Spermatogonial stem cells in higher primates: are there differences from those in rodents?

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Abstract

Spermatogonial stem cells (SSCs) maintain spermatogenesis throughout the reproductive life of mammals. While a single spermatogonia comprise the rodent SSC pool, the identity of the stem cell pool in the primate spermatogenic lineage is not well established. The prevailing model is that primate spermatogenesis arises from a dark and a pale spermatogonia, which are considered to represent reserve and active stem cells respectively. However, there is limited information about how the a dark and a pale descriptions of nuclear morphology correlate with the clonal (a single, a paired and a aligned), molecular (e.g. GFRα1 (GFRA1) and PLZF), and functional (SSC transplantation) descriptions of rodent SSCs. Thus, there is a need to investigate primate SSCs using criteria, tools, and approaches that have been used to investigate rodent SSCs over the past two decades. SSCs have potential clinical application for treating some cases of male infertility, providing impetus for characterizing and learning to manipulate these adult tissue stem cells in primates (nonhuman and human). This review recounts the development of a xenotransplant assay for functional identification of primate SSCs and progress dissecting the molecular and clonal characteristics of the primate spermatogenic lineage. These observations highlight the similarities and potential differences between rodents and primates regarding the SSC pool and the kinetics of spermatogonial self-renewal and clonal expansion. With new tools and reagents for studying primate spermatogonia, the field is poised to develop and test new hypotheses about the biology and regenerative capacity of primate SSCs.

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Progress characterizing SSCs and development of the spermatogenic lineage in rodents may provide insights about the identity and characteristics of nonhuman primate and human SSCs. Here, we provide a review of tools and strategies used to characterize rodent SSCs, and summarize classical and contemporary approaches for studying primate SSCs. We will conclude with comments on the evolutionary conservation of SSC phenotype and function from rodents to primates and future studies that may help to elucidate the mode of stem cell renewal and differentiation in primates.

Spermatogonial stem cells in rodents

Spermatogonial stem cells (SSCs) are undifferentiated germ cells that balance self-renewing and differentiating divisions to maintain spermatogenesis throughout adult life. This is a productive stem cell system that produces millions of sperms each day while also maintaining rigorous quality control to safeguard germline integrity. Investigating the biological properties of SSCs that achieve this delicate balance in vivo will expand the understanding of stem cell/niche interactions in a variety of adult tissues and may also have implications for treating male infertility. Despite their critical importance to spermatogenesis and male fertility, the cellular and molecular characteristics of SSCs remain largely undefined. Experimental determination of the basic characteristics of SSCs requires a standardized biological assay that detects the capacity to initiate and maintain spermatogenesis. A SSC transplantation technique, developed for mice in 1994, measures this endpoint, and, thus, functionally evaluates stem cell activity in any mouse testis cell preparation (Brinster & Avarbock 1994, Brinster & Zimmermann 1994). Briefly, germ cells are isolated from the testes of donor animals and transplanted into the seminiferous tubules of infertile recipients where they produce colonies of spermatogenesis and functional sperm. Only a stem cell can produce

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and maintain a colony of spermatogenesis, and each colony in recipient testes arises from the clonogenic proliferation and differentiation of a single SSC (Dobrinski et al. 1999b, Nagano et al. 1999, Zhang et al. 2003, Kanatsu-Shinohara et al. 2006). Application of this technique in rodents revealed that SSCs from donors of all ages (newborn to adult) are competent to produce complete spermatogenesis in the testes of infertile males (Brinster & Avarbock 1994, Ogawa et al. 2000, Ohta et al. 2000, Nagano et al. 2001a, Shinohara et al. 2001, Brinster et al. 2003, Ryu et al. 2003). In addition to rodents, SSC transplantation has successfully generated complete spermatogenesis in other higher species, including goats (Honaramooz et al. 2003), pigs (Mikkola et al. 2006), and dogs (Kim et al. 2008). These results may have future implications for treating some cases of human male infertility (reviewed by Orwig & Schlatt (2005), Brinster (2007) and Schlatt et al. (2009)).

In addition, fluorescence-activated cell sorting (FACS) combined with SSC transplantation has also enabled systematic characterization of mouse SSCs as a sub-population of mouse testis cells defined by the phenotype α6-INTGRIN⁺, B1-INTGRIN⁺, THY1⁺, CD9⁺, Hoechst side population⁺, Rho123low, αv-INTGRIN−, KIT− (ckIT⁻), major histocompatibility complex class I (MHC-I)−, and CD45− (Shinohara et al. 1999, 2000, Kubota et al. 2003, Falciatori et al. 2004, Kanatsu-Shinohara et al. 2004, Lassalle et al. 2004, Fujita et al. 2005, Lo et al. 2005). Rodent SSCs can also be identified in whole mount preparations of testicular seminiferous tubules (initially described by Clermont & Bustos-Obregon (1968)) as isolated A single spermatogonia and probably some A paired spermatogonia. These A single SSCs can be distinguished in whole mount from committed progenitor spermatogonia (some A paired and A aligned chains of 4–16 cells) on the basement membrane of seminiferous tubules because committed cells exist as clonal chains connected by intercellular cytoplasmic bridges. Here, we define progenitors as undifferentiated spermatogonia that are committed to differentiate and can undergo a finite number of self-renewing divisions. Although no SSC-specific marker has been identified, whole mount analyses indicate that GFRα1 (GFRα1), PLZF (ZBTB16), CDH1, NGN3 (NEUROG3) and POU5F1 (OCT3/4) are expressed by undifferentiated stem and progenitor spermatogonia, including A single, A paired, and A aligned (4–16) (Buaas et al. 2004, Yoshida et al. 2004, Greenbaum et al. 2006, Nakagawa et al. 2007, Tokuda et al. 2007, Schlesser et al. 2008). In contrast, the KIT receptor tyrosine kinase is absent from A single, A paired, and most A aligned spermatogonia, but initiates expression in larger A aligned clones (8 and 16 cells) and continues in differentiating types A1–4, intermediate, and B spermatogonia (Manova et al. 1990, Sorrentino et al. 1991, Yoshinaga et al. 1991, Tajima et al. 1994, Dym et al. 1995, Schrans-Stassen et al. 1999). Initiation of KIT expression marks the transition from undifferentiated A aligned spermatogonia to differentiating A1 spermatogonia (Schrans-Stassen et al. 1999). Thus, based on whole mount analyses and molecular phenotyping in rodents, it is possible to distinguish stem/progenitor spermatogonia (A single, A paired, and A aligned; GFRα1⁺, PLZF⁺, NGN3⁺/−, and KIT⁻) and differentiating spermatogonia (A1–4, intermediate, and B: GFRα1⁻, PLZF⁻, NGN3⁺/−, and KIT⁺).

