimes acquire functions over evolutionary time that are useful to the host organism. For example, some transposons have been shown to evolve into regulatory sequences and protein-coding domains that have been co-opted to serve the purposes of the host (Payer and Burns, 2019). But on the negative side, transposons can be highly deleterious. This follows from the fact that transposable elements transpose in a relatively random manner in the genome. Thus, they can disrupt gene function or alter gene expression, contributing to genetic disease and cancer (Payer and Burns, 2019; Hancks and Kazazian, 2016).

DNA methylation is a major mechanism that curbs transposable element transposition and thus maintains genome stability (Greenberg and Bourc'his, 2019). In the male germline, the methylation of transposons occurs in ProSG, primarily between E13.5 to E16.5 (Tan and Wilkinson, 2023). However, many transposons evade *de novo* DNA methylation and thus their transposition must be suppressed by other mechanisms. Histones decorated with specific marks may play this role. Repressive histone marks enriched in ProSG include H2A (H4R3me2S) and H3K9me2 (Seki *et al.*, 2007; Ng *et al.*, 2013; Kim *et al.*, 2014). Several specific proteins also influence DNA methylation and transposon defense in ProSG, including SWI/SNF family members, as described below (Ito *et al.*, 2021).

Another major mechanism that protects against transposons in ProSG (as well as other germ cell stages) is the Piwi-interacting (pi) RNA pathway. The pathway is mediated by short (~24–31 nt) non-coding RNAs called "piRNAs" that associate with members of the PIWI RNA-binding Argonaute protein family to negatively regulate gene expression, either transcriptionally or post-transcriptionally (Iwasaki *et al.*, 2015). piRNAs are segregated into different classes, with one class—called pre-pachytene piRNAs—that are primarily expressed in ProSG and mainly target (through complementarity) retrotransposons (Kawase and Ichiyanagi, 2022). In mice, there are three PIWI family members—PIWIL1, PIWIL2, and PIWIL4 (also known as MIWI, MILI, and MIWI2, respectively)—the latter two of which are expressed in ProSG. The gene encoding PIWIL2 is first detectably expressed in gonadal tissue at E12.5 when PGC transition to form M-ProSG (Kuramochi-Miyagawa *et al.*, 2001), whereas the gene encoding PIWIL4 is not detectably expressed until E15.5 in T1-ProSG (Kuramochi-Miyagawa *et al.*, 2008). Both PIWIL2 and PIWIL4 are critical for suppression of long interspersed nuclear elements (LINE) and other retrotransposons in ProSG, based on analysis of *Piwil2*- and *Piwl4*-KO mice (Kuramochi-Miyagawa *et al.*, 2008; Kojima-Kita *et al.*, 2016). Both of these KO strains suffer from male sterility (Kuramochi-Miyagawa *et al.*, 2008; Kojima-Kita *et al.*, 2016). Given the severe long-term negative effects of *LINE1* elements, it was a surprise when a recent study found that *LINE1* expression correlated with M-ProSG survival and differentiation rather than apoptosis (Nguyen *et al.*, 2020). It remains to be determined whether this reflects a positive role of *LINE1* in germ cells or instead a priming role of *LINE1* expression to initiate piRNA-mediated defense against *LINE1* elements later in ProSG development.

Studies have examined the relative importance of the piRNA pathway and DNA methylation in suppressing transposons. Inoue *et al.* found that the piRNA pathway and DNA methylation act differentially in suppressing transposons, with the piRNA

pathway more important in the early stage of developing germ cells (in ProSG), and DNA methylation becomes important in later stages (during meiosis). One line of evidence for this comes from their comprehensive omics analyses of developing male germ cells from *Pld6*/*Mitopld* and *Dnmt3l* knockout mice, which are defective in piRNA biogenesis and *de novo* DNA methylation, respectively (Inoue *et al.*, 2017). Manakov *et al.* found that the differential effects of piRNA pathway and DNA methylation might be due, in part, to different functions of PIWI members. For example, they found that PIWIL2 and PIWIL4 have differential effects on piRNA biogenesis and DNA methylation in ProSG (Manakov *et al.*, 2015). Although PIWIL4 influences the biogenesis of fewer piRNAs than does PIWIL2, PIWIL4 is nonetheless required to generate high levels of piRNAs targeting several *LINE1* families. Conversely, PIWIL2 is responsible for DNA methylation of a larger subset of transposon families than is PIWIL4.

