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Spermatogonial stem cells and spermatogenesis in mice, monkeys and men

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ABSTRACT

Continuous spermatogenesis in post-pubertal mammals is dependent on spermatogonial stem cells (SSCs), which balance self-renewing divisions that maintain stem cell pool with differentiating divisions that sustain continuous sperm production. Rodent stem and progenitor spermatogonia are described by their clonal arrangement in the seminiferous epithelium (e.g., A_{single}, A_{paired} or $A_{aligned}$ spermatogonia), molecular markers (e.g., ID4, GFRA1, PLZF, SALL4 and others) and most importantly by their biological potential to produce and maintain spermatogenesis when transplanted into recipient testes. In contrast, stem cells in the testes of higher primates (nonhuman and human) are defined by description of their nuclear morphology and staining with hematoxylin as A_{dark} and A_{pale} spermatogonia. There is limited information about how dark and pale descriptions of nuclear morphology in higher primates correspond with clone size, molecular markers or transplant potential. Do the apparent differences in stem cells and spermatogenic lineage development between rodents and primates represent true biological differences or simply differences in the volume of research and the vocabulary that has developed over the past half century? This review will provide an overview of stem, progenitor and differentiating spermatogonia that support spermatogenesis; identifying parallels between rodents and primates where they exist as well as features unique to higher primates.

1. Introduction

Spermatogonial stem cells (SSCs) are the adult tissue stem cells in the testis that are at the foundation of spermatogenesis and essential for male fertility (Phillips et al., 2010). SSCs are defined by their dual potentials: 1) self-renew to maintain the stem cell pool and 2) differentiate to maintain continuous sperm production in post-pubertal males (de Rooij & Grootegoed, 1998). Similar to other adult tissue stem cells, SSCs are rare, comprising only 0.03% of total germ cells in mice (Tegelenbosch & de Rooij, 1993). However, numerous transit amplifying mitotic divisions in progenitor and differentiating spermatogonia, followed by two meiotic divisions give rise to millions of sperm each day (Phillips et al., 2010).

2. Spermatogonial stem cells and spermatogenic lineage development: lessons from the rodent

In the post-natal rodent testis, SSC activity is broadly believed to reside in the population of isolated (single) spermatogonia located on the basement membrane of the seminiferous tubules (Huckins, 1971; Oakberg, 1971a; de Rooij, 1973). These rare cells are called the A_{single} spermatogonia (A_s), which divide once every three days and make up about 0.03% of the total germ cells in the mouse testis (Tegelenbosch & de Rooij, 1993; Huckins & Oakberg, 1978a). Mitotic division of A_s produces a pair of spermatogonia (A_{paired}; A_{pr}) that will either complete cytokinesis to produce two new A_s (self-renewing division) or remain joined by an intracytoplasmic bridge and produce a chain of four $A_{aligned}$ spermatogonia (A_{al4}) at the next division (Phillips et al., 2010; de Rooij & Griswold, 2012) (Fig. 1A). The A_{al4} spermatogonia may undergo one or more mitotic divisions to form larger chains of 8, 16 and sometimes 32 A_{al} spermatogonia. Collectively, A_s, A_{pr} and A_{al} make up the population of undifferentiated spermatogonia that comprises 0.3% of germ cells in the rodent testis; A_s make up 10% of undifferentiated spermatogonia (0.03% of germ cells; Fig. 2A) (Phillips et al., 2010; Huckins, 1971; Oakberg, 1971a; Valli et al., 2015; Oakberg, 1971b). Larger clones of A_{al} spermatogonia differentiate to A1 spermatogonia. In this context, a clone is defined as the group of interconnected cells that arise from a single A_s spermatogonia. In Rodents, the clones become so large that they fill entire segments of seminiferous tubule due to sequential mitotic divisions from A1 spermatogonia that produce types A2, A3, A4, Intermediate and B spermatogonia, which divide to produce primary spermatocytes. Two meiotic divisions from primary spermatocytes give rise to secondary spermatocytes and round spermatids, which undergo spermiogenesis (morphological differentiation)