Mammalian spermatogenesis occurs in a synchronized, cyclic pattern where the cellular associations of differentiating germ cells and Sertoli cells are maintained in a progressive and repeated fashion (de Rooij & Russell 2000). Using this information, the seminiferous epithelium can be categorized into numerous discrete ‘stages’ based upon the cellular complement observed in a given segment of seminiferous tubule. Through evaluation of these cellular associations has identified 12 discrete stages of the seminiferous epithelium in mice and 14 stages in rats (Table 1; Leblond & Clermont 1952, Oakberg 1956).

Morphometric whole mount studies have demonstrated that the numbers of A single in mice, rats, and Chinese hamsters remain relatively constant throughout the spermatogenic cycle, apparently due to the balanced renewal of A single and formation of A paired (Fig. 1A; Huckins 1971, Oakberg 1971, de Rooij 1973, Lok et al. 1982, Tegelenbosch & de Rooij 1993). Likewise, numbers of A paired are relatively constant across the seminiferous cycle (Fig. 1A). In contrast, the density of A aligned is cyclic and is lowest at stages IX–XI, after large A aligned clones produce differentiating A1 spermatogonia, and highest at stage VI of the subsequent cycle, as A aligned clones become larger prior to recruitment to A1 (Fig. 1A). Morphometric quantification of total undifferentiated spermatogonial numbers per testis indicates that there are roughly 35 000 A single per testis, representing roughly 0.03% of all testicular germ cells (1.3% of spermatogonia or 10.6% of undifferentiated spermatogonia; Tegelenbosch & de Rooij 1993).

Identity and arrangement of undifferentiated spermatogonia in primates

Clermont & Leblond (1959) initially described two morphologically distinct types of undifferentiated spermatogonia in the testes of rhesus macaques, and designated these cells A bright and A pale (later renamed A dark and A pale respectively; Clermont & Antar 1973). Both cell types are present on the basement membrane of primate seminiferous tubules, but differ based on nuclear architecture and staining intensity with hematoxylin. Clermont proposed that A dark were SSCs, which undergo self-renewing divisions to maintain the stem cell pool and give rise to A pale that subsequently generate differentiating type-B spermatogonia (Clermont & Leblond 1959).
Ten years after the initial description of $A_{dark}$ and $A_{pale}$ spermatogonia, Clermont (1969) revised his linear ‘$A_{dark}$ stem cell–$A_{pale}$ progenitor model’ based on the observations in the vervet monkey (*Cercopithecus aethiops*) that $A_{dark}$ failed to label with $^{3}$H-thymidine. Thus, since $A_{dark}$ did not appear to self-renew under steady-state conditions, Clermont (1969) proposed that $A_{dark}$ and $A_{pale}$ represent reserve and active stem cells respectively. In this ‘reserve stem cell’ model, spermatogenesis is maintained by the ‘active’ pool of $A_{pale}$ SSCs under normal circumstances. A similar model has been proposed for human spermatogenesis, where both $A_{dark}$ and $A_{pale}$ are present, and active $A_{pale}$ proliferation maintains spermatogenesis by balancing the production of differentiating $B$ spermatogonia and by renewing the $A_{pale}$ pool ((Clermont 1972) and Clermont & Antar (1973)).

Alternatively, others have proposed that the low mitotic index of $A_{dark}$ is specifically indicative of a ‘true SSC’ phenotype, while the regular divisions of nearly all $A_{pale}$ demonstrate that these cells are ‘renewing progenitors’ that amplify spermatogonial output to $B^1$ (reviewed by Ehmcke & Schlatt (2006) and Ehmcke et al. (2006)).

Similar to rodents, 12 distinct stages of the seminiferous epithelium have been described for stump-tailed macaques, cynomolgus monkeys, rhesus macaques, and baboons, while marmosets and chimpanzees stages are reported to have nine and six stages respectively (Table 1; Clermont & Leblond 1959, Clermont & Antar 1973, Chowdhury & Steinberger 1976, Holt & Moore 1984, Fouquet & Dadoune 1986, de Rooij et al. 1996). Similar to chimpanzees, morphological examination of human spermatogenesis identified only six stages based on discrete cellular associations (Table 1; Clermont 1963, 1966a, Heller & Clermont 1963; reviewed by Amann (2008)). The topological arrangement of stages in human seminiferous tubules is described as spiral or patchy where one cross section may contain germ cells in two to four different stages (Amann 2008). This arrangement is different from the ‘linear’ arrangement described for rodents where a given seminiferous tubule cross section contains only one stage (Table 1). Different nonhuman primate species exhibit varying degrees of ‘linear’ or ‘patchy’ stage topography (Table 1).

As for rodents, numbers and distribution of undifferentiated spermatogonia along the seminiferous epithelium have been described for primates. Morphometric studies indicate that the adult rhesus testis contains roughly equal numbers of $A_{dark}$ and $A_{pale}$ (Marshall & Plant 1996). In the rhesus testis, $A_{dark}$ are equally distributed along the length of the seminiferous tubule epithelium and do not fluctuate significantly between stages (Fig. 1B, red line; Clermont & Leblond 1959, Fouquet & Dadoune 1986). Cells with an indeterminate ‘transition’ phenotype also do not vary

Table 1 Stages of the cycle of the seminiferous epithelium in rodents and primates.

<table>
<thead>
<tr>
<th>Species</th>
<th>Stages (#)</th>
<th>One cycle</th>
<th>Spermatogenesis</th>
<th>Stages per cross section</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pan troglodytes</em> (chimpanzee)</td>
<td>6</td>
<td>14</td>
<td>62.5</td>
<td>2–4 (1–5)</td>
<td>Smithwick &amp; Young (1996) and Smithwick et al. (1996)</td>
</tr>
<tr>
<td><em>Macaca arctoides</em> (stump-tailed macaque)</td>
<td>12</td>
<td>11.6</td>
<td>46.4</td>
<td>1</td>
<td>Clermont (1972) and Clermont &amp; Antar (1973)</td>
</tr>
<tr>
<td><em>Macaca fascicularis</em> (cynomolgus monkey)</td>
<td>12</td>
<td>10.5</td>
<td>42</td>
<td>1 to several</td>
<td>Dietrich et al. (1986) and Fouquet &amp; Dadoune (1986)</td>
</tr>
<tr>
<td><em>Macaca mulata</em> (rhesus macaque)</td>
<td>12</td>
<td>10.5</td>
<td>42</td>
<td>1</td>
<td>Clermont &amp; Leblond (1959), de Rooij et al. (1986) and Rosiepen et al. (1997)</td>
</tr>
<tr>
<td><em>Callithrix jacchus</em> (common marmoset)</td>
<td>9</td>
<td>10</td>
<td>37</td>
<td>1–5</td>
<td>Holt &amp; Moore (1984) and Millar et al. (2000)</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>12</td>
<td>8.62</td>
<td>34.5</td>
<td>1</td>
<td>Oakberg (1956, 1971), Clermont &amp; Trot (1969) and Kluin et al. (1982)</td>
</tr>
<tr>
<td><em>Rattus norvegicus</em></td>
<td>14</td>
<td>13</td>
<td>51.6</td>
<td>1</td>
<td>Leblond &amp; Clermont (1952), Clermont &amp; Perey (1957) and Huckins (1971)</td>
</tr>
<tr>
<td><em>Cricetulus griseus</em> (Chinese hamster)</td>
<td>12</td>
<td>17</td>
<td>68</td>
<td>1</td>
<td>Clermont &amp; Trot (1969) and Oud &amp; de Rooij (1977)</td>
</tr>
<tr>
<td><em>Mesocricetus brandti</em> (Turkish hamster)</td>
<td>8</td>
<td>8</td>
<td>32</td>
<td>1</td>
<td>Myoga et al. (1991)</td>
</tr>
<tr>
<td><em>Mesocricetus auratus</em> (Golden hamster)</td>
<td>13</td>
<td>8.74</td>
<td>35</td>
<td>1</td>
<td>Leblond &amp; Clermont (1952) and Miething (1998)</td>
</tr>
</tbody>
</table>

