REPRODUCTION
REVIEW

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*. 1999*b*, 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*. 2001*a*, 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 subpopulation of mouse testis cells defined by the phenotype α6-INTEGRIN^{+}, β1-INTEGRIN^{+}, THY1^{+}, CD9^{+}, Hoechst side population^{+}, Rho123^{low}, αv-INTEGRIN^{-}, 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 (GFRA1), 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 $A_{aligned}$; GFRA1^{+}, PLZF^{+}, NGN3$^{+/-}$, and KIT^{-}) and differentiating spermatogonia (A1–4, intermediate, and B; GFRA1^{-}, 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. Thorough 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_1 and A_2 (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).

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

Species	Stages (#)	Duration (days)		Stages per cross section[a]	References
		One cycle	Spermatogenesis		
Homo sapiens	6	16	64	2–4	Clermont (1963, 1966*a*, 1966*b*), Heller & Clermont (1963) and Amann (2008)
Pan troglodytes (chimpanzee)	6	14	62.5	2–4 (1–5)	Smithwick & Young (1996) and Smithwick *et al.* (1996)
Papio anubis (olive baboon)	12	11	42	1–3	Chowdhury & Marshall (1980) and Chowdhury & Steinberger (1976)
Macaca arctoides (stump-tailed macaque)	12	11.6	46.4	1	Clermont (1972) and Clermont & Antar (1973)
Macaca fascicularis (cynomolgus monkey)	12	10.5	42	1 to several	Dietrich *et al.* (1986) and Fouquet & Dadoune (1986)
Macaca mulata (rhesus macaque)	12	10.5	42	1	Clermont & Leblond (1959), de Rooij *et al.* (1986) and Rosiepen *et al.* (1997)
Callithrix jacchus (common marmoset)	9	10	37	1–5	Holt & Moore (1984) and Millar *et al.* (2000)
Mus musculus	12	8.62	34.5	1	Oakberg (1956, 1971), Clermont & Trott (1969) and Kluin *et al.* (1982)
Rattus norvenicus	14	13	51.6	1	Leblond & Clermont (1952), Clermont & Perey (1957) and Huckins (1971)
Cricetulus griseus (Chinese hamster)	12	17	68	1	Clermont & Trott (1969) and Oud & de Rooij (1977)
Mesocricetus brandti (Turkish hamster)	8	8	32	1	Myoga *et al.* (1991)
Mesocricetus auratus (Golden hamser)	13	8.74	35	1	Leblond & Clermont (1952) and Miething (1998)

[a]Evidence of discrete stages of the seminiferous epithelium in individual cross sections of seminiferous tubules.

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} spermatogonia 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 1966*a*, 1966*b*), reviewed in Amann (2008) and Dym *et al.* (2009)). 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 B1 (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.* 1986, Smithwick & Young 1996). Similar to chimpanzees, morphological examination of human spermatogenesis identified only six stages based on discrete cellular associations (Table 1; Clermont 1963, 1966*a*, 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