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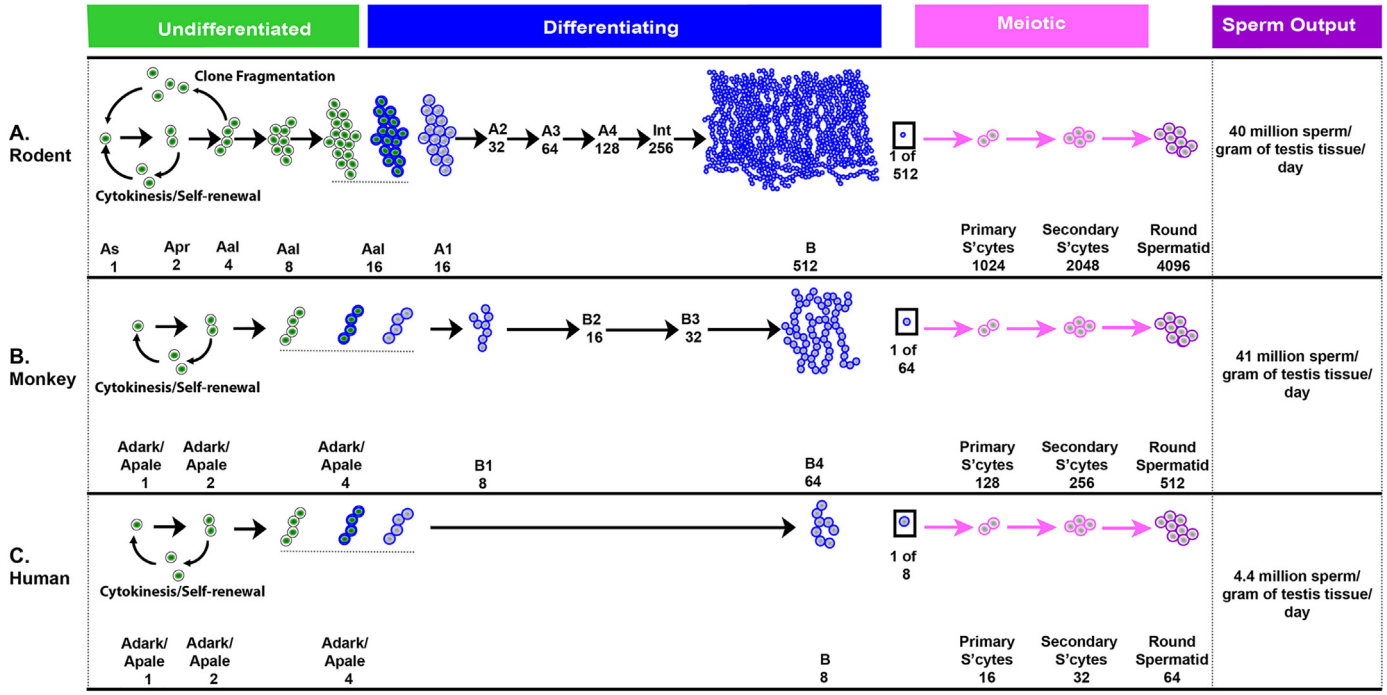

Fig. 1. Clonal development in the spermatogenic lineages of rodents, monkeys and humans. Undifferentiated spermatogonia are described as A_s, A_{pr} or A_{al} in the rodents and A_{dark} or A_{pale} in monkey and human. During spermatogenic development, A_{single} (A_s) and A_{dark} and/or A_{pale} undergo one or more mitotic divisions to give rise to cells of larger clones (chains) of interconnected cells sizes through transit-amplifying mitotic divisions. A) Clonal development in rodents features 3–4 transit amplifying divisions in the pool of undifferentiated A_s, A_{pr} and A_{al} spermatogonia followed by 6 amplifying divisions in the pool of differentiated spermatogonia (A1–A4, Intermediate, B), which give rise to primary spermatocytes. Two additional meiotic divisions produce round spermatids that undergo spermiogenesis to produce sperm. B) Clonal development of spermatogonia in monkeys features 0, 1 or 2 transit amplifying divisions in the pool of undifferentiated A_{dark}/A_{pale} spermatogonia, followed by 4 transit amplifying divisions of differented spermatogonia (B1–B4), which give rise to primare spermatocytes. C) Clonal development of spermatogonia in humans features 0, 1 or 2 transit amplifying divisions in the pool of undifferentiated A_{dark}/A_{pale} spermatogonia followed by a single a single transit amplyfying division in differentiated B spermatogonia that give rise to primary spermatocytes. Thus, there are typically 12 transit amplifying divisions in rodents; 8 in monkeys and 5 in humans between stem cell and sperm. The reduced number of transit amplyfing divisions in monkeys and humans is compensated in part by a larger stem cell pool (see Fig. 2).

to produce mature sperm. Thus, through a series of transit amplifying mitotic and meiotic divisions, a relatively small pool of stem cells in the rodent testis produces 40 million sperm per gram of testis parenchyma each day (Figs. 1A and 2A) (Tegelenbosch & de Rooij, 1993; Valli et al., 2015; Thayer et al., 2001).

Undifferentiated stem and progenitor spermatogonia in rodent testes are defined in part by clone size, as indicated above, and in part by molecular markers (e.g., ID4, PAX7, BMI1, EOMES, GFRa1, NANOS2, UTF1, ZBTB16, SALL4, LIN28, FOXO1 and others). Markers can be observed by immunohistochemistry in histological cross sections; in whole mount preparations of seminiferous tubules or by fluorescence-activated cell sorting (FACS), but clone size can only be observed in whole mount preparations of seminiferous tubules. Based on whole mount immunohistochemistry, ID4, PAX7, BMI1 and EOMES appear to have the most restricted pattern of expression, which is limited to A_s spermatogonia (Oatley et al., 2011; Aloisio et al., 2014; Komai et al., 2014; Braun et al., 2017). GFRa1, NANOS2 and UTF1 have expression limited to A_s, A_{pr} and A_{al4} (Suzuki et al., 2009; van Bragt et al., 2008; Meng et al., 2000), while ZBTB16, SALL4, LIN28, CDH1 and FOXO1 are expressed by most or all undifferentiated A_s, A_{pr} and A_{al} spermatogonia (Costoya et al., 2004; Buaas et al., 2004; Hobbs et al., 2012; Eildermann et al., 2012a; Gassei & Orwig, 2013; Tokuda et al., 2007; Goertz et al., 2011), including some overlap with cKIT+ differentiating spermatogonia (Fig. 3A). Based on the restricted pattern of expression, some have suggested that cells expressing ID4, PAX7 and/or BMI1 might be the ultimate spermatogonial stem cells ($SSC_{ultimate}$) (Helsel et al., 2017; Lord & Oatley, 2017; de Rooij, 2017). Indeed, the expression of each marker on functional stem cells has been confirmed by SSC transplantation and/or lineage tracing. However, there is little information about the extent of overlap among these markers; whether any of these proteins mark the entire population of A_s spermatogonia or whether the entire population of functional stem cells resides in the population of A_s spermatogonia. In fact, molecular heterogeneity among undifferentiated spermatogonia of all clone sizes has been repeatedly documented (Suzuki et al., 2009; Gassei & Orwig, 2013; Hermann et al., 2015; Nakagawa et al., 2010; Zheng et al., 2009).

It seems reasonable to suppose that the stem cell pool also extends to some A_{pr} spermatogonia because A_s must transit through an A_{pr} state in the process of self-renewal (see Fig. 1); this concept has been described as "false pairs" and is nicely reviewed in (de Rooij & Griswold, 2012). Furthermore, Hara and colleagues provided live video imaging of GFRa1-GFP spermatogonia data to suggest that fragmentation of larger clones (eg., A_{al4} fragmenting to A_{al3} + A_s or A_{pr} + 2 A_s or 4 A_s) was an important contributor to maintenance of the A_s pool ((Hara et al., 2014); Fig. 1A). While the fate of the fragmenting clones could not be documented in that study, clones of A_{al3} (possibly resulting from clone fragmentation) have been observed by others (Suzuki et al., 2009; Gassei & Orwig, 2013; Tokuda et al., 2007; Hara et al., 2014). However, one concern with the clone fragmentation model is that it is based entirely on observations of GFRa1 positive cells and does not account for the contribution of GFRa1 negative cells that are known to exist in the pool of A_s (Suzuki et al., 2009; Gassei & Orwig, 2013) and the pool of transplantable stem cells (Grisanti et al., 2009; Garbuzov et al., 2018). Any model that considers only a part of the stem cell pool defined by a single molecular marker is likely to be incomplete.

To date, the only way to definitively identify a spermatogonial stem cell is by observing its capacity to produce and maintain spermatogenesis long-term, by transplantation (Brinster & Zimmermann, 1994;

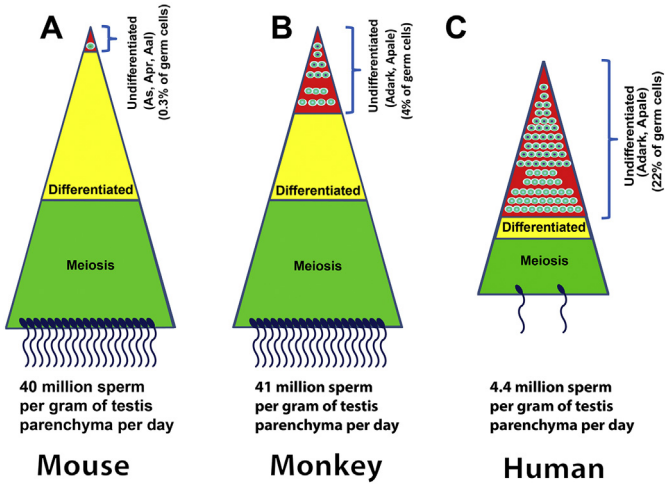

Fig. 2. Schematic comparison of the stem cell pools and sperm output in mice, monkeys and humans. A) The spermatogenic lineage in mice features a relatively small pool of A_s, A_{pr} and A_{al} undifferentiated spermatogonia (0.3% of germ cells). However, with about 12 transit amplifying divisions between stem cells and sperm (see Fig. 1), the small pool of stem cells produced 40 million sperm per gram of testicular tissue per day. B) The spermatogenic lineage in monkeys features a relatively larger pool of Adark/Apale spermatogonia (4% of germ cells) and this compensates for the reduced number of transit amplying divisions between stem cell and sperm. Sperm output in monkeys is similar to mice: 41 million sperm per gram of testicular tissue per day. C) The spermatogenic lineage in humans features the largest pool of undifferentiated Adark/Apale spermatogonia (22% of germ cells). However, with only one transit amplifying division of differentiated spermatogonia, sperm output in humans is reduced to 4.4 million sperm per gram of testicular tissue per day. Thus distinguishing features of spermatogenic lineage development in mice, monkeys and men include 1) the size of the pool of undifferentiated stem/progenitor spermatogonia; 2) the number of transit amplifying divisions in differentiated spermatogonia and 3) sperm output.

Brinster & Avarbock, 1994) or lineage tracing (Aloisio et al., 2014; Komai et al., 2014; Nakagawa et al., 2007). These are retrospective assays. There is no evidence that the mouse SSCs can be prospectively defined completely and exclusively by a specific clone size or molecular marker (see discussion above). However, it is generally agreed that smaller clones are more undifferentiated while larger clones are more differentiated and that cKIT marks the transition to differentiated type A1 spermatogonia. Differentiated type A1 spermatogonia in rodents appear to be equivalent to type B1 in nonhuman primates and type B in humans based on appearance of heterochromatin and initiation of cKIT expression (Figs. 1 and 3). Co-staining with cKIT and a marker of undifferentiated spermatogonia (e.g., PLZF, SALL4, CDH1, UCHL1, etc) in whole mount preparations of seminiferous tubules can help to define the clone size where undifferentiated stem/progenitor spermatogonia transition to differentiated spermatogonia. In mice, this transition occurs most frequently at a clone size of 16 (Fig. 1A), but can also happen at clones sizes of 8 and less frequently at smaller clone sizes (Suzuki et al., 2009; Gassei & Orwig, 2013; Tokuda et al., 2007; Hara et al., 2014). In nonhuman primates and humans, this transition occurs at smaller clone sizes (Fig. 1B & C; see discussion below).

3. Stem cells and spermatogenic lineage development in higher primates

Nonhuman primate and human testes contain two morphologically distinct types of undifferentiated spermatogonia, identified as A_{dark} and A_{pale}, based on differences in nuclear morphology and staining intensity with hematoxylin (Clermont & Leblond, 1959; Clermont & Antar, 1973; Clermont, 1966). A_{dark} spermatogonia are "relatively small, spherical or slightly ovoid" cells on the basement membrane of seminiferous tubules having dark, dense chromatin in their "uniformly stained" nuclei. A_{pale} spermatogonia are identified as "relatively larger, oval" or almost round cells on the basement membrane of the seminiferous tubules having pale, elongated nuclei with "coarser" or more "granular

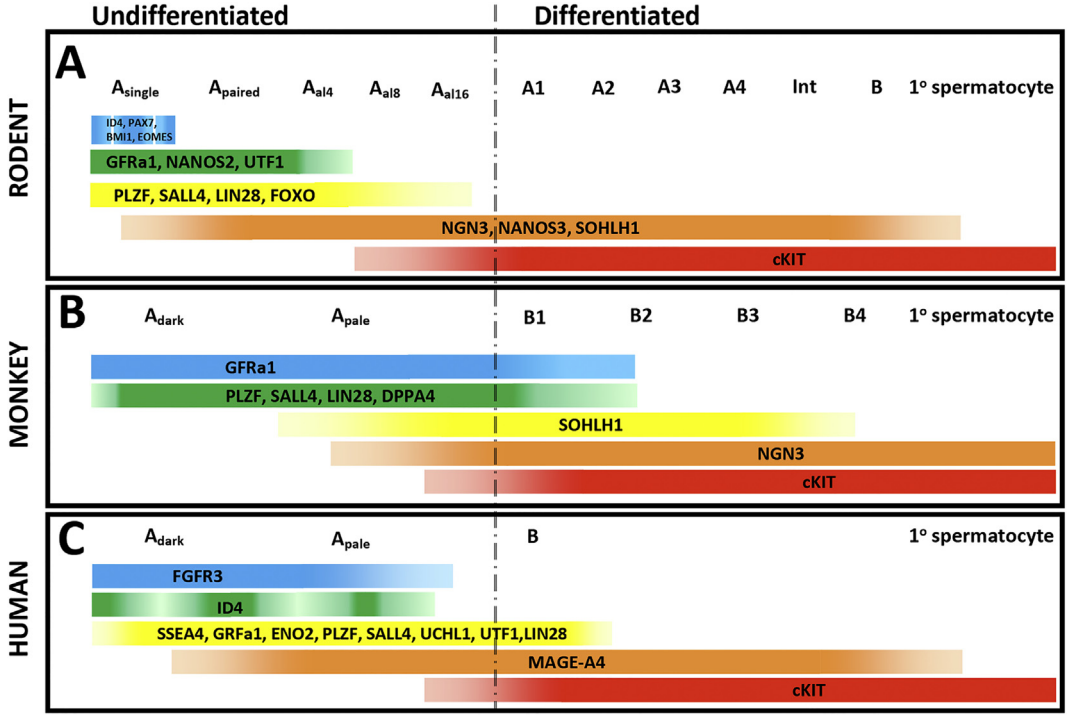

Fig. 3. Spermatogonial markers in rodents, monkeys and humans. A) Rodents; B) Monkeys; C) Humans. Several markers are conserved from rodents to monkeys to humans, suggesting their importance in spermatogenic lineage development. GFRa1, PLZF, SALL4 and LIN28 are conserved markers of undifferentiated spermatogonia. cKIT is a conserved marker of differentiating/differentiated spermatogonia. The following references describe markers in this figure that were not referenced elsewhere in the text: NGN3 (Yoshida et al., 2004); SOHLH1 (Ballow et al., 2006).