*Evidence of discrete stages of the seminiferous epithelium in individual cross sections of seminiferous tubules.*
between stages (Fig. 1B, orange line; Fouquet & Dadoune 1986). In contrast, \( A_{\text{pale}} \) numbers are not constant due to their more active stage-dependent proliferation resulting in peak numbers at mid-cycle (stages VII–VIII) and a nadir late in the cycle (stages X–XII) (Fig. 1B, blue line; Fouquet & Dadoune 1986). \( A_{\text{pale}} \) divide between stages VII and IX of the seminiferous epithelium (Clermont 1969, Clermont & Antar 1973, Fouquet & Dadoune 1986, Ehmcke et al. 2005b, Simorangkir et al. 2009), although there is disagreement in the literature about whether \( A_{\text{pale}} \) divide once (Clermont & Leblond 1959, Clermont 1969, Simorangkir et al. 2009) or twice (Clermont & Antar 1973, Ehmcke et al. 2005a) during a cycle of the seminiferous epithelium. The profile of rhesus \( A_{\text{pale}} \) numbers across the cycle of the seminiferous epithelium (Fig. 1B, blue line) appears more similar to mouse \( A_{\text{aligned}} \) spermatogonia (Fig. 1A, green and blue lines), although there appears to be a difference between species in the timing of divisions.

There is limited information about the clonal arrangement of \( A_{\text{dark}} \) and \( A_{\text{pale}} \) spermatogonia in nonhuman primate seminiferous. Clermont & Leblond (1959) first reported pairs and ‘quartets’ of \( A_{\text{dark}} \) and \( A_{\text{pale}} \) spermatogonia using maps of rhesus macaque spermatogonia from individual seminiferous tubules generated by plotting the relative positions of spermatogonia in numerous serial testis sections. Using the camera lucida technique, Clermont (1969) subsequently reported the clonal arrangement of \( A_{\text{dark}} \) and \( A_{\text{pale}} \) spermatogonia in hematoxylin-stained segments of intact seminiferous tubules from adult vervet monkeys (Cercopithecus aethiops). This approach enabled the visualization of \( A_{\text{dark}} \) and \( A_{\text{pale}} \) spermatogonia and their topological arrangement. Clermont concluded from these studies that \( A_{\text{dark}} \) and \( A_{\text{pale}} \) existed only as clearly demarcated even-numbered clusters, with the highest frequency being pairs (20.7%) and groups of 4 (40.7%). It is difficult to draw any broad conclusions about the clonal organization of \( A_{\text{dark}} \) and \( A_{\text{pale}} \) in primates using these spermatogonial map and camera lucida data for two reasons. First, intercellular cytoplasmic bridges, which would definitively identify clones, are not reproduced in camera lucida drawings. Secondly, topographical guidelines to help establish clonality, such as the 25 \( \mu \)m criteria employed later in rodents (see Huckins 1971), were not applied to these camera lucida analyses.

De Rooij and colleagues reported that clonal arrangement of type-A spermatogonia in seminiferous tubules recovering after low-dose radiation in a large number of rhesus macaques (van Alphen et al. 1988). Clonal analysis was performed using morphological and topographical criteria similar to those employed for rodents (Huckins 1971, Oakberg 1971, de Rooij 1973). Results in the repopulating seminiferous epithelium demonstrated clones of 1, 2, 4, 8, and \( \geq 16 \) of both \( A_{\text{dark}} \) and \( A_{\text{pale}} \) (van Alphen et al. 1988), but it is not clear whether these data equate to the normal clonal arrangement of spermatogonia during steady-state spermatogenesis. As proposed recently, the clonal arrangement of \( A_{\text{dark}} \) and \( A_{\text{pale}} \) in primate testes during steady-state spermatogenesis may relate to whether these cells exhibit functional identity as stem cells or progenitor spermatogonia (reviewed by Ehmcke & Schlatt (2006) and Ehmcke et al. (2006)). The observation that isolated, single spermatogonia in S-phase were exceedingly rare in the rhesus testis prompted the theory that there are many nonproliferating single type-A spermatogonia.
spermatogonia, which may be \( A_{\text{dark}} \) spermatogonia (Ehmcke et al. 2005b). Thus, additional studies are needed to clarify the kinetics of type-A spermatogonial expansion in primates, including defining \( A_{\text{dark}} \) and \( A_{\text{pale}} \) clone size as it relates to the stage of the seminiferous epithelium.

### Kinetics of type-A spermatogonial proliferation in primate testes

In support of the ‘reserve stem cell’ model discussed above, a few studies have proposed mechanisms for how \( A_{\text{pale}} \) maintain spermatogenesis in the absence of significant \( A_{\text{dark}} \) proliferation. Based on morphometric evaluation in the vervet monkey, Clermont (1969) reported that all \( A_{\text{pale}} \) divide between stages IX and X of the seminiferous epithelium. Half of the daughter population remains as \( A_{\text{pale}} \) (self-renews) and the other half differentiates to B1 spermatogonia (Clermont 1969). In the stump-tailed macaque (Macaca arctoides), Clermont observed two divisions of \( A_{\text{pale}} \) in each cycle of the seminiferous epithelium. A doubling \( A_{\text{pale}} \) division occurs at stage VII, and a second differentiating division occurs at stage IX to produce B1 spermatogonia (Clermont 1972, Clermont & Antar 1973). Similarly, using whole mount preparations of rhesus seminiferous tubules, Ehmcke et al. (2005a) observed two mitotic \( A_{\text{pale}} \) events per cycle and proposed a ‘clone splitting’ model of \( A_{\text{pale}} \) renewal and differentiation. In this model, larger clones of \( A_{\text{pale}} \) (e.g. eight cells) produced after the first division at stage VII disintegrate into multiple smaller \( A_{\text{pale}} \) clones (e.g. two clones of two \( A_{\text{pale}} \) and one clone of four \( A_{\text{pale}} \)) that will again divide (at stage IX) to form clones of four or eight \( B_{1} \) spermatogonia or more clones of eight \( A_{\text{pale}} \) (self-renewal). These models attempt to explain how \( A_{\text{pale}} \) spermatogonia might balance self-renewing and differentiating divisions to maintain spermatogenesis with little contribution from \( A_{\text{dark}} \).