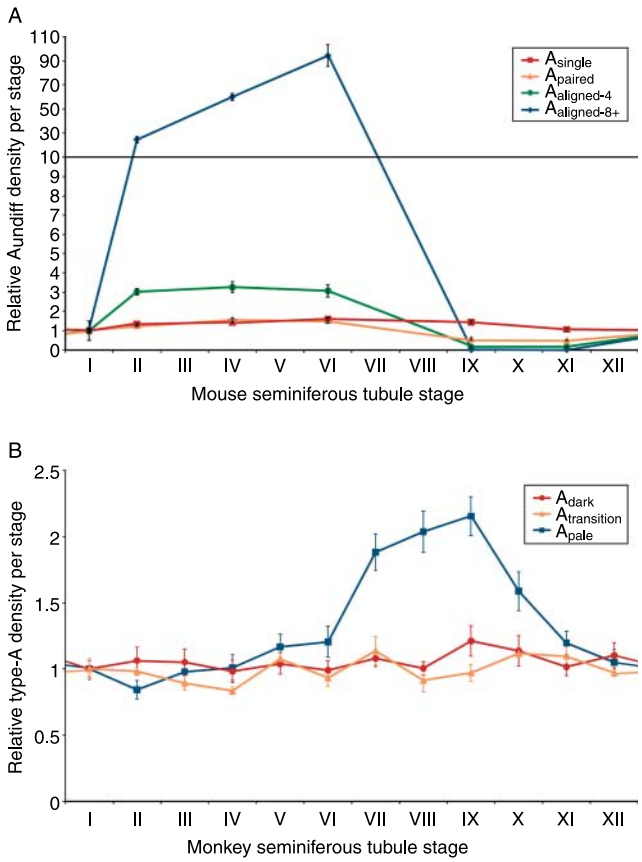

Figure 1 Relative distribution of undifferentiated type-A spermatogonia in mice and monkeys. The relative number of undifferentiated spermatogonia in any given segment of seminiferous tubules at each stage of the seminiferous cycle is shown for (A) mice (Tegelenbosch & de Rooij 1993) and (B) monkeys (Fouquet & Dadoune 1986). For A, data for the relative number of A_{single} (red squares, line), A_{paired} (orange triangles, line), and $A_{aligned}$ clones of 4 and 8+ cells (green circles, blue diamonds) are shown for C3H-101 F1 hybrid mice (Tegelenbosch & de Rooij 1993). The calculated relative density of undifferentiated type-A spermatogonia at each stage was determined by dividing the value for each stage (number of spermatogonia per 1000 Sertoli cells) by the same value for stage I (Tegelenbosch & de Rooij 1993). In B, data for the relative density of A_{dark}, $A_{transition}$, and A_{pale} per stage are as reported for cynomolgus monkeys (Fouquet & Dadoune 1986). The calculated relative density of type-A spermatogonia in each stage (number of spermatogonia per 100 Sertoli cells) was normalized to the value for stage I (Fouquet & Dadoune 1986).

between stages (Fig. 1B, orange line; Fouquet & Dadoune 1986). In contrast, A_{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_{pale} divide between stages VII and IX of the seminiferous epithelium (Clermont 1969, Clermont & Antar 1973, Fouquet & Dadoune 1986, Ehmcke *et al*. 2005*b*, Simorangkir *et al*. 2009), although there is disagreement in the literature about whether A_{pale} divide once (Clermont & Leblond 1959, Clermont 1969, Simorangkir *et al*. 2009) or twice (Clermont & Antar 1973, Ehmcke *et al*. 2005*a*) during a cycle of the seminiferous epithelium. The profile of rhesus A_{pale} numbers across the cycle of the seminiferous epithelium (Fig. 1B, blue line) appears more similar to mouse $A_{aligned}$ 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_{dark} and A_{pale} spermatogonia in nonhuman primate seminiferous. Clermont & Leblond (1959) first reported pairs and 'quartets' of A_{dark} and A_{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_{dark} and A_{pale} spermatogonia in hematoxylin-stained segments of intact seminiferous tubules from adult vervet monkeys (*Cercopithecus aethiops*). This approach enabled the visualization of A_{dark} and A_{pale} spermatogonia and their topological arrangement. Clermont concluded from these studies that A_{dark} and A_{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_{dark} and A_{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 μm 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 ≥16 of both A_{dark} and A_{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_{dark} and A_{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, which may be A_{dark} spermatogonia (Ehmcke *et al*. 2005*b*). Thus, additional studies are needed to clarify the kinetics of type-A spermatogonial expansion in primates, including defining A_{dark} and A_{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_{pale} maintain spermatogenesis in the absence of significant A_{dark} proliferation. Based on morphometric evaluation in the vervet monkey, Clermont (1969) reported that all A_{pale} divide between stages IX and X of the seminiferous epithelium. Half of the daughter population remains as A_{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_{pale} in each cycle of the seminiferous epithelium. A doubling A_{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*. (2005*a*) observed two mitotic A_{pale} events per cycle and proposed a 'clone splitting' model of A_{pale} renewal and differentiation. In this model, larger clones of A_{pale} (e.g. eight cells) produced after the first division at stage VII disintegrate into multiple smaller A_{pale} clones (e.g. two clones of two A_{pale} and one clone of four A_{pale}) that will again divide (at stage IX) to form clones of four or eight B1 spermatogonia or more clones of eight A_{pale} (self-renewal). These models attempt to explain how A_{pale} spermatogonia might balance self-renewing and differentiating divisions to maintain spermatogenesis with little contribution from A_{dark}.