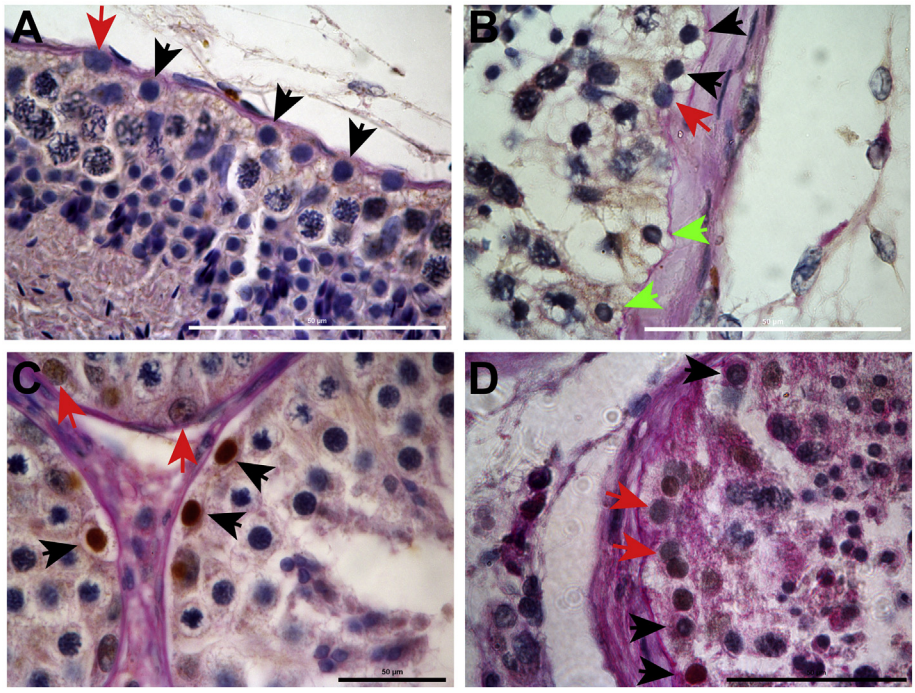

Fig. 4. Histological and immunohistochemical evaluation of A_{dark} and A_{pale} spermatogonia in monkeys and humans. Periodic acid, Schiffs' and Hematoxylin (PAS-H) staining in monkey (A and C) and human (B and D) testis section reveals A_{dark} (black arrows) and A_{pale} (red arrows) spermatogonia on the basement membrane of the seminiferous tubules. The subpopulation of A_{dark} spermatogonia with a rarefraction zone are indicated by a green arrow in (B). Colorimetric staining for UTF1 (brown color) with PAS-H staining confirms that UTF1 is a conserved marker of most, but not all A_{dark} (black arrow) and A_{pale} (red arrows) spermatogonia in monkey (C) and Human (D) testes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

chromatin". Nucleoli may be visible in both A_{dark} and A_{pale} spermatogonia (Fig. 4A). B-type spermatogonia are identified by their relatively larger size, location on or close to the basement membrane of the seminiferous tubules; clear and roundish nuclei and they are differentiated from one another by the granulation and density of heterochromatin staining. B1 spermatogonia are least heterochromatic and B4 spermatogonia are most heterochromatic (Clermont & Leblond, 1959) Some studies have identified a "rarefraction zone" (chromatin free zone) in a subpopulation of A_{dark} spermatogonia (Fig. 4B indicates examples of A_{dark} with rarefaction zone and A_{dark} without rarefaction zone). The observation of a rarefaction zone may be fixation-dependent and is used more frequently to describe A_{dark} spermatogonia in humans than in nonhuman primates (see review from von Kopylow et al. in this special issue) (von Kopylow et al., 2010; Lim et al., 2011; Paniagua & Nistal, 1984; Schulze, 1978). There are currently few researchers with the experience or patience to use the classic A_{dark} and A_{pale} descriptors of primate spermatogonia. However, researchers who do so provide a valuable link between contemporary molecular readouts and the histological descriptions in the classic literature.