Work from the de Rooij laboratory provided evidence that \( A_{\text{dark}} \) are mobilized following cytotoxic insult to the testis (van Alphen & de Rooij 1986). During the first 11 days after X-irradiation of rhesus testes, these investigators observed a near depletion of \( A_{\text{pale}} \) spermatogonia, with no significant change in the number of \( A_{\text{dark}} \). A significant decrease in \( A_{\text{dark}} \) spermatogonia was observed 14 days after X-irradiation with a corresponding increase in \( A_{\text{pale}} \) spermatogonia. The authors concluded that the resting \( A_{\text{dark}} \) were activated into proliferating \( A_{\text{pale}} \) spermatogonia (van Alphen & de Rooij 1986). Thus, \( A_{\text{dark}} \) appear to fulfill the role of a ‘reserve stem cell’. However, whether \( A_{\text{dark}} \) spermatogonia also participate in steady-state spermatogenesis remains an open question.

Since Clermont’s (1969) initial observation that \( A_{\text{dark}} \) spermatogonia do not divide, six additional studies have been conducted in various nonhuman primate species to describe the proliferating fraction of type-A spermatogonia (Table 2). Four of the five studies observed \(^{3}\text{H}-\text{thymidine} \) or BrdU label in \( A_{\text{dark}} \) spermatogonia within a few hours and/or several days after the administration of a pulse label (Clermont & Antar 1973, Kluin et al. 1983, Fouquet & Dadoune 1986, Ehmcke et al. 2005b, Simorangkir et al. 2009). Similar to Clermont’s observation in the vervet monkey, Simorangkir et al. (2009) did not observe labeling in \( A_{\text{dark}} \). However, these investigators observed labeling in a new ‘unclassified’ category of type-A spermatogonia (\( A_{\text{unc}} \)), which they acknowledged might be classified as \( A_{\text{dark}} \) by other investigators. It is not clear whether \( A_{\text{unc}} \) are the same cells as those previously defined with an intermediate phenotype as A-transition (\( A_{t} \)), some of which also label with \(^{3}\text{H}-\text{thymidine} \) (Fouquet & Dadoune 1986). Immuno-histochemistry for the proliferating cell nuclear antigen (PCNA) has also determined that rhesus and human \( A_{\text{dark}} \) spermatogonia failed to label, while only a fraction of \( A_{\text{pale}} \) in late stages of the cycle of the seminiferous epithelium were PCNA\(^{+} \) (Schlatt & Weinbauer 1994). Considering the lack of consensus about the cell cycling characteristics of \( A_{\text{dark}} \) spermatogonia, additional studies are needed to confirm whether 1) \( A_{\text{dark}} \) are quiescent and serve as reserve stem spermatogonia or 2) \( A_{\text{dark}} \) divide with sufficient (albeit low) frequency to maintain spermatogenesis under normal, steady-state conditions.

The \( A_{\text{dark}}/A_{\text{pale}} \) ‘reserve stem cell’ model of nonhuman primate SSCs is very similar to the \( A_{0}/A_{1} \) ‘reserve stem cell’ model that was originally advanced for rodents (Clermont & Bustos-Obregon 1968, Dym & Clermont 1970, Clermont & Hermo 1975, Bartmanska & Clermont 1983). However, this model was supplanted by an alternative model (Huckins 1971, Oakberg 1971), in which there is a single population of stem cells (\( A_{\text{single}} \) spermatogonia) that divides regularly, but infrequently, and gives rise to the entire spermatogenic lineage. This \( A_{\text{single}} \) model has gained wide (but not universal) acceptance in the field. Spermatogenesis is highly conserved (Fritz 1986), and thus, it is tempting to extrapolate results from the studies of rodent SSCs to primates.

The identity, characteristics, and behavior of primate SSCs, however, must be determined experimentally. As reviewed above, rodent SSCs can be identified using three approaches: 1) transplantation to observe functional capacity to establish and maintain spermatogenesis (Ogawa et al. 1997, Nagano & Brinster 1998), 2) molecular phenotype (expression of some or all of a battery of specific molecular markers), and 3) clonal arrangement (\( A_{\text{single}} \) de Rooij & Russell 2000). Until recently, almost nothing was known about SSCs in primate testes. Progress in the last few years has begun to address this deficit using rhesus-to-nude mouse xenotransplantation, molecular phenotyping of spermatogonia, and clonal analysis of spermatogonia in whole mount aided by immuno-histochemistry.
An assay for studying primate SSCs

In rodents, SSC transplantation is the experimental ‘gold standard’ for detecting SSC activity because it demonstrates that a cell has the biological capacity to initiate and maintain spermatogenesis by balancing self-renewal and differentiation (Brinster & Avarbock 1994, Brinster & Zimmerman 1994). While this functional assay has been a powerful tool for characterizing SSCs in rodents, monkey-to-monkey SSC transplantation as a routine biological assay is not feasible. Nonhuman primates are a limited resource, and studies on these large animals are confounded by their large size, long lifespan, high cost, and variability among outbred individuals. Pioneering work from the Brinster, de Rooij, Dobrinski, and Griswold laboratories, however, provided the proof-in-principle that SSCs from a variety of species can be transplanted to the testes of immune-deficient nude mice where they migrate to the seminiferous tubule basement membrane and proliferate to form chains of spermatogonia that persist long term (Clouthier et al. 1996, Dobrinski et al. 1999a, 2000, Ogawa et al. 1999, Nagano et al. 2001b, 2002, Honaramooz et al. 2002, Oatley et al. 2002, Izadyar et al. 2003). Germ cells from closely related species (e.g. mouse, rat, and hamster) produce chains or networks of spermatogonia by 2 weeks after transplantation (Fig. 2A and B), which give rise to extensive colonies of complete spermatogenesis by 2 months (Fig. 2C and D). In contrast, germ cells from primate species including rhesus macaques (Fig. 2E and F; Hermann et al. 2007, 2009), baboons (Nagano et al. 2001b), and humans (Fig. 2G and H; Nagano et al. 2002) produce chains and patches of spermatogonia, similar to rodent colonies at 2 weeks, but do not produce complete spermatogenesis. The ability of mouse Sertoli cells to support the early stages of spermatogenesis from distantly related species represents remarkable evolutionary conservation. These patches of spermatogonia are maintained long term and have been observed for several months to a year after transplantation (Nagano et al. 2001b, 2002, Hermann et al. 2007, 2009). As detailed below, these rudimentary spermatogonial patches in the xenotransplant paradigm may constitute an experimentally tractable bioassay for primate SSCs.

To enable this xenotransplant assay, immune-deficient nude mice are treated with busulfan to eliminate endogenous spermatogenesis as described previously (Brinster & Avarbock 1994, Brinster & Zimmerman 1994). Donor testes cell suspensions can be generated by two-step enzymatic digestion (Bellve et al. 1977, Hermann et al. 2007) and transplanted into seminiferous tubules of nude mouse recipient testes by efferent duct injection (Nagano et al. 2001b, 2002, Hermann et al. 2007, Maki et al. 2009). Donor-derived patches of

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**Table 2** Experimental evidence for $A_{\text{dark}}$ and $A_{\text{apale}}$ proliferation.

<table>
<thead>
<tr>
<th>Study</th>
<th>Species*</th>
<th>Label</th>
<th>Animals, X-sections</th>
<th>Time to analysis</th>
<th>Percentage of $A_{\text{dark}}$ labeled (stage)</th>
<th>Percentage of $A_{\text{apale}}$ labeled (stage)</th>
<th>Percentage of $A_{\text{Aunc}}$ labeled (stage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clermont (1969)</td>
<td>Cercopithecus aethiops / Macaca arctoides</td>
<td>$^{3}$H-Thy</td>
<td>1, N/A</td>
<td>2.5 h</td>
<td>0</td>
<td>36.1% (VII–X)</td>
<td>N/A</td>
</tr>
<tr>
<td>Clermont &amp; Antar (1973)</td>
<td>Macaca arctoides</td>
<td>$^{3}$H-Thy</td>
<td>3, $\geq$400</td>
<td>3 h</td>
<td>0.06–0.09 (VII–X)</td>
<td>25.6–41.9 (VII–X)</td>
<td>N/A</td>
</tr>
<tr>
<td>Kluin et al. (1983)</td>
<td>Macaca fascicularis</td>
<td>$^{3}$H-Thy</td>
<td>4, $\geq$400</td>
<td>12 days 3 h</td>
<td>0</td>
<td>5.9–11.8 (VII–X)</td>
<td>N/A</td>
</tr>
<tr>
<td>Fouquet &amp; Dadoune (1986)</td>
<td>Macaca fascicularis</td>
<td>$^{3}$H-Thy</td>
<td>5, 60</td>
<td>1 h&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.5 (9 of $\sim$600 cells)</td>
<td>14.9 (88 of $\sim$591 cells)</td>
<td>N/A</td>
</tr>
<tr>
<td>Schlatt &amp; Weinbauer (1994)</td>
<td>Macaca mulata</td>
<td>PCNA</td>
<td>2, 800</td>
<td>9–10 days</td>
<td>N/A</td>
<td>2.3–10.8 (IV–IV)</td>
<td>X–XI only</td>
</tr>
<tr>
<td>Ehmcke et al. (2005a, 2005b)</td>
<td>Homo sapiens / Macaca mulata</td>
<td>BrdU + PCNA</td>
<td>2, not indicated</td>
<td>3 h</td>
<td>N/A</td>
<td>V only</td>
<td>N/A</td>
</tr>
<tr>
<td>Simorangkir et al. (2009)</td>
<td>Macaca mulata</td>
<td>BrdU</td>
<td>4, N/A&lt;sup&gt;g&lt;/sup&gt;</td>
<td>3 h</td>
<td>N/A</td>
<td>27.52 (VII only)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4, N/A&lt;sup&gt;g&lt;/sup&gt;</td>
<td>11 days 3 h</td>
<td>N/A</td>
<td>Yes (VII–X&lt;sup&gt;h&lt;/sup&gt;)</td>
<td>Yes (V–VI)</td>
</tr>
</tbody>
</table>

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* Cercopithecus aethiops, vervet monkey; Macaca arctoides, stump-tailed macaque; Macaca fascicularis, cynomolgus monkey (aka: crab-eating monkey, java monkey, Macaca irus); Macaca mulata, rhesus monkey.<sup>a</sup> Number of seminiferous tubule cross sections is noted per animal evaluated if available. <sup>b</sup> Testicular fragments (1–8 mm<sup>3</sup>) were incubated in medium containing $^{3}$H-thymidine for 1 h at 32 °C after a 30 min equilibration period. <sup>c</sup> Label was administered into the testicular artery followed by two intratesticular injections of label at positions 2 cm apart. Biopsy at 1 h recovered tissue at one intratesticular injection site, hemicastration at 9–10 days was used to remove tissue at second injection site. <sup>h</sup> Although the precise number of seminiferous cross sections was not reported, the number of microscopic fields was reported per animal (not discriminating between the two analysis points). Spermatogenesis of two animals included in this study was maintained by exogenous gonadotropin administration following a GnRH clamp for a separate study; $A_{\text{dark}}$ labeling was classified as weak and strong, representing the two values noted respectively. Number of seminiferous tubule cross sections were not reported, but 1053–1299 cells of each type were evaluated per testis. The labeling index of these cells was not reported at 11 days 3 h after label administration.
spermatogonia can be identified 1–2 months after transplantation by staining with a donor species-specific antiserum as described previously (Nagano et al. 2001b, 2002, Hermann et al. 2007, 2009). Alternatively, donor testis cells can be preloaded with a fluorescent marker (e.g. PKH26 or CFDA) prior to transplantation (Honaramooz et al. 2002, Maki et al. 2009). With the fluorescent loading approach, recipient testes are typically analyzed within 2–3 weeks after transplantation to minimize the dilution of the fluorescent dye through cell divisions.

While evolutionary distance between primates and mice presumably precludes the establishment of complete spermatogenesis in the xenotransplant assay, colonization foci consisting of spermatogonial patches exhibit several features of SSCs. They arise from transplanted cells that migrate to the basement membrane of recipient seminiferous tubules and produce chains of spermatogonia that persist long term (Nagano et al. 2001b, 2002, Hermann et al. 2007, 2009). For our studies in rhesus macaques, we have defined SSC-derived spermatogonial patches as four or more cells in discrete patches (without a gap of ≥ 100 μm between adjacent cells) on the basement membrane of recipient mouse seminiferous tubules that exhibit characteristic spermatogonial features (i.e. ovoid shape with high nuclear to cytoplasmic ratios). Nagano et al. (2001b, 2002) used similar criteria for baboon and human SSCs, but defined patches as containing ten or more cells within a 150 μm length of seminiferous tubule. Co-staining for VASA or RBMY has been used to confirm that the xenotransplant spermatogonial patches were composed of germ cells (Nagano et al. 2001b, Hermann et al. 2007). However, it is important to interpret xenotransplant results cautiously because some donor cell foci fail to exhibit spermatogonial features, and therefore, are not considered to arise from stem cells (Hermann et al. 2007). As more information becomes available, it may be reasonable to revise the criteria for defining spermatogonial patches in the xenotransplant assay.

Using the criteria detailed above, we employed rhesus-to-nude mouse xenotransplantation to characterize SSC activity in adult rhesus testes. Analysis of nude mouse recipient seminiferous tubules 2 months after transplantation revealed that 4.64 patches/10⁶ viable donor adult rhesus testis cells transplanted (Hermann et al. 2007). In contrast, testis cells from males treated with busulfan (8 or 12 mg/kg) failed to produce any patches of spermatogonia. These data suggested that high-dose alkylating chemotherapy caused depletion of SSCs in rhesus testes, consistent with previous observations for mice (Kanatsu-Shinohara et al. 2003, Orwig et al. 2008). In a separate study, the xenotransplant assay demonstrated that spermatogonial patches were enriched in the THY1⁺ fraction of rhesus testis cells (Hermann et al. 2009), with corresponding depletion in THY1⁻ testis cells, similar to observations of mouse and rat SSCs (Kubota et al. 2003, Ryu et al. 2004). Evolutionary conservation of these biological readouts provides a partial validation of the xenotransplantation assay (Hermann et al. 2007, 2009). Maki et al. (2009) have also utilized the xenotransplant technique and reported enhanced colonizing activity in an SSEA4⁺ subpopulation of rhesus testes cells. Continued FACS and xenotransplantation experiments will enable the systematic characterization of primate SSCs and potentially lead to enrichment strategies with implications for future SSC-based treatments of male infertility.

Figure 2 SSC transplantation from different donor species into busulfan-treated mouse testes. (A and B) Donor GFP mouse testis cells (green) at 2 weeks after transplantation. Margins of recipient seminiferous tubules are marked by a dashed white line. (C and D) Donor GFP mouse testis cells (green) at 2 months after transplantation. Patches of transplanted donor (E and F) rhesus and (G and H) human testis cells in immune-deficient nude mouse seminiferous tubules were detected by whole mount immunohistochemistry using the rhesus testis cell antibody. Scale bars = 50 μm. Adapted from Hermann et al. (2007, 2009) and unpublished data.
Marker analysis for functional categorization of rhesus spermatogonia and identification of the putative stem cell pools

Until recently, little was known about the molecular characteristics of nonhuman primate spermatogonia, including SSCs. This contrasts with rodents, where decades of studies provide an extensive molecular phenotype of cell surface, cytoplasmic, and nuclear proteins that are expressed by rodent SSCs. Although no SSC-specific marker has been identified to date for any species, the combined expression profiles of multiple markers provide composite phenotypic information about stem, progenitor, and differentiating spermatogonia in rodents that may be used to identify similar cells in other species, including primates. To this end, several recent studies have evaluated nonhuman primate primary testes for the expression of various proteins known to mark SSCs and other stem cells (reviewed in Table 3).

However, there is limited information about how these markers correlate with spermatogenic cell types in primates (e.g. $A_{\text{dark}}$, $A_{\text{pale}}$, and B spermatogonia).

In order to bridge the gap between molecular phenotype data and spermatogenic cell types, we recently investigated expression of rodent spermatogonial markers (GFRA1, PLZF, NGN3, and KIT) in the rhesus testis and related our findings to classical descriptions of nuclear morphology (i.e. $A_{\text{dark}}$, $A_{\text{pale}}$, and B spermatogonia; Fig. 3A–E; Hermann et al. 2009).

The expression profile of each marker in the rhesus testis was correlated with the functional categories of rodent spermatogonia exhibiting similar phenotypes, including stem ($A_{\text{single}}$ and some $A_{\text{paired}}$; GFRA1$^+$, PLZF$^+$, NGN3$^{+/+}$, and KIT$^+$), transit-amplifying progenitor (some $A_{\text{paired}}$ and $A_{\text{aligned}}$; GFRA1$^+$, PLZF$^+$, NGN3$^+$, and KIT$^{+/+}$), and differentiating (AI–4, intermediate).

### Table 3 Molecular markers of germ cells and spermatogonial stem cells expressed in nonhuman primate testes.

<table>
<thead>
<tr>
<th>Marker (aliases)</th>
<th>References</th>
<th>Species$^a$</th>
<th>Approach</th>
<th>Expressed in spermatogonia?</th>
<th>$A_{\text{dark}}$</th>
<th>$A_{\text{pale}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIT (cKIT)</td>
<td>Hermann et al. (2009)</td>
<td>Macaca mulata</td>
<td>IHC</td>
<td>Yes</td>
<td>0%</td>
<td>22.8%</td>
</tr>
<tr>
<td>DAZL</td>
<td>Hermann et al. (2007)</td>
<td>Macaca mulata</td>
<td>FCM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GFRA1 (GFRz1)</td>
<td>Hermann et al. (2007)</td>
<td>Macaca mulata</td>
<td>IHC</td>
<td>Staining weak/absent in spermatogonia</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>α6-INTEGRIN</td>
<td>Hermann et al. (2007)</td>
<td>Macaca mulata</td>
<td>IHC</td>
<td>Rare BM cells$^b$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MAGEA4</td>
<td>Mitchell et al. (2008)</td>
<td>Callithrix jacchus</td>
<td>IHC</td>
<td>Yes in 0–6 weeks testis</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NANO51</td>
<td>Mitchell et al. (2008)</td>
<td>Callithrix jacchus</td>
<td>IHC</td>
<td>Yes in 0–6 weeks testis</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NGN3 (NEUROG3)</td>
<td>Hermann et al. (2009)</td>
<td>Macaca mulata</td>
<td>IHC</td>
<td>Rare cells in 0–6 weeks testis</td>
<td>0%</td>
<td>48.5%</td>
</tr>
<tr>
<td>POU5F1 (OCT-4)</td>
<td>Mitchell et al. (2008)</td>
<td>Macaca mulata</td>
<td>IHC</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PLZF (ZBTB16)</td>
<td>Hermann et al. (2007)</td>
<td>Macaca mulata</td>
<td>IHC</td>
<td>Rare BM cells$^b$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SSEA4</td>
<td>Hermann et al. (2009)</td>
<td>Macaca mulata</td>
<td>IHC</td>
<td>Yes</td>
<td>82%</td>
<td>100%</td>
</tr>
<tr>
<td>SALL4</td>
<td>Hermann et al. (2008)</td>
<td>Macaca mulata</td>
<td>IHC</td>
<td>Rare BM cells$^b$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>THY1 (CD90)</td>
<td>Hermann et al. (2009)</td>
<td>Macaca mulata</td>
<td>FCM+ICC/XenoTP</td>
<td>Yes$^c$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TRA-1-81</td>
<td>Muller et al. (2008)</td>
<td>Macaca mulata</td>
<td>IHC</td>
<td>Rare BM cells$^b$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>VASA (DDX4)</td>
<td>Hermann et al. (2007)</td>
<td>Macaca mulata</td>
<td>IHC</td>
<td>Weak in adult</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Hermann et al. (2009)</td>
<td>Macaca mulata</td>
<td>IHC</td>
<td>Yes in juvenile</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

IHC, immunohistochemistry; FCM, flow cytometry/fluorescence-activated cell sorting; XenoTP, xenotransplantation; ICC, immunocytochemistry; qPCR, quantitative PCR.

$^a$Macaca mulata, rhesus monkey; Macaca silenus, lion-tailed macaque; Callithrix jacchus, common marmoset.

$^b$Rare cells positive for the marker located on the basement membrane of testicular seminiferous tubule cross sections.

$^c$Expression by spermatogonia determined by immunocytochemical staining for GFRA1 in sorted fractions of adult rhesus testis cells.

$^d$Expression by spermatogonia determined by immunocytochemical staining for VASA and qPCR for VASA, GFRA1, and PLZF in juvenile rhesus testis cells sorted for THY1.
and B; GFRA1\textsuperscript{−}, PLZF\textsuperscript{−}, NGN3\textsuperscript{+}, and KIT\textsuperscript{+}). We observed a remarkable degree of evolutionary conservation from rodents to primates in the succession of spermatogonial marker expression and their correlation with differentiation state (Fig. 3E). Assuming that molecular characteristics correlate with function and that these relationships are evolutionarily conserved, it may be possible to identify stem spermatogonia in primates. Note that most A\textsubscript{dark} and \sim 50% of A\textsubscript{pale} in the adult rhesus testis exhibit the phenotype GFRA1\textsuperscript{+}, PLZF\textsuperscript{+}, NGN3\textsuperscript{−}, and KIT\textsuperscript{−} (Fig. 3E). As far as we can ascertain from the literature, this phenotype is restricted to A\textsubscript{single} spermatogonia, which most will agree comprise at least part of the stem cell pool in mice. Moreover, since NGN3 marks 11.7% of functional stem cells in mice as well as A\textsubscript{aligned} progenitor spermatogonia (Nakagawa et al. 2007), rhesus A\textsubscript{pale} spermatogonia with the phenotype GFRA1\textsuperscript{+}, PLZF\textsuperscript{+}, NGN3\textsuperscript{+}, KIT\textsuperscript{−} could be either stem or progenitor (shaded as transition cells in Fig. 3E). Lastly, KIT expression begins in longer chain progenitors (i.e. A\textsubscript{aligned} 8–16) in rodents and continues in differentiating types A1–4 spermatogonia (Schrans-Stassen et al. 1999). Some A\textsubscript{pale} in the adult rhesus testis exhibit a transition phenotype (GFRA1\textsuperscript{+}, PLZF\textsuperscript{+}, NGN3\textsuperscript{+}, KIT\textsuperscript{−}, like longer chain rodent A\textsubscript{aligned}; Fig. 3E).

Based on the conservation of molecular markers from rodents to primates, we have proposed that the stem cell pool in the rhesus testis comprises all A\textsubscript{dark} and at least 50% of A\textsubscript{pale} spermatogonia, and that
the stem cell pool is considerably larger in rhesus than mouse testes (see Hermann et al. 2009 for detailed discussion). In contrast to the large SSC pool, the relative size of the progenitor pool (GFRA1⁺, PLZF⁺, NGN3⁺, and KIT) appeared much smaller in adult macaques than in rodents. Thus, it appears that rodents and primates employ different strategies to meet a similar biological demand (adult rodent and adult rhesus testes have similar sperm output per gram of testis per day (Sharpe 1994, Gupta et al. 2000, Thayer et al. 2001)). Rodents may have few SSCs and more transit-amplifying progenitors, while rhesus testes may have more SSCs and fewer transit-amplifying progenitors.

Progress studying SSCs and the spermatogenic lineage in rodents and nonhuman primates has begun to stimulate investigations of the biology and regenerative potential of human SSCs (reviewed by Dym et al. (2009)). One recent study confirmed that α6-INTEGRIN, CD133, SSEA4, VASA, DAZL, and TSPYL2 are expressed in human testis cells or histological section (Conrad et al. 2008). For this review, we have also conducted a comparative analysis of the consensus SSC marker, PLZF, in mouse (Fig. 4A and B), rhesus monkey (Fig. 4C and D), and human (Fig. 4E and F) testes. PLZF staining in human testes was restricted to a subpopulation of cells on the basement membrane of the seminiferous epithelium (like mouse and monkey), and the frequency of PLZF⁺ cells was more similar to monkey than mouse. Other differences (e.g. expression of POU5F1, TSPY, and KIT) have been reported in marker expression between rodent and human spermatogonia, suggesting phenotypic differences in markers of stem cells and their progeny (see review by Dym et al. (2009)). Perhaps, this suggests that the dynamics of the stem/progenitor spermatogonial pools in humans is similar to monkeys. Additional studies are necessary to elaborate on these findings. For future studies, it appears that human-to-nude mouse xenotransplantation can also be optimized as a bioassay for human SSCs (Nagano et al. 2002; Fig. 2G and H).

Figure 4 Comparative immunohistochemical analysis reveals species-specific staining profiles for the stem/progenitor marker PLZF. To begin translating knowledge of rodent and monkey SSCs to humans, we have initiated comparative marker analysis using immunohistochemistry for the transcription factor PLZF in sections from (A and B) mouse, (D and E) rhesus macaque, and (G and H) human testes. Images of sections incubated with nonimmune isotype control IgGs are also shown for (C) mouse, (F) monkey, and (I) human to demonstrate nonspecific background staining and tissue autofluorescence. White asterisks in A and C indicate the nonspecific fluorescent signal observed in interstitial space between seminiferous tubules. Images are shown from (A, C, D, F, G and I) low magnification and (B, D and F) high magnification. PLZF immunoreactivity was observed as a nuclear fluorescent signal (green) in all three species. Sections were counterstained with DAPI (blue). Scale bar = 50 μm. (BP Hermann, MC Hansel & KE Orwig, unpublished observations).

Future directions
The considerable degree of phenotypic similarity between Adark and Apale spermatogonia in the adult rhesus testis raises questions about the distinct functional classification of Adark and Apale as reserve and renewing stem cells respectively (Hermann et al. 2009). One possibility is that dark and pale nuclear morphologies correspond with the stage of the cell cycle (i.e. G0 versus G1/S/G2/M) and not with the distinct stem cell populations. Experiments are ongoing to determine whether nuclear morphology correlates with cell cycle stage. In addition, expression of NGN3 encompassed a transition from KIT⁻ to KIT⁺ within Apale spermatogonia, suggesting that the initiation of NGN3 coincides with monkey spermatogenic differentiation. Future studies
will begin to dissect this transition in \( A_{\text{pale}} \) to identify regulatory networks that instruct spermatogonial differentiation in primates.

In rodents, spermatogonial clone size is associated with spermatogenic differentiation state; a spermatogonial clone differentiates, as it becomes larger. As discussed above (see Section ‘Identity and arrangement of undifferentiated spermatogonia in primates’), there is limited information about the clonal arrangement of type-A spermatogonia in primates (Clermont 1969). We and others have begun to characterize the markers of undifferentiated (e.g. PLZF and GFRA1) versus differentiating (KIT) spermatogonia in primates. Thus, coupling immunohistochemical staining with spermatogonial clone size analysis in whole mount preparations of seminiferous tubules would provide valuable information correlating differentiation state with clone size in primates. Clonal analyses of this nature could elucidate the point in spermatogonial amplification (i.e. clone size) at which a differentiated phenotype begins to emerge. To this end, we have initiated studies to evaluate spermatogonial clones that exhibit undifferentiated (PLZF\(^+\); Fig. 5A and B) or differentiating (KIT\(^+\); Fig. 5C and D) phenotypes using whole mount immunohistochemistry. We have taken the liberty of labeling some of these clones as single (S), paired (P), and aligned (AI) to stimulate thinking about how rhesus spermatogenic lineage development might compare/contrast with the rodent (Fig. 5).

Figure 5 Clonal organization of undifferentiated and differentiating spermatogonia in adult rhesus seminiferous tubules. Determining the clonal arrangement of undifferentiated and differentiating spermatogonia may be possible using whole mount immunohistochemistry in intact seminiferous tubules. In separate experiments, (A and B) undifferentiated (PLZF\(^+\), green) or (C and D) differentiating (KIT\(^+\), red) spermatogonia were detected in adult rhesus seminiferous tubules. Some clones are identified in each panel as single (S), pairs (P), or aligned (AI). Scale bars = 25 \( \mu \)m. (BP Hermann & KE Orwig, unpublished observations). Note: this is not a co-staining experiment.

There is a growing body of literature suggesting that rodent SSCs exhibit some degree of phenotypic, and perhaps functional, heterogeneity. For instance, several studies have shown that neurogenin 3 is expressed by a subset of spermatogonia with stem cell properties, suggesting subpopulations of SSCs with different functional roles or degrees of differentiation (Nakagawa et al. 2007, Yoshida et al. 2007, Zheng et al. 2009). A recent report suggests that GFRA1 expression is heterogeneous among \( A_{\text{single}} \) spermatogonia in mice and among human \( A_{\text{dark}} \) and \( A_{\text{pale}} \) (Grisanti et al. 2009). Moreover, a recent study suggested that some spermatogonia expressing the differentiation marker KIT retained some degree of stem cell capacity and could repopulate the seminiferous epithelium under certain circumstances (Barroca et al. 2009). Additional studies are needed to validate these suggestions of stem cell heterogeneity and flexibility, expound on their implications for spermatogenesis, and define whether similar phenomena occur in primate testes.

Another emerging concept in the field is that there may be intraclonal heterogeneity among the earliest progeny of SSCs. Striking results have been observed in three separate studies where some undifferentiated spermatogonial clones (\( A_{\text{paired}} \) and \( A_{\text{aligned}} \)) exhibit this type of heterogeneity and some individual cells within a clone appear different from their clonal partners (Grisanti et al. 2009, Luo et al. 2009, Zheng et al. 2009). One interpretation of these data is that individual clones demonstrate asymmetry that could produce new SSCs when larger spermatogonial clones divide. These are infrequent observations (1.7–5%) but reminiscent of the ‘clone splitting’ model of type-A spermatogonial self-renewal and differentiation in primates (reviewed by Ehmcke & Schlatt (2006)). To date, there are no other data supporting a mechanism of asymmetric division among clones of undifferentiated spermatogonia in rodents, but asymmetric division of germline stem cells is well established in flies (reviewed by Fuller & Spradling (2007)).

Conclusions

For several decades, rodent spermatogenesis and primate spermatogenesis have been considered largely dissimilar with important biological differences in the identity and behavior of SSCs (Plant & Marshall 2001). Concepts of rodent and primate spermatogenesis and SSC biology, however, were based largely on different experimental methodologies (Fig. 6A–C). Knowledge of primate testis biology lagged behind rodents, due in part to the dramatic differences in the relative volume of research conducted in these species, which are experimentally and evolutionarily disparate. Research on primate spermatogenesis (nonhuman and human) is poised for accelerated growth with improved access to validated reagents (e.g. antibodies for SSC markers) and
experimental tools (e.g. xenotransplantation and xenografting; Jahnukainen et al. 2006, Hermann et al. 2007, Muller et al. 2008, Hermann et al. 2009, Maki et al. 2009). The molecular characteristics of A dark and A pale spermatogonia are beginning to emerge, allowing their alignment with subpopulations of undifferentiated rodent spermatogonia (i.e. A single, A paired, A aligned; Fig. 6D and E), and identification of putative monkey SSCs (Fig. 3E). A dark spermatogonia exhibit the most undifferentiated phenotype (GFRA1⁺, PLZF⁺, and KIT⁻). Additional studies will be required to determine whether A dark spermatogonia are truly reserve stem cells or whether these cells divide with sufficient frequency to participate in steady-state spermatogenesis of the adult monkey testis. While many A pale also exhibit this undifferentiated phenotype (GFRA1⁺, PLZF⁺, and KIT⁻), some A pale appear phenotypically more similar to committed progenitor spermatogonia in rodents (i.e. A aligned 8–16; GFRA1⁺, PLZF⁺, and KIT⁺). This linear developmental ordering bears some resemblance to the ‘A single’ model of rodent spermatogenesis (Fig. 6D and E). Questions remain about whether A dark and A pale are 1) different stem cell populations, 2) parts of the same cell population, perhaps at different stages of the cell cycle, or 3) stem cells and progenitors respectively (Fig. 6E). In addition, more studies are needed to determine whether clone size correlates with differentiation state in primates, as it does in rodents (Fig. 6A) and whether clone size correlates with ‘dark’ and ‘pale’ descriptions of spermatogonial nuclear morphology (Fig. 6B and C). There is now increasing experimental momentum toward identifying and characterizing primate SSCs and the mechanisms by which they self-renew and differentiate to produce spermatogenesis.
This momentum is fueled by both the biological insights that it provides and the possible implications for treating human male infertility.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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