Work from the de Rooij laboratory provided evidence that A_{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_{pale} spermatogonia, with no significant change in the number of A_{dark}. A significant decrease in A_{dark} spermatogonia was observed 14 days after X-irradiation with a corresponding increase in A_{pale} spermatogonia. The authors concluded that the resting A_{dark} were activated into proliferating A_{pale} spermatogonia (van Alphen & de Rooij 1986). Thus, A_{dark} appear to fulfill the role of a 'reserve stem cell'. However, whether A_{dark} spermatogonia also participate in steady-state spermatogenesis remains an open question.

Since Clermont's (1969) initial observation that A_{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}H-thymidine or BrdU label in A_{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*. 2005*b*, Simorangkir *et al*. 2009). Similar to Clermont's observation in the vervet monkey, Simorangkir *et al*. (2009) did not observe labeling in A_{dark}. However, these investigators observed labeling in a new 'unclassified' category of type-A spermatogonia (A_{unc}), which they acknowledged might be classified as A_{dark} by other investigators. It is not clear whether A_{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}H-thymidine (Fouquet & Dadoune 1986). Immunohistochemistry for the proliferating cell nuclear antigen (PCNA) has also determined that rhesus and human A_{dark} spermatogonia failed to label, while only a fraction of A_{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_{dark} spermatogonia, additional studies are needed to confirm whether 1) A_{dark} are quiescent and serve as reserve stem spermatogonia or 2) A_{dark} divide with sufficient (albeit low) frequency to maintain spermatogenesis under normal, steady-state conditions.

The A_{dark}/A_{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_{single} spermatogonia) that divides regularly, but infrequently, and gives rise to the entire spermatogenic lineage. This A_{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_{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 immunohistochemistry.

Table 2 Experimental evidence for A_{dark} and A_{pale} proliferation.

Study	Species[a]	Label	Animals, X-sections[b]	Time to analysis	Percentage of A_{dark} labeled (stage)	Percentage of A_{pale} labeled (stage)	Percentage of A_t/A_{unc} labeled (stage)
Clermont (1969)	*Cercopithecus aethiops*	^{3}H-Thy	1, N/A	2.5 h	0	36.1% (VII–X)	N/A
Clermont & Antar (1973)	*Macaca arctoides*	^{3}H-Thy	3, ≥400	3 h	0.06–0.09 (VII–X)	25.6–41.9 (VII–X)	N/A
			4, ≥400	12 days 3 h	0	5.9–11.8 (VII–X)	N/A
Kluin *et al.* (1983)	*Macaca fascicularis*	^{3}H-Thy[c]	5, 60	1 h[c]	1.5 (9 of ∼600 cells)	14.9 (88 of ∼591 cells)	N/A
Fouquet & Dadoune (1986)	*Macaca fascicularis*	^{3}H-Thy[d]	2, 800	1 h	0	4–34 (VI–IX)	0.5–2.2 A_t (VII–IX)
			2, 800	9–10 days	2.3–10.8 (IV–IV)	8.4–47.6 (IV–IX)	2.9–36.3 A_t (V–VIII)
Schlatt & Weinbauer (1994)	*Macaca mulata*	PCNA	2, 1.5× 10^6 μm^2	N/A	0	X–XI only	N/A
	Homo sapiens	PCNA	2, not indicated	N/A	0	V only	N/A
Ehmcke *et al.* (2005*a*, 2005*b*)	*Macaca mulata*	BrdU	4, N/A[e]	3 h	0.77 or 18.39 (all stages)[f]	27.52 (VII only)	N/A
Simorangkir *et al.* (2009)	*Macaca mulata*	BrdU	4, N/A[g]	3 h	0 (IV–IX)	∼13–24 (VI–XII)	∼2% (VI–XII) A_{unc}
			4, N/A[g]	11 days 3 h	0	Yes (VII–X)[h]	Yes (VI–XI) A_{unc}[h]

[a]*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. [b]Number of seminiferous tubule cross sections is noted per animal evaluated if available. [c]Testicular fragments (1–8 mm^3) were incubated in medium containing ^{3}H-thymidine for 1 h at 32 °C after a 30 min equilibration period. [d]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. [e]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. [f]A_{dark} labeling was classified as weak and strong, representing the two values noted respectively. [g]Numbers of seminiferous tubule cross sections were not reported, but 1053–1299 cells of each type were evaluated per testis. [h]The labeling index of these cells was not reported at 11 days 3 h after label administration.