A transcription factor that acts to suppress retrotransposons in ProSG is RHOX10. This transcriptional regulator is encoded by a large homeobox gene cluster on the X chromosome. In mice, all 33 of these *Rhox* family members are primarily expressed in reproductive organs and placenta. *Rhox10* is the only *Rhox* family member known to be highly expressed in ProSG (Song *et al.*, 2012). Tan *et al.* (2021a) found that RHOX10 suppress the transposition of *LINE1* elements, suggesting that this homeobox gene family member functions to silence parasitic DNA in the germline. Some other mouse RHOX family members, as well as human RHOX family members, each with different expression patterns *in vivo*, suppressed *LINE1* transposition *in vitro*. Using a *SN1* single-copy *LINE1* transgene reporter mouse line (Newkirk *et al.*, 2017), Tan *et al.*, (2021a) found that *SN1 LINE1* copy number was significantly higher in the testes in *Rhox10*-null mice as compared to littermate wt mice, indicative of increased *LINE1* transposition as a result of *Rhox10* loss. This effect was specific to testes, not the other adult tissues tested. Time-course analysis showed that the increased *SN1 LINE1* copy number first occurs at ∼E13.5 and is maximal at ∼E16.5, indicating that RHOX10 suppresses *LINE1* transposition in ProSG. Bisulfite analysis of the *LINE1* transgene promoter in testes from E16.5 *Rhox10*-null mice revealed that this promoter exhibited significantly decreased methylation upon *Rhox10* loss. This suggests that one mechanism by which RHOX10 suppresses *LINE1* transposition in ProSG is by promoting *LINE1* promoter methylation. Tan *et al.* also showed that RHOX10 suppresses *LINE1* transposition through direct transcriptional activation of the *Piwil2* gene. Thus, RHOX10 appears to blunt retrotransposon activation by not only promoting methylation of retrotransposon DNA but also by activating the piRNA pathway.

Specific Genes Operating in ProSG

Analysis of mouse KO models has led to the identification of many genes critical for ProSG. As comprehensively reviewed by Hamer and de Rooij (2018), these genes function in the following processes: inclusion of ProSG into the seminiferous cords (*Kit*), ProSG cytokinesis (*Cit*), ProSG proliferation and apoptosis (*Bcl-x*, *Slx4*, *Brip1*, *Lin28a*, *Mirlet7a-1*, *Prmt5*, *Setdb1*, *Spi1*, *Tgfbr2*, and *Zfp148*), ProSG cell cycle regulation and meiotic entry (*Bnc2* and *Nanos2*), and ProSG migration and differentiation (*Aips1*, *Ccnb1*, *Dazl*, *Dmrt1*, *Glis3*, *Huwe1*, and *Rhox10*). Below, we describe studies defining genes functional in ProSG that have been published since the Hamer and de Rooij review.

The M- to T1-ProSG transition. Recent studies have implicated or provided more insight into the roles of several genes in the M- to T1-ProSG transition, whose hallmark is proliferative quiescence. Genes encoding both transcriptional and post-transcriptional regulators have been shown to be involved.

RB1. This transcriptional co-receptor (also known as simply "RB") serves to inhibit the proliferation of a wide array of cell types (Boward *et al.*, 2016) and was suggested to have a role in the proliferative quiescence of T1-ProSG, based on results from *ex vivo* organ culture of testicular cells from *Rb1*-null and control mouse fetuses (Spiller *et al.*, 2010). To ask the role of RB1 of ProSG quiescence *in vivo*, Du *et al.* (2021) conditionally knocked out *Rb1* at the stage proceeding the ProSG stage–in PGCs—using *Blimp1*-Cre mice. This cKO blunted the drop in proportion of germ cells becoming quiescent at the beginning of the T1-ProSG stage (E14.5) and caused an even more striking effect later (at E16.5, only 35% of *Rb1*-cKO^{Blimp1} ProSG were quiescent as compared with 98% of ProSG in control mice). Du *et al.* also found that a 6-fold higher proportion of *Rb1*-cKO^{Blimp1} ProSG undergo apoptosis at E16.5 than do control ProSG. This result suggests that RB1 not only drives mitotic arrest of ProSG, but it promotes their survival.

TAF4B. This component of the Polymerase II-associated TFIID protein complex is highly expressed in gonadal tissues and is required for both male and female fertility. *Taf4b*-null male mice are initially fertile, but they exhibit a progressive germ cell loss that is likely due to deficient SSCs. In turn, the SSC deficiency may be due, in part, to a ProSG defect, as *Taf4b*-null male mice have ∼4 fold reduced number of T1-ProSG at E18.5 (Lovasco *et al.*, 2015). A recent study—Gura *et al.* (2022)—obtained more evidence that TAF4B functions in ProSG; they found that the ProSG in *Taf4b*-null mice exhibit delayed entry into quiescence, such that they have significantly more proliferating ProSG at E14.5 than do control mice. Interestingly, this is a transient defect, as virtually all *Taf4b*-null ProSG are quiescent by E16.5. To probe into underlying mechanism, Gura *et al.* identified TAF4B-regulated genes and TAF4B-direct target genes in ProSG. In the future, it will be critical to determine the biological importance of TAF4B's ability to promote the timely quiescence of ProSG. In addition, it will be important to determine how loss of TAF4B reduces the number of ProSG at E18.5 given that TAF4B loss allows for *more* ProSG proliferation at E14.5 (since TAF4B loss delays conversion of M-ProSG to T1-ProSG). A likely explanation is that TAF4B not only promotes ProSG quiescence, but also ProSG survival, by analogy with RB1 (see above). Indeed, these two functions may be connected, such that timely ProSG quiescence may be required for ProSG survival.

SMARCB1. SMARCB1 plays a critical role in regulating the transcription of a wide variety of genes through its role as a core subunit of the SWI/SNF chromatin remodeling complex that alters nucleosome structure. Ito *et al.* (2021) showed that cKO of *Smarcb1* (also called *Snf5*) causes delayed mitotic arrest of ProSG. This was accompanied by DNA hypomethylation of transposons and imprinted genes, suggesting that the SWI/SNF complex is critical for normal epigenetic reprogramming in ProSG.

DND1. This RNA-binding protein has long been known to drive the proliferative quiescence that occurs at the M-T1 transition. In mice homozygous for the Ter mutation (a point mutation that introduces a premature stop codon in *Dnd1*), many ProSG fail to enter G1/G0 and instead form teratomas (Stevens, 1973). Gu *et al.* (2018) showed that this occurs through DND1 upregulating the expression of the methyltrasferase EZH2, which, in turn, deposits the repressive mark, H3K27me3, at the *Ccnd1* locus that encodes a proliferation-promoting cyclin protein. Recently, Ruthig *et al.* (2023) defined direct DND1 target mRNAs at different time points of ProSG development. Interestingly, many of these mRNA targets encode chromatin-modifying enzymes and other epigenetic regulators, raising the possibility that DND1 acts on the M-to-T1 transition through epigenetic regulation. These investigators also identified two subsets of ProSG with different expression levels of DND1 expression. The DND1-high subset peaked at E16.5 and was enriched for the expression of epigenetic regulators. It remains to be determined the functional significance of this DND1-high ProSG subset, including in teratoma formation.

The T1- to T2-ProSG transition. The program that triggers re-activation of ProSG proliferation and the temporally-associated process—migration to the SSC niche—is poorly understood. Recent studies have identified some factors involved in this developmental program.

RHOX10. As described above, RHOX10 is a transcription factor encoded by the X-linked *Rhox* gene cluster. Mutational inactivation of *Rhox10* causes a SSC establishment defect phenotype that is secondary to a T1-to-T2 ProSG transition defect, based on several lines of evidence, including scRNAseq analysis (Song *et al.*, 2016). In addition to a defect in ProSG progression, loss of *Rhox10* reduces migration of ProSG to the periphery of the seminiferous tubule. The notion that RHOX10 drives ProSG progression was further supported by a recent paper (Tan *et al.*, 2021b). Using newly identified ProSG markers—DNMT3L and ETV4 (Tan *et al.*, 2020)—Tan *et al*. demonstrated that *Rhox10*-null mice accumulate ProSG at P2 and P3 *in vivo*, a phenotype they recapitulated *in vitro*. Using a battery of genome-wide approaches, Tan *et al*. also identified high-confidence RHOX10 target genes that are candidates to drive ProSG differentiation. Through "rescue" experiments, the authors obtained causal evidence that at least two of these genes (encoding the transcription factors DMRT1 and ZBTB16) act downstream of RHOX10 in a molecular circuit to drive ProSG progression (Tan *et al.*, 2021b).

hnRNPU. hnRNP proteins are a large family of RNA-binding proteins that influence many post-transcriptional processes. One family member—hnRNPU—was recently found to be critical for ProSG progression through the T1-to-T2 transition (Wen *et al.*, 2024). Using *Ddx4-Cre* mice to conditionally KO the *Hnrnpu* gene in fetal germ cells, Wen *et al*. found that this causes a series of defects, including an accumulation of T1-ProSG, a reduction in T2-ProSG, an almost complete failure of ProSG migration to the periphery of the seminiferous tubule at P5, and only a few germ cells remaining in the testis at P10 as a result of massive apoptosis. Since defects in the T1-to-T2 transition and ProSG migration are also a feature of *Rhox10*-KO mice (see above), this raises the possibility that hnRNPU and RHOX10 collaborate in some manner to drive these two processes. Interestingly, Wen *et al*. obtained evidence that the molecular mechanism by which hnRNPU acts is through its influence on RNA splicing. They found that *Hnrnpu*-cKO mice testes are enriched in mRNAs harboring skipped exons (759 of 955 alternatively splicing events), strongly suggesting that hnRNPU promotes exon inclusion. This leads to the possibility that hnRNPU is important for ProSG because it is required for the normal RNA splicing of a large number transcripts in ProSG. In addition, hnRNPU may also regulate key alternative RNA splicing events in ProSG, as *Hnrnpu*-cKO testes have altered levels of some alternatively spliced mRNAs; e.g., those with alternative 3′ and 5′ splice site usage.

DDX20. DEAD-box (DDX) proteins are RNA helicases that influence post-transcriptional events. Zou *et al.* (2024) recently reported that one DDX family member—DDX20—is a translational regulator that is critical for the proliferative re-activation of ProSG that occurs at the T1-to-T2 transition. Interestingly, while the authors found that conditional mutation of *Dddx20* in ProSG (via *Ddx4-Cre*) prevents proliferative reactivation of ProSG and thus reduces the number of ProSG, it does not prevent the downregulation of the G1-phase marker, cyclin D1, nor does it prevent the later induction of SSC markers (e.g., GFRA1 and FOXC2) and progenitor markers (e.g., ZBTB16). This suggests that DDX20 is critical for proliferation of ProSG, but not their ability to initiate the progression program to form SSCs and progenitors. In addition, DDX20 does not appear to be involved in the migration of ProSG to the SSC niche, as *Ddx20*-cKO ProSG were able to efficiently migrate to the seminiferous tubule periphery. This is important, as this migration event occurs approximately at the same time as ProSG proliferative reactivation and thus these two events have sometimes been regarded as being necessarily coordinated. The ability to genetically separate these two events will be instrumental in dissecting underlying mechanisms. Together, the data from Zou *et al*. support a model in which DDX20 is specifically involved in the proliferative-reactivation of ProSG. Given that earlier work had shown that global loss of DDX20 causes lethality during the early zygotic period when the early embryo begins proliferating (Mouillet *et al.*, 2008), this raises the possibility that DDX20 has a general function in driving cell cycle-arrested cells to re-enter the cell cycle.

CHEK1. Another factor that may be involved in the T1- to T2-ProSG transition is the checkpoint protein kinase CHEK1 (also called CHK1), based on the finding that neonatal mice conditionally mutant for *Chek1* in germ cells (using *Ddx4-Cre* mice) lack dividing germ cells, the key characteristic of T2-ProSG (Abe *et al.*, 2018). However, interpretation is clouded by the finding that at P3, when dividing T2-ProSG should be abundant, the authors found that even control mice lacked detectable dividing germ cells. Furthermore, while *Chek1*-mutant mice have abundant germ cells at P3, few were present at P5, raising the possibility that CHEK1 is also involved in germ cell survival.

The ProSG-to-SG transition. The ultimate goal of ProSG is to give rise to SSCs and differentiating SG. Little is known about the factors that drive ProSG to become SSCs, but progress has been made in defining factors involved in promoting ProSG to become differentiating SG.

CYP26. Pharmacological inhibition studies conducted by Velte *et al.* (2019) indicated that the cytochrome P450 family member, CYP26, has a role in the ability of ProSG to respond to RA and thereby convert into SG. The authors found that the CYP26 inhibitor, talarozole, increases the proportion of ProSG that differentiate into SG at P1, suggesting that the ability of CYP26 to catabolize RA helps prevent ProSG from prematurely differentiating.

JMJD1A and JMJD1B. The Jumonji family of proteins encode histone demethylases that function in a wide variety of biological processes. Kuroki *et al.* (2020) discovered that two Jumonji family members—JMJD1A and JMJD1B—are responsible for the extremely low levels of H3K9me2 in ProSG. The authors went on to show that cKO of *Jmjd1a* and *Jmjd1b* in germ cells (using *Ddx4-Cre* mice) leads to reduced migration of ProSG to the seminiferous tubule periphery as well as a defect in the T2-ProSG-to-SG transition. Given that JMJD1A and JMJD1B remove the repressive H3K9me2 chromatin mark, this leads to the hypothesis that these histone demethylases promote ProSG processes through transcriptional activation of specific genes. Kuroki *et al.* identified candidate target genes involved through RNAseq analysis of *Jmjd1a/Jmjd1b* cKO mice vs. control mice germ cells.

Perspective

In this review, we have summarized research that has shed light on cellular events and molecular mechanisms operating in ProSG. We suggest that, in the future, it will be important to continue to study mice mutants with defects in ProSG. To gain molecular insight, it will also be important to use cutting-edge tools, including single-cell multi-omic methods and spatial transcriptomics, in combination with traditional approaches. A major challenge for the field in the future will be to understand human ProSG. Given the difficulty in obtaining live human ProSG for study, efforts may need to be directed towards studying human ProSG derived *in vitro* from pluripotent cells (Saitou and Hayashi, 2021). Towards this goal, Hwang *et al.* (2020) recently developed a method to generate cells resembling M- and T1-ProSG from human pluripotent cells. In summary, we suggest that by integrating different approaches using different model systems, the field will be able to define—in detail—how ProSG (i) first form, (ii) differentiate, (iii) undergo epigenetic reprogramming, (iv) safeguard the male germline from mutations (v) migrate to the SSC niche, and (vi) generate SSCs and differentiating spermatogonia. In turn, these discoveries will have the potential to provide insights into how ProSG defects lead to male infertility and malignancy.

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