In 1959, Clermont and Leblond proposed that A1 (A_{dark}) are the stem cells, which divide to either self–renew and maintain the stem cell pool or give rise to the A2 (A_{pale}) progenitor cells that may undergo one or more transit-amplifying divisions before giving rise to differentiated B1 spermatogonia. Clermont revised his model 10 years later based on in vivo labeling with ^{3}H-thymidine, which indicated that A_{pale}, but not A_{dark}, incorporated ^{3}H-thymidine in Vervet monkeys (*Cercopithecus aethiops*). Since A_{dark} did not appear to self-renew under steady state conditions, he proposed that A_{pale} are the "active" stem cells that maintain spermatogenesis in the adult testis while A_{dark} are "reserve" stem cells that regenerate spermatogenesis when it is destroyed by noxious insult (e.g., chemotherapy or radiation) (Clermont, 1969). Experimental evidence supporting this model are derived from observations in nonhuman primates and men that X-irradiation caused a striking depletion of spermatogenesis, including the entire population of A_{pale}, which were subsequently replenished from the surviving pool of A_{dark} spermatogonia (Clifton & Bremner, 1983; Oakberg, 1968; Oakberg, 1975; van Alphen et al., 1988). Ehmcke and Schlatt argued that low mitotic index and regenerative capacity of A_{dark} is consistent with the characteristics of a "true stem cell" and the regular proliferation of A_{pale} is indicative of "renewing progenitors" (Ehmcke & Schlatt, 2006). During the past 50 years, eight studies have reported on the acute labeling index of A_{dark} and A_{pale} spermatogonia. While four studies observed no labeling in the A_{dark} spermatogonia (Buageaw et al., 2005; Schlatt & Weinbauer, 1994; de Rooij et al., 1986; Simorangkir et al., 2009), consistent with the results of Clermont; four studies reported a wide range (0.06% to 18%) of A_{dark} labeling (Clermont & Antar, 1973; Fouquet & Dadoune, 1986; Ehmcke et al., 2005; Kluin et al., 1983). In all of those studies, ^{3}H-thymidine or BrdU was administered as a single bolus, and this may not effectively label a very slow cycling population of stem cells. Chronic labeling studies are needed to determine whether A_{dark} are indeed quiescent or whether A_{dark} are slow-cycling, active stem cells in steady state spermatogenesis. We have proposed that A_{dark} and at least some A_{pale} are the same population of cells that are simply at different stages of the cell cycle (i.e., A_{dark}: G0 versus A_{pale}: G1/S/G2/M) (Hermann et al., 2010). The concept that A_{dark} are in prolonged G0 is supported by observations of von Kopylow and colleagues, who found the Ki67 was expressed by A_{pale}, but not A_{dark} spermatogonia of the human testis (von Kopylow et al., 2012a). We believe this indicates that A_{dark} are slow cycling (long G0 phase), not quiescent or reserve, because when we treated adult Rhesus macaques with BrdU in the drinking water for three weeks, nearly 15% of A_{dark} incorporate label (Fayomi and Orwig, In Preparation).

4. Clonal expansion in higher primates

Three dimensional reconstruction mapping of serial cross sections and camera lucida drawings indicated that A_{dark} and A_{pale} spermatogonia in monkey testes are arranged in clones of 1, 2 or 4 cells, suggesting that there are only 1 or 2 transit amplifying divisions before differentiation to B1 spermatogonia (Clermont & Leblond, 1959; Clermont, 1969). This is fewer than the 3–4 transit amplifying divisions that occur in undifferentiated rodent spermatogonia before differentiation to type A1 spermatogonia (See Fig. 1A & B; Fig. 2A & B). Subsequent divisions from B1 spermatogonia in nonhuman primates produce types B2, B3 and B4 spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids that undergo spermiogenesis to produce mature sperm (Clermont & Leblond, 1959). Thus, the number of transit amplifying divisions from B1 spermatogonia to

spermatocyte in monkeys (four) is less than the six divisions from A1 spermatogonia to spermatocytes in rodents (Fig. 1A & B; Fig. 2A & B). Despite these differences in spermatogonial transit amplifying divisions, sperm output in rodents and monkeys is about the same (~40 million sperm per gram of testicular parenchyma per day; see Figs. 1 & 2). In contrast, men have only one generation of differentiated type B spermatogonia and sperm output is reduced to 4.4 million sperm per gram of testicular parenchyma per day (Fig. 1C; Fig. 2C; reviewed in (Valli et al., 2015)).

To summarize, there are a total of 12 transit amplifying divisions from the isolated A_s spermatogonia in rodents to the terminally differentiated sperm, which should yield 4096 sperm per stem cell that commits to differentiate (12 doublings = 2^{12} = 4096) (Russell et al., 1990). The actual yield is considerably less due to massive apoptosis (~50%) that occurs in the differentiated type A2-A4 spermatogonia (de Rooij, 1973; Huckins & Oakberg, 1978b; Huckins, 1978). By comparison, there appear to be only 8 transit amplifying divisions in nonhuman primates and 5 transit amplifying divisions in humans between the isolated A_{dark}/A_{pale} undifferentiated spermatogonia and terminally differentiated sperm (Fig. 1). Assuming similar stem cell pool sizes and spermatogenic lineage development dynamics, one might expect that sperm output in nonhuman primates (2^8 = 256) and humans (2^5 = 32) to be reduced 16-fold and 128-fold, respectively, compared with mice. However, as indicated above and in Figs. 1 and 2, this is not the case. Sperm output in monkeys is equivalent to rodents and sperm output in humans is reduced by only 10-fold compared with rodents. The contribution of apoptosis to sperm output in higher primates is not known, but the size of the stem cell pool is likely to be major contributor to differences in sperm output among species.

5. The pool of stem/progenitor spermatogonia in higher primates is larger than rodents

As described above, the precise molecular or clone size definition of functional stem cells in the rodent testis is subject to debate. However, the broader pool of stem & transit amplifying progenitors in rodents is understood to include A_s, A_{pr} and A_{al} spermatogonia with a cKIT negative phenotype. Similarly, the precise definition of functional stem cells in primate testes are subject to debate (Valli et al., 2015; Hermann et al., 2009), but the broader pool of stem/progenitor spermatogonia resides in the population of A_{dark} and A_{pale} spermatogonia with a cKIT negative phenotype. In rodents, the population of A_s, A_{pr} and A_{al} undifferentiated spermatogonia comprises 0.3% of germ cells in the testis (Fig. 2A). In nonhuman primates, A_{dark} and A_{pale} spermatogonia are present in equal numbers and comprise 4% of germ cells in the testis (Marshall & Plant, 1996). Like nonhuman primates, A_{dark} and A_{pale} spermatogonia are present in equal numbers in the human testis (Clermont, 1966; Schulze, 1978; Paniagua et al., 1987) and constitute 22% of germ cells in the testis (Paniagua et al., 1987). Thus, the larger pool of stem/progenitor cells in the testes of higher primates compensates, in part, for the reduced number of transit amplifying divisions (Figs. 1 & 2). The large pool of stem/progenitor cells in higher primates may also be a mechanism to reduce the replicative demand on each individual stem cells in longer lived species.

6. Molecular description of spermatogonia in higher primates

Based on expression of conserved molecular markers, A_{dark} and some A_{pale} spermatogonia in nonhuman primates and humans exhibit an undifferentiated phenotype, similar to A_s, A_{pr} and some A_{al} rodent spermatogonia (GFRa1+, PLZF+, SALL4+, cKIT−). Some A_{pale} have a transition phenotype similar to larger chain A_{al} spermatogonia in rodents (e.g., GFRa1+/SOHLH1+/NGN3+/cKIT+) (Hermann et al., 2009; Ramaswamy et al., 2014). Markers of undifferentiated spermatogonia that are conserved from rodents to nonhuman primates to humans include GFRa1, UTF1, PLZF, SALL4 and LIN28 (van Bragt et al., 2008; Meng et al., 2000; Costoya et al., 2004; Buaas et al., 2004; Hobbs et al., 2012; Eildermann et al., 2012a; Gassei & Orwig, 2013; Zheng et al., 2009; Hermann et al., 2009; Ramaswamy et al., 2014; Aeckerle et al., 2012; Lin et al., 2015; Di Persio et al., 2017; Valli et al., 2014; Zheng et al., 2014; Sachs et al., 2014) (See UTF1 staining of monkey and human testis cross sections in Fig. 4C and D). ID4 is conserved in the undifferentiated spermatogonia of rodents (Oatley et al., 2011) and humans (Sachs et al., 2014), but has not been described in nonhuman primates. cKIT appears to be a conserved marker of differentiated spermatogonia, marking the transition to A1 spermatogonia in rodents; B1 spermatogonia in nonhuman primates and B spermatogonia in humans (Hermann et al., 2009; Valli et al., 2014). There are no molecular markers that distinguish the entire population of A_{dark} from the entire population of A_{pale}, perhaps because both are elements of the same stem cell pool that are in different stages of the cell cycle (Hermann et al., 2010). However, a few markers have been identified that are restricted to the subpopulation of A_{dark} with a rarefaction zone (EXOSC10, FGFR3, OCT2) (Lim et al., 2011; von Kopylow et al., 2012a; von Kopylow et al., 2012b) and a few markers are restricted to A_{pale} (DMRT1, Ki67, SSX2-4) or a subpopulation of A_{pale} (NGN3, cKIT) (von Kopylow et al., 2012a; Hermann et al., 2009).

7. Cell surface markers of undifferentiated spermatogonia in higher primates

To date, no cell surface marker has been identified in any species with expression restricted to functional spermatogonial stem cells. GFRa1 is a conserved marker that appears to be most restricted to undifferentiated spermatogonia (i.e., A_s, A_{pr}, A_{al4} in rodents and A_{dark} and A_{pale} in higher primates). This marker has been used to isolate and enrich undifferentiated spermatogonia (Garbuzov et al., 2018; Buageaw et al., 2005; Gassei et al., 2010; He et al., 2012), but many investigators have reported difficulty sorting SSCs using GFRa1 antibodies (personal communications and unpublished data). It is also now clear that half of functional stem cells in the adult mouse testis are in the GFRa1 negative fraction (Garbuzov et al., 2018). This may indicate that stem cells oscillate between GFRa1+ and GFRa1− states depending on cell cycle status, signals from the SSC niche, density of germ cells on the basement membrane or other circumstances. In contrast, GFRa1 appears to be expressed by all A_{dark} and A_{pale} spermatogonia in the Rhesus macaques (Hermann et al., 2009), which presumably include the entire population of functional stem cells. ITGA6 is another robust and conserved marker that can be used to isolate and enrich SSCs from rodent, monkey and human testis cell suspensions (Valli et al., 2014; Shinohara et al., 2000; Maki et al., 2009). ITGA6 expression is not restricted to SSCs or even germ cells, but the entire population of functional SSCs can be recovered and are significantly enriched in the ITGA6+ fraction of rodent (Shinohara et al., 2000) and human testis cells (Valli et al., 2014). SSEA4 has not been used to isolate mouse SSCs, but is expressed by undifferentiated spermatogonia in monkey and human testes and has been used effectively to isolate transplantable SSCs (Zheng et al., 2014; Muller et al., 2008; Izadyar et al., 2011; Eildermann et al., 2012b; Smith et al., 2014). CD9 is expressed by a subpopulation of MAGEA4+ spermatogonia in human testes and can be used to isolate transplantable stem cells (Zohni et al., 2012). ITGA6, SSEA4 and CD9 are effective single markers for isolating primate spermatogonia because they clearly segregate the heterogeneous testis cell suspension into positive and negative fractions and have been tested functionally by xenotransplantation into infertile mouse recipients. Thus, the majority of functional SSCs are captured in the positive fractions with limited loss to the negative fractions. Other cell surface markers that have been used to isolate and enrich functional SSCs, alone or in combination with other markers, include CD90, EpCAM and GPR125 (Hermann et al., 2009; Valli et al., 2014; Kubota et al., 2003; Ryu et al., 2004; Nickkholgh et al., 2014).

8. SSC transplantation bioassay in higher primates

Similar to rodents, transplantation is the established method to quantify functional stem cells in higher primates. Of course, homologous species transplantation is not possible in humans. Homologous species SSC transplantation is possible in primates (Hermann et al., 2012; Jahnukainen et al., 2011), but not practical as a routine biological assay. Therefore, xenotransplantation to the testes of infertile, immune deficient mice has emerged as the gold standard to quantify functional stem cells from monkey or human cells populations. Human and monkey SSCs do not regenerate complete spermatogenesis when transplanted into mouse testes. However, they do migrate to the seminiferous tubule basement membrane and produce chains or networks of spermatogonia that persist for many months after transplantation (Hermann et al., 2009; Valli et al., 2014; Izadyar et al., 2011; Zohni et al., 2012; Nagano et al., 2001; Nagano et al., 2002; Hermann et al., 2007; Wu et al., 2009; Sadri-Ardekani et al., 2009; Sadri-Ardekani et al., 2011; Dovey et al., 2013; Clark et al., 2017; Durruthy Durruthy et al., 2014; Ramathal et al., 2014). It is not currently possible to recapitulate complete spermatogenesis from monkey or human cells using the xenotransplantation assays. Perhaps one day this challenge will be overcome by transplantation to more closely related species and/or using an organ culture system similar to that described for producing eggs or sperm from primordial germ cell-like cells (PGCLCs) in mice (Hayashi et al., 2012; Zhou et al., 2016).

9. Concluding remarks

Although different vocabularies have evolved to describe spermatogonial stem cells and spermatogenic lineage development in rodents, monkeys and humans, many features are conserved between species. For example, spermatogenesis emerges from isolated spermatogonia that give rise to clones of interconnected chains or networks of cells that become progressively more differentiated with each successive transit amplifying division. In all species, smaller clones are the more undifferentiated elements while larger clones are the more differentiated elements. Many markers are conserved from rodents to primates to humans as well as their association with undifferentiated versus transition versus differentiated spermatogonia. Some markers appear to be more species specific, but in some cases, this may be an artifact of antibody quality or availability for different species. There are important differences between rodents and higher primates. Rodents have more transit amplifying divisions in the pool of undifferentiated and differentiated spermatogonia than nonhuman primates or humans. Based on sperm output data, the difference in transit amplifying divisions appears to be fully compensated by a much larger pool of stem/progenitor spermatogonia in nonhuman primates. In contrast, the large pool of stem/progenitor spermatogonia in humans does not compensate for the reduced number of transit amplifying divisions and consequently, sperm output is reduced. Understanding similarities and differences between species will help to explain challenges in translating technologies such as SSC culture and SSC transplantation to higher primates and ultimately to the human clinic.

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