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 & Zimmermann 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.* 1999*a*, 2000, Ogawa *et al.* 1999, Nagano *et al.* 2001*b*, 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.* 2001*b*), 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.* 2001*b*, 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 & Zimmermann 1994). Donor testis 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.* 2001*b*, 2002, Hermann *et al.* 2007, Maki *et al.* 2009). Donor-derived patches of

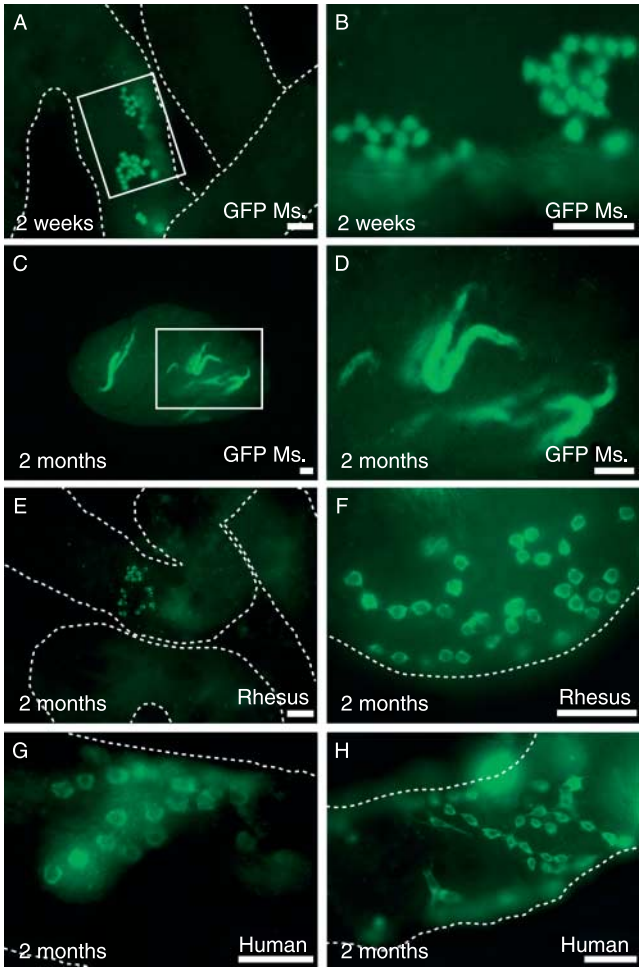

Figure 2 SSC transplantation from different donor species into busulfan-treated mouse testes. (A and B) Donor GFP mouse testis cells (green) at 2 weeks after transplantation. Margins of recipient seminiferous tubules are marked by a dashed white line. (C and D) Donor GFP mouse testis cells (green) at 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.

spermatogonia can be identified 1–2 months after transplantation by staining with a donor species-specific antiserum as described previously (Nagano *et al.* 2001*b*, 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.* 2001*b*, 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 $\geq$ 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.* (2001*b*, 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.* 2001*b*, 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/10^6 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$^-$ rhesus 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 testis 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.

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 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_{dark}, A_{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_{dark}, A_{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_{single} and some A_{paired}; GFRA1$^+$, PLZF$^+$, NGN3$^{+/-}$, and KIT$^-$), transit-amplifying progenitor (some A_{paired} and $A_{aligned}$; GFRA1$^+$, PLZF$^+$, NGN3$^+$, and KIT$^{+/-}$), and differentiating (A1–4, intermediate,

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

					Overlap with	
Marker (aliases)	**References**	**Species**[a]	**Approach**	**Expressed in spermatogonia?**	A_{dark}	A_{pale}
KIT (cKIT)	Hermann *et al.* (2009)	*Macaca mulata*	IHC	Yes	0%	22.8%
	Maki *et al.* (2009)	*Macaca mulata*	FCM	ND	ND	ND
DAZL	Hermann *et al.* (2007)	*Macaca mulata*	IHC	Staining weak/absent in spermatogonia	ND	ND
GFRA1 (GFRα1)	Hermann *et al.* (2007)	*Macaca mulata*	IHC	Rare BM cells[b]	ND	ND
	Hermann *et al.* (2009)	*Macaca mulata*	IHC	Yes	100%	100%
	Maki *et al.* (2009)	*Macaca mulata*	ICC/IHC	Rare BM cells[b]	ND	ND
α6-INTEGRIN (INTGA6)	Maki *et al.* (2009)	*Macaca mulata*	FCM+ICC/XenoTP	Yes[c]	ND	ND
MAGEA4	Mitchell *et al.* (2008)	*Callithrix jacchus*	IHC	Yes in 0–6 weeks testis	ND	ND
NANOS1	Mitchell *et al.* (2008)	*Callithrix jacchus*	IHC	Yes in 0–6 weeks testis	ND	ND
NGN3 (NEUROG3)	Hermann *et al.* (2009)	*Macaca mulata*	IHC	Yes	0%	48.5%
POU5F1 (OCT-4)	Mitchell *et al.* (2008)	*Callithrix jacchus*	IHC	Rare cells in 0–6 weeks testis	ND	ND
PLZF (ZBTB16)	Hermann *et al.* (2007)	*Macaca mulata*	IHC	Rare BM cells[b]	ND	ND
	Hermann *et al.* (2009)	*Macaca mulata*	IHC	Yes	82%	100%
SSEA4	Muller *et al.* (2008)	*Macaca mulata*	IHC	Rare BM cells[b]	ND	ND
	Muller *et al.* (2008)	*Macaca silenus*	IHC	Rare BM cells[b]	ND	ND
	Muller *et al.* (2008)	*Callithrix jacchus*	IHC	Rare BM cells[b]	ND	ND
	Maki *et al.* (2009)	*Macaca mulata*	FCM+ICC/XenoTP	Yes[c]	ND	ND
SALL4	Hermann, Marshall & Orwig unpublished	*Macaca mulata*	IHC	Yes	Yes	Yes
THY1 (CD90)	Maki *et al.* (2009)	*Macaca mulata*	FCM+ICC/XenoTP	Yes[c]	ND	ND
	Hermann *et al.* (2009)	*Macaca mulata*	FCM+ICC/qPCR/XenoTP	Yes[d]	ND	ND
TRA-1-81	Muller *et al.* (2008)	*Macaca mulata*	IHC	Rare BM cells[b]	ND	ND
	Muller *et al.* (2008)	*Macaca silenus*	IHC	Rare BM cells[b]	ND	ND
	Muller *et al.* (2008)	*Callithrix jacchus*	IHC, FCM	Rare BM cells[b]	ND	ND
VASA (DDX4)	Hermann *et al.* (2007)	*Macaca mulata*	IHC	Weak in adult	ND	ND
	Mitchell *et al.* (2008)	*Callithrix jacchus*	IHC	Yes in 0–6 weeks testis	ND	ND
	Hermann *et al.* (2009)	*Macaca mulata*	IHC	Yes in juvenile	100%	100%

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.

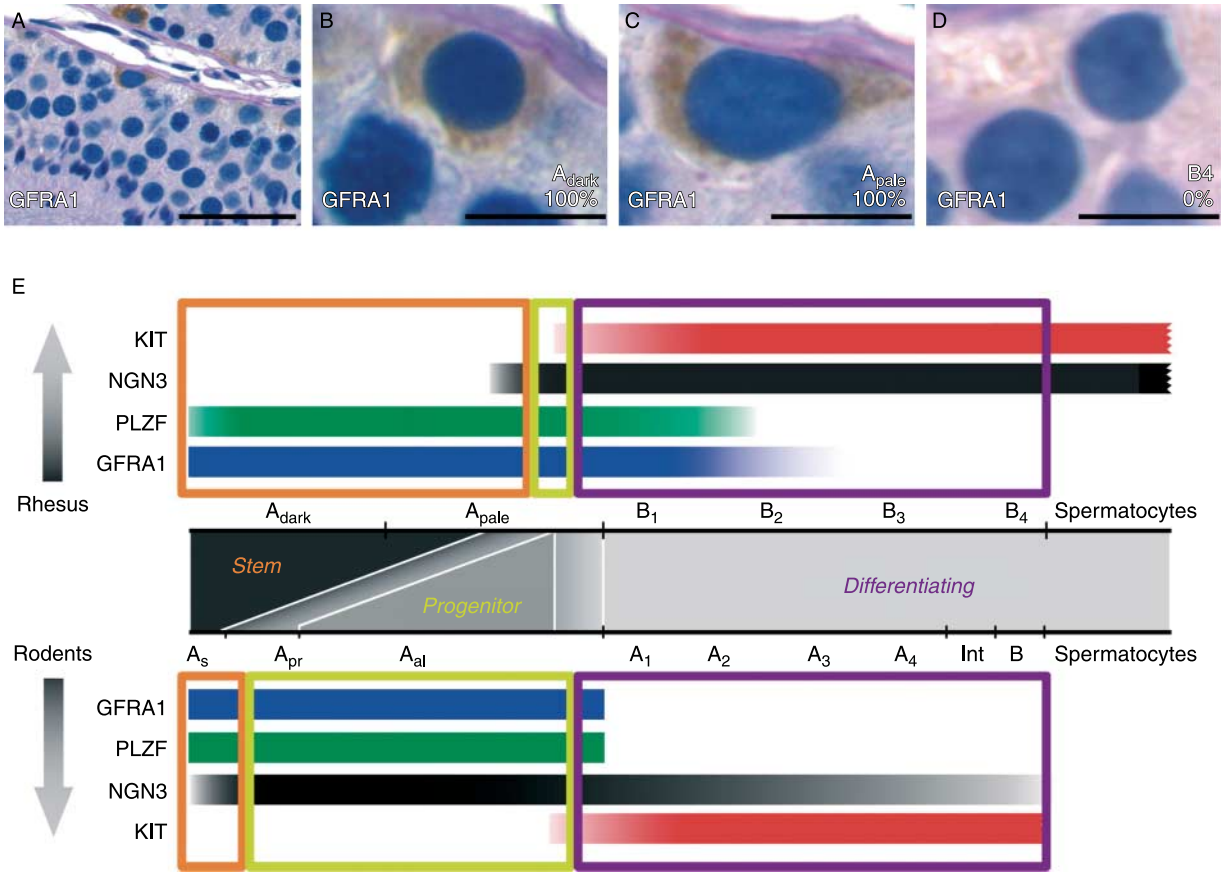

Figure 3 Correlation between molecular markers of rodent SSCs (GFRA1, PLZF, NGN3, and KIT) and morphological descriptions (A_{dark}, A_{pale}, and B) of spermatogonia in the adult rhesus testis. Sections of adult rhesus testes were evaluated by immunohistochemistry for GFRA1, PLZF, NGN3, and KIT (Hermann *et al.* 2009). Subsequently, sections were counterstained by the PAS–hematoxylin method to reveal nuclear morphology and identify A_{dark} and A_{pale} spermatogonia, as well as differentiating type-B spermatogonia. (A and D) Representative staining for GFRA1 is shown. The image in (A) shows part of one seminiferous tubule (scale bar=50 μm). Enlargements are also shown of representative A_{dark} (B), A_{pale} (C), and B4 spermatogonia (D) (scale bar=10 μm). For all spermatogonia that could be definitively classified as A_{dark} or A_{pale}, the percentage that were labeled for GFRA1 is shown. (E) Colored bars (GFRA1, blue; PLZF, green; NGN3, black; KIT, red) indicate the extent of marker expression in the adult spermatogenic lineage based on recently published data (Hermann *et al.* 2009; rhesus, top) or previously published mouse studies (reviewed in Hermann *et al.* (2009); mice, bottom). Colored boxes indicate functional descriptors 'stem' (orange), 'progenitor' (yellow), and 'differentiating' (violet), based on rodent data and may identify rhesus spermatogonia with corresponding phenotype and function. The transitions from stem to progenitor or progenitor to differentiating are noted by gradient shading in the middle gray bar between these functional categories. The following abbreviations are used for rodents: A_s, A_{single}; A_{pr}, A_{paired}; A_{al}, $A_{aligned}$. Adapted from Hermann *et al.* (2009).

and B; GFRA1$^-$, PLZF$^-$, NGN3$^+$, and KIT$^+$). 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_{dark} and ~50% of A_{pale} in the adult rhesus testis exhibit the phenotype GFRA1$^+$, PLZF$^+$, NGN3$^-$, and KIT$^-$ (Fig. 3E). As far as we can ascertain from the literature, this phenotype is restricted to A_{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 $A_{aligned}$ progenitor spermatogonia (Nakagawa *et al.* 2007), rhesus A_{pale} spermatogