Cycling to and from a stem cell niche: the temporal and spatial odyssey of mitotic male germ cells

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ABSTRACT  The development of male germ cells within the prenatal and prepubertal periods in mammals combines multiple biological events that integrate cell cycle regulation, epigenetic reprogramming, and cell migration along temporally and spatially dynamic lines. Germ cells arise from their precursor primordial germ cells in the mid-gestation embryo, forming gonocytes that enter G₀ phase cell cycle arrest within the fetal testis. Cyclin-dependent kinase inhibitors, activated retinoblastoma 1 protein, and increased levels of transforming growth factor beta 2 collectively influence this cell cycle arrest. Gonocyte quiescence persists until shortly after birth, whereupon the cells concomitantly re-enter the cell cycle and migrate towards a niche that establishes and maintains self-renewing spermatogonial stem cells and balances them with differentiating spermatogonia. Platelet-derived growth factor signaling is one of the mechanisms that regulates both mitotic activation and migration in neonatal gonocytes, along with mitogens, 17β-estradiol and retinoic acid, and chemoattractants C-C-motif ligand 9 and members of the ADAM, integrin, and tetraspanin families. Numerous germ cell-intrinsic proteins have been identified that ensure the retention of germ cells within the spermatogonial stem cell niche. Sertoli cells are a significant component of this niche, contributing essential growth factors and chemokines to spermatogonia. This review focuses on the dynamic events that occur to mitotic male germ cells before and during their arrival at this niche, with an emphasis on the cell cycle and directed migration.

KEY WORDS: spermatogonia, cell cycle, germ cell, testis, gonocyte, stem cell niche

Introduction

Male germ cell development in mammals is a remarkable process that depends upon precise migratory and cell cycle dynamics. From their precursor primordial germ cells (PGCs) that proliferate while migrating to the fetal gonad, male germ cells undergo a period of mitotic arrest until shortly after birth. They then re-enter the cell cycle and migrate once more to the basement membrane of seminiferous tubules within the testis – a niche that establishes and maintains self-renewing spermatogonial stem cells (SSCs) throughout the lifespan of individual organisms. When appropriate differentiation signals are processed, a subset of spermatogonia then leave the stem cell niche and commit to a multitude of differentiation events comprising spermatogenesis. This balance of SSC self-renewal with differentiation must continue throughout adulthood to ensure the uninterrupted production of spermatozoa.

Nearly six decades of research into how mammalian SSCs, their precursor cells and differentiating progeny are regulated from a cell cycle perspective have contributed to our current understanding of this highly orchestrated process. Beginning with cell counts and ³H-thymidine labeling (Clermont and Leblond, 1953, Monesi, 1962), and progressing to transgenic pulse-labeling and live imaging experiments (Nakagawa et al., 2007, Yoshida et al., 2007), the field of male germ cell biology has uncovered the complexity of the journey taken by these cells from PGCs to differentiating spermatogonia. This development spans fetal and neonatal stages through puberty and into adulthood, with temporal and spatial dynamics that reveal alternating periods of mitotic activity and cell movement, quiescence and repose (Fig. 1). This review will encompass the developmental events that occur during these periods, highlighting the experimental approaches taken to

Abbreviations used in this paper: PGC, primordial germ cell; SSC, spermatogonial stem cell.
elucidate their regulatory mechanisms. While some studies have been performed using non-human primate and human samples, the majority of experimental analysis has been accomplished with mouse and rat male germ cells. Accordingly, this review will focus on rodent male germ cell mechanisms.

**From the site of origin to the gonadal ridge**

Mouse primordial germ cells (PGCs) were first identified by light microscopy at 8.5 days post coitum [embryonic day (E)8.5] in the posterior region of the embryo (Chiquoine, 1954). These cells express high levels of alkaline phosphatase (AP), which facilitated their subsequent discovery at the base of the allantois at E8.0 (Ozdenski, 1967). Cell counting identified approximately 100 PGCs per embryo at E8.5, which increase in number to approximately 25,000 germ cells per embryo at E13.5 (Tam and Snow, 1981). Detection of PGCs at E7.0 was then enabled through the use of whole-mount immunostaining for AP activity (Ginsburg et al., 1990). PGCs specification is initiated from signals coming from the extraembryonic ectoderm and visceral endoderm that encompass the epiblast. These signals include bone morphogenetic proteins (BMPs) 2, 4, and 8b (Lawson et al., 1999, Ying et al., 2000, Ying and Zhao, 2001). BMP signaling within epiblast cells results in the upregulation of *Fragilis* and the expression of *Blimp1* in approximately six PGC precursor cells at E6.25 (Ohinata et al., 2005). *Blimp1* encodes a transcription factor that leads to the upregulation of genes characteristic of germ cells, including *Stella* and Nanos3, and the downregulation of mesodermal genes such as fibroblast growth factor 8 (*Fgf8*) and brachyury (*T*).

By E7.5 the proliferating PGCs have begun to migrate, although the specifics of their cellular behavior between E7.25 and E8.5 are not well understood. PGC migration depends upon the localization of the kit receptor (KIT) and the chemokine (C-X-C motif) receptor 4 (CXCR4) in PGCs, responding to kit ligand (Steel, stem cell factor) and C-X-C-motif ligand 12 (CXCL12, Sdf1), respectively (Ara et al., 2003, Doitsidou et al., 2002, Godin et al., 1991, Molynieux et al., 2003). PGCs respond to the chemoattractants as they traverse their path to the developing gonads. By E8.5, ~100 or so PGCs distribute near the hindgut diverticulum and become transcriptionally silent, undergoing a transient G2 phase cell cycle arrest. Seki and colleagues performed 5-bromo-2’-deoxyuridine (BrdU) labeling and found that most PGCs did not enter S-phase between E7.5 and E9.0 (Seki et al., 2007). Assessment of cyclin B1 levels, which accumulate in the cytoplasm specifically in the G2 phase, revealed that 80% of PGCs exhibited cyclin B1 between E8.5 and E8.75, decreasing in number after E9.75. Coinciding with this quiescence is extensive epigenetic reprogramming, including the erasure of methylated histone lysine H3K9me2, increase in H3K27me3, and decrease in DNA methylation (Seki et al., 2007). DNA methyltransferases DNMT1, DNMT3A, and DNMT3B are suppressed, along with RNA polymerase II transcription. Between E9.0 and E11.0 the transient cell cycle arrest is reversed, and by E11.0 the migrating PGCs have entered the genital ridge of the developing gonads. PGCs actively proliferate upon their arrival in the gonad, from E11.5 to E12.5 (Tam and Snow, 1981). During this same period, the germ cells are once more subjected to extensive epigenetic reprogramming, including DNA demethylation at imprinted gene loci (Sato et al., 2003). DNMT3A and DNMT3B are suppressed, but unlike during the earlier reprogramming between E7.5 and E9.0, DNMT1 remains active during this post-migratory

**Fig. 1. Overview of mitotic male germ cell development in mammals.**

Primordial germ cells (PGCs) originate from epiblast tissue proximal to the extraembryonic ectoderm. Following their specification, PGCs begin active proliferation while migrating through the hindgut to the site of the presumptive gonad. A transient G2 phase cell cycle arrest occurs in rodent PGCs between embryonic days (E)8-E9.5. Mitotic activity then resumes in rodents after E9.5 as PGCs continue their migration. Upon their arrival at the gonadal ridge, male germ cells continue to proliferate and are now classified as gonocytes. Then, at approximately E13.5 in rodents and 18-20 weeks in humans, male gonocytes undergo G2 phase cell cycle arrest. These germ cells remain quiescent until shortly after birth. At this time they reside in the lumen of the seminiferous tubules in the testis, surrounded by mitotic Sertoli cells that extend inward from the basement membrane. Upon re-entry into the cell cycle at approximately postnatal day (P)3 in rodents, gonocytes begin to migrate from the lumen to the basement membrane. Once the germ cells have completed their migration, they are classified as spermatogonia and form self-renewing spermatogonial stem cells (SSCs) at approximately P5 in rodents and 8-12 weeks in humans. Sertoli cells comprise a key component of the stem cell niche, which balances SSC maintenance with differentiating spermatogonia in response to multiple germ cell-intrinsic and extrinsic factors.
Developing male germ cells: mitotic and quiescent

Fig. 2. Mitotic arrest of gonocytes within the fetal mouse testis. Male gonocytes within the E12.5 testis proliferate actively, expressing the marker Ki67. Unlike female germ cells, they do not respond to retinoic acid (RA) due to the presence of CYP26B1, which degrades RA and prevents meiotic entry. E12.5 gonocytes contain inactive phosphorylated RB1 protein, phosphorylated ATR, and phosphorylated CHK1. When gonocytes are exposed to unknown signals beginning after E12.5, an upregulation in Cdkn1b (p27\(^\text{Kip1}\)) and Cdkn2b (p15\(^\text{INK4b}\)) inhibits Cyclin E-cdk2 complexes, which in turn inhibits the phosphorylation of RB1. The G1-to-S phase checkpoint is activated by hypophosphorylated RB1, leading to cell cycle arrest in the G0 phase. This quiescence is accompanied by an increase in TGF\(_\beta\) levels. As the majority of gonocytes become arrested in G0 by E14.5, elevated p27\(^{\text{Kip1}}\) and p15\(^{\text{INK4b}}\) expression is augmented with upregulated Cdkn1a (p21\(^{\text{Waf1/Cip1}}\)), Cdkn1c (p57\(^{\text{Kip1}}\)), and Cdkn2a (p16\(^{\text{INK4a}}\)), maintaining the block to Cyclin E-cdk2 activity and RB1 phosphorylation. G0 arrest persists until shortly after birth. TGF\(_\beta\) levels remain high, and these quiescent gonocytes are Ki67-negative.

period (Hajkova et al., 2002). PGC arrival at the gonadal ridge also exposes them to inductive signals that results in sex determination. Expression of Sry in the surrounding somatic tissue, which develops into Sertoli cells, leads to a cascade of signaling events that culminates in the formation of male germ cells from the PGCs (Wilhelm and Koopman, 2006). Within the developing testis levels of retinoic acid (RA), which are high in the developing ovary and induce female germ cells to enter meiosis, are kept low by the activity of CYP26B1, an enzyme that degrades RA (Bowles et al., 2006, Koubova et al., 2006). This action prevents meiosis from occurring in the fetal testis, and assists in the commitment of male germ cells to the spermatogenic pathway. Other factors involved in this commitment of germ cells to the male program include FGF9 and NANOS2 (Barrios et al., 2010, Bowles et al., 2010, Suzuki and Saga, 2008). Beginning at E11.5, male germ cells rely upon FGF9 for survival, and it has been proposed that FGF9 supports NANOS2 production in these cells to prevent meiotic entry and the activation of events downstream of RA (Barrios et al., 2010, Bowles et al., 2010, DiNapoli et al., 2006, Suzuki and Saga, 2008). Collectively, these factors help to ensure that male germ cells establish their own developmental program that is distinct from that of female germ cells.

Dormancy within the fetal testis

Between E12.5 and E14.5, male germ cells exit the cell cycle and enter a prolonged phase of G0 arrest until birth (Fig. 2). Now classified as gonocytes, male germ cells during this period respond to unknown signal(s) that induce this quiescence. Various studies had reported that E13.5 gonocytes were partially or fully in cell cycle arrest (Adams and McLaren, 2002, Hilscher et al., 2005), while others had shown gonocytes continuing to divide up to E16.5 (Vergouwen et al., 1991). \(^{3}\)H-thymidine labeling demonstrated that approximately 8% of gonocytes were cycling at E14.5, with almost no mitotic activity at E16.5 (Vergouwen et al., 1991). More recent quantitative studies of BrdU incorporation by Western and colleagues revealed that while roughly 70% of E13.5 gonocytes progressed through S-phase, this percentage dropped to near zero by E14.5 (Western et al., 2008). Indeed, flow cytometry of E14.5 gonocytes revealed that approximately 95% of cells were in G0, a marker of cycling cells, was observed at E12.5 but no longer detected by E14.5 (Western et al., 2008). Two additional proteins localized in E12.5 gonocytes were phosphorylated ATR (ataxia telangiectasia and Rad3 related), a molecule upstream in cell cycle checkpoint pathways, and its target CHK1 (checkpoint kinase 1), which acts upon cell cycle regulators when phosphorylated (Rhind and Russell, 2000, Shiloh, 2001, Western et al., 2008). These proteins were likewise undetected by E14.5. Direct examination of the cell-cycle-dependent kinase inhibitors Cdkn1a (p21\(^{\text{Cip1}}\)), Cdkn1b (p27\(^{\text{Kip1}}\)), Cdkn1c (p57\(^{\text{Kip1}}\)), Cdkn2a (p16\(^{\text{INK4a}}\)), and Cdkn2b (p15\(^{\text{INK4b}}\)) revealed that p27\(^{\text{Kip1}}\) and p15\(^{\text{INK4b}}\) undergo enrichment in E13.5 gonocytes, leading to the inhibition of cyclin E1/E2 and cdk2 (cyclin-dependent kinase 2) and cell cycle arrest (Western et al., 2008). By E14.5, the additional inhibitors p21\(^{\text{Cip1}}\), p57\(^{\text{Kip1}}\), and p16\(^{\text{INK4a}}\) join p27\(^{\text{Kip1}}\) and p15\(^{\text{INK4b}}\) in maintaining the cell cycle arrest. Interestingly, the G1/S phase checkpoint protein retinoblastoma 1 (RB1) is inactive at E12.5, localized in its phosphorylated form (Western et al., 2008). By E13.5 it becomes dephosphorylated, leading to its activation (Fig. 2). This activity is transient, however, and RB1 is degraded once the majority of gonocytes are quiescent (Western et al., 2008). In Rb1-deleted male mice, gonocytes show a higher percentage of cycling cells at E14.5, though this activity subsides and delayed quiescence occurs by E16.5 in mutant animals (Spiller et al., 2010). Thus, the temporal role of RB1 in mediating G0 arrest is compensated a short time later by additional factors, including p27\(^{\text{Kip1}}\) and p15\(^{\text{INK4b}}\).

The mitotic arrest of fetal male gonocytes has also been investigated in the context of transforming growth factor (TGF) signaling. TGF\(_\beta\) was shown to target rat gonocytes through immunohisto-chemical studies as well as in vitro functional assays (Gautier et al., 1994, Olaso et al., 1998, Teerds and Dorrington, 1993). TGF\(_\beta1\) also exhibits anti-proliferative properties on mouse PGCs (Godin and Wylie, 1991). More recently, Moreno and colleagues analyzed a conditional Tgbr2 gene-targeted mouse and ex vivo organ cultures and observed higher levels of gonocyte quiescence when TGF\(_\beta2\) expression was elevated (Moreno et al., 2010). Addition of exogenous TGF\(_\beta2\) to gonocytes in vitro significantly diminished the number of BrdU-positive gonocytes. Thus, TGF\(_\beta2\) appears to
influence the G0 arrest in gonocytes after E12.5 (Fig. 2). Activin signaling may also play a role, as inhibin-beta A (Inhba) knockout male mice exhibited increased numbers of gonocytes at E15.5 (Mendis et al., 2011). Taken altogether, these results suggest that growth factors, in addition to cyclin-dependent kinase inhibitors, exert anti-mitotic activity on gonocytes from E13.5 until just after birth to maintain male germ cells in their appropriate state. Further research is necessary to identify the signal(s) that initiate these anti-proliferative events, triggering the upregulation of such growth factors and cell cycle inhibitors.

From the lumen to the basement membrane: entering the stem cell niche

Upon birth, perinatal male gonocytes remain quiescent and reside within the lumen of the seminiferous tubules in the testis. Then, at approximately postnatal day (P) 3 in both mice and rats, gonocytes re-enter the cell cycle and begin to proliferate once more (Fig. 3). The identity of the factor(s) responsible for this resumption of mitotic activity remained unknown until the past two decades. Li and colleagues discovered that platelet-derived growth factor (PDGF)-BB as well as 17β-estradiol (E2) induced in vitro cycling of P3 rat gonocytes by measuring BrdU incorporation (Li et al., 1997). When an estrogen receptor antagonist was introduced to the cell co-cultures, and the number of BrdU-positive P2 gonocytes increased after one day of RA exposure in gonocyte-Sertoli cell co-cultures, and the number of BrdU-positive P2 gonocytes increased after one day of RA treatment in vitro (Bougogne et al., 2003, Zhou et al., 2008a). Thus, a combination of in vivo, ex vivo, and in vitro experiments have demonstrated that levels of PDGFs, E2, and RA increase shortly after birth and act upon gonocytes to stimulate cell cycle progression (Fig. 3).

Accompanying the resumption of proliferation in P3 rodent gonocytes is a migration from the lumen to the basement membrane of seminiferous tubules (Fig. 3; Clermont and Perey, 1957). Such migration is necessary for the further development of male germ cells, as gonocytes that remain in the lumen after P5 undergo apoptosis (Tres and Kierszenbaum, 2005). Studies on KIT suggest that it may be one of many factors that mediate this process (Orth et al., 1997). Antibodies that blocked KIT on gonocytes in vitro decreased the number of cells with detectable pseudopods available for motility (Orth et al., 1997). Additional factors that may influence gonocyte migration include members of the ADAM (A Disintegrin And Metalloprotease), integrin, and tetraspanin families. Specifically, ADAMs 1 and 2, integrins α3β1 and α6β1, and tetraspanins CD9, CD81, and CD98 have been proposed to mediate this cell movement based upon their localization and increasing levels in migratory gonocytes, as well as their known interactions (Tres and Kierszenbaum, 2005). PDGFRβ and V1-PDGFRβ, in addition to regulating gonocyte proliferation, also appear to influence migration.

Exploitation of neural crest-derived cells as a source of bone marrow cells for hematopoietic reconstitution has been a long-sought goal in regenerative medicine. However, the difficulty in isolating neural crest-derived cells and the lack of a reliable in vitro model for the study of neural crest development have hampered progress in this field. To address these challenges, researchers have turned to the study of gonocyte migration, as gonocytes are a well-characterized population of cells that share many similarities with neural crest-derived cells. By understanding the factors that regulate gonocyte migration, researchers hope to gain insights into the mechanisms that govern neural crest development and to develop new strategies for the manipulation of neural crest-derived cells for regenerative medicine.

Collectively, these findings suggest that levels of ADAMs 1/2,
CD9, CD81, CD98, and PDGFRβ increase in pre-migratory and migratory neonatal gonocytes, mediating their movement away from the lumen. Recent experiments performed using P5-P6 male germ cells, comprising the transition period between gonocytes and spermatogonia, showed chemotaxis of undifferentiated germ cells expressing the glial cell line derived neurotrophic factor family receptor alpha 1 (GFRα1) towards the chemokine C-C-motif ligand 9 (CCL9) in vitro (Simon et al., 2010). CCL9 is strongly expressed by neonatal Sertoli cells, and its receptor, CCR1, is localized to GFRα1+ germ cells (Simon et al., 2010). Furthermore, neonatal Sertoli cells lacking the gene Sin3a exhibit significantly diminished levels of CXCL12, while Sin3a-deleted perinatal gonocytes have reduced levels of its receptor, CXCR4, and fail to migrate from the lumen to the basement membrane (Payne et al., 2010; Gallagher et al., 2013). Thus, it is possible that migratory neonatal gonocytes also utilize CCR1 and CXCR4 to migrate towards high levels of CCL9 and CXCL12, respectively, present at the basal surface of Sertoli cells along the basement membrane. In this case, postnatal gonocytes would utilize some of the same molecules that embryonic PGCs use to mediate their migration. Upon reaching the basement membrane, male germ cells enter the spermatogonial stem cell niche, where establishment and self-renewal of the germ line stem cell pool occurs in balance with spermatogenesis. The gonocytes have now become spermatogonia.

Balancing self-renewal with differentiation

Spermatogonia were originally classified by their nuclear morphology. The so-called Type A spermatogonia had undetectable levels of heterochromatin using light microscopy, while Intermediate spermatogonia had some heterochromatin, and Type B had larger amounts (Clermont and Leblond, 1953, Monesi, 1962, Roosen-Runge and Giesel, 1950). Two primary models of spermatogonial stem cell (SSC) regulation and maintenance have been proposed during the past forty years. In the first model, the examination of rodent seminiferous tubules by whole-mount microscopy revealed that within the Type A class, the putative stem cell A^stem cell (A^st) divides to generate either two additional A^stem cell spermatogonia or A^paired (A^pa) daughter cells that do not undergo complete cytokinesis and remain connected with intercellular cytoplasmic bridges (Huckins, 1971, Oakberg, 1971). Additional divisions of the A^pa cells generate chains of 4, 8, 16, and even 32 interconnected Type A spermatogonia (A^linked, or A^linked) that are still classified as undifferentiated. That is, they continue to harbor a similar transcriptome to the A^stem cell spermatogonia, and even retain stem cell potential (Nakagawa et al., 2010). The large chains of A^pr then become A^0 differentiating spermatogonia, which further divide to generate A^2, A^4, A^8, Intermediate, and Type B spermatogonia. The spermatogonial cell divisions beginning with A^stem cell are all synchronous, unlike the A^0, A^4, and A^8 cell divisions.

In contrast to the aforementioned description, the second model of SSC regulation proposes a so-called A^1/A^0 model. Observations of single or pairs of cells with little to no mitotic activity classified them as A^stem cell spermatogonia, or reserve stem cells much like hematopoietic stem cells (Clermont and Bustos-Obregon, 1968, Clermont and Hermo, 1975, Dym and Clermont, 1970). Active stem cells, then, are comprised of A^1, A^4, and A^8 spermatogonia (no relation to the first model). A^1 cell division either gives rise to another A^stem cell spermatogonium as SSC self-renewal, or generates a differentiating intermediate spermatogonium. While similarities exist between the A^1/A^0 model and the so-called A^stem cell and A^paired model of SSC regulation in non-human primates and humans, most studies examining the molecular mechanisms of SSC self-renewal and differentiation rely upon the A^1/A^0-A^4 model.

Within the SSC niche, undifferentiated spermatogonia are exposed to growth factors, cytokines, and other molecules that promote the maintenance of the actual and potential germline stem cells in their undifferentiated state (Fig. 4). The predominant niche component is the Sertoli cell, producing glial cell line derived neurotrophic factor (GDNF) that acts upon GFRA1 and its co-receptor Ret in undifferentiated A^1, A^4, and A^8 spermatogonia (Meng et al., 2000). When Gdnf, Gfra1, or Ret are deleted in male mice, SSCs establishment fails and germ cells are rapidly depleted (Meng et al., 2000, Naughton et al., 2006). Sertoli cells also release basic FGF (bFGF) and stem cell factor (SCF, kit ligand, Steel) into the milieu to ensure SSC survival (Mullaney and Skinner, 1992, Tajima et al., 1991). Peritubular myoid cells and Leydig cells, meanwhile, exhibit colony-stimulating factor 1 (CSF1) localization, with the CSF1 receptor restricted to the undifferentiated spermatogonia expressing cell surface antigen THY1 (Oatley et al., 2009). Additional germ cell-intrinsic molecules enriched in SSCs and important for their self-renewal include POU domain, class 5, transcription factor 1 (Pou5f1, Oct4), zinc finger and BTB domain containing 16 (Zbtb16, Plzf), lin-28 homolog A (Lin28), B cell CLL/lymphoma 6, member B (Bcl6b), neuregulin 3 (Neureg3, Ngn3), inhibitor of DNA binding 4 (Id4), sal-like 4 (Sal4), cadherin 1 (Cdh1), and undifferentiated embryonic cell transcription factor 1 (Utf1) (Buaas et al., 2004, Costoya...
In contrast to the many factors that promote SSC self-renewal, only a handful of differentiation markers have been identified in A1, A2, Intermediate, and Type B spermatogonia. Increased levels of RA appear to coincide with the timing of SSC differentiation, with both A1 and A2 spermatogonia expressing RA receptor gamma (RARγ), critical for transducing RA signals and essential for spermatogonial differentiation (Gely-Pernet et al., 2012, Snyder et al., 2010). In response to RA, spermatogonia upregulate stimulated by retinoic acid gene 8 (Stra8), whose expression persists until the germ cells enter meiosis (Zhou et al., 2008a, Zhou et al., 2008b). KIT levels also increase in differentiating spermatogonia, responding to elevated SCF activity in the microenvironment (Ohta et al., 2000, Schrans-Stassen et al., 1999). Two additional factors enriched upon differentiation are SOHLH1 and SOHLH2 (spermatogenesis and oogenesis specific basic helix-loop-helix 1 and 2), which were recently shown to upregulate KIT (Barrios et al., 2012). Genetic ablation of either family member results in a loss of KIT+ spermatogonia (Ballow et al., 2006, Toyoda et al., 2009). Thus, when SSCs commit to the spermatogenic process, levels of RARγ, STRA8, SOHLH1, SOHLH2, and KIT increase within spermatogonia, responding to elevated RA and SCF in the surrounding milieu, and the germ cells leave the stem cell niche (Fig. 4). SSC maintenance occurs from shortly after birth to the extent of adulthood, while spermatogonial differentiation from those undifferentiated cells commences at the onset of puberty.

A unique first wave: rodent spermatogenesis initiation

Once the pool of self-renewing SSCs is established, differentiating spermatogonia emanate from those SSCs. In other words, in an adult mouse, STRA8+ spermatogonia originate from OCT4+, PLZF+, NGN3+ spermatogonia (Fig. 5). An unanswered question within the field, however, was whether the initial set of differentiating spermatogonia, or mitotic cells initiating the ‘first wave’ of spermatogenesis in rodents, emanated from SSCs like in adults, or directly from gonocytes prior to SSC establishment. This was an important question to address as the first functional mouse spermatooza appear at P40, and the entire mouse spermatogenic process beginning from A1 cells takes approximately 35 days. Yoshida and colleagues answered this question through elegant lineage tracing and pulse-labeling experiments, examining mice harboring both Ngn-cre and CAG-CAT-Z reporter transgenes (Yoshida et al., 2006). They found that KIT+ spermatogonia contributing to the first wave never expressed Ngn3. Few NGN3+ germ cells exist prior to P4, suggesting that these first-wave differentiating spermatogonia arise directly from NGN- gonocytes (Fig. 5). This finding supports the view of two distinct subsets of rodent gonocytes at P3, when mitotic activity resumes: one subset that remains undifferentiated and establishes the self-renewing SSC pool that is maintained throughout adulthood, and one subset that immediately expresses KIT at P3 and differentiates, initiating the first wave of spermatogenesis (Fig. 5). How these differential subsets of gonocytes are established and regulated is poorly understood, but evidence supports the possibility that events within the fetal testis, prior to or during gonocyte quiescence from E13.5 to P2, may influence these differential cell fate changes (Payne et al., 2010). Mice harboring Sin3a-deleted Sertoli cells, in which the gene is rendered non-functional by E13.5, exhibit a normal initiation of the spermatogenic first wave but do not establish SSCs during the first postnatal week (Payne et al., 2010). Thus, there could be an uncharacterized factor expressed by fetal and perinatal Sertoli cells that influence gonocyte cell fate. Galectin 1 has been proposed to influence whether gonocytes generate SSCs or establish first-wave differentiating spermatogonia, but there are likely other contributing factors (Yoshida et al., 2006).

Fig. 5. The establishment of the first spermatogenic wave in rodents. Fetal male germ cells express pluripotency genes such as Pou5f1 (Oct4), Nanog, and Sox2. At P3 in mice, a subset of male germ cells continues to express Oct4 and additional stem cell markers Gfra1, Zbtb16 (Plzf), Lin28, Bcl6b, Sall4, Id4, Cdh1, Utf1, and Ngn3. These cells collectively are classified as undifferentiated spermatogonia, with Type A single (A1) spermatogonia comprising the actual stem cells; Type A paired (A2) and Type A aligned (A3) spermatogonia exhibit stem cell potential and can regenerate the germ cell population of testes if called upon to do so. Another subset of male germ cells at P3 begins to immediately differentiate, upregulating Kit, Stra8, Sohlh1, Sohlh2, and Rary. These differentiating cells form Type A1, A2 spermatogonia, which then further differentiate to become Intermediate and Type B spermatogonia, before finally entering meiosis as spermatocytes.
Conclusion

Mitotic male germ cells undergo a remarkable journey from their initial development from PGCs to their entry into meiosis. Recurring waves of cell cycle dynamics, epigenetic reprogramming, and migration play fundamental, overlapping roles in shaping the identity of these unique cells. Like the epic journey of adventure described by Homer, prenatal male germ cells are, in a sense, destined for the stem cell niche that will house them between birth and adulthood. It is there they will reside until they are called into service once more, to differentiate and begin the spermatogenic process. The critical window of fetal gonocyte quiescence is also where dysregulation can occur, leading to the formation of carcinoma in situ, the precursor of testicular germ cell tumors that develop after puberty. Such events will be covered in more detail in the accompanying articles within this special issue. Suffice it to say that both gonocyte quiescence and SSC niche establishment are crucially important biological events, yet remain insufficiently characterized with respect to the molecular mechanisms that initiate and regulate them. When these events do not proceed properly, cancer or infertility can result. Many decades of innovative, exciting research have provided insight into the cellular events of the premeiotic male germ line. Undoubtedly, further investigation of these events will continue for many years to come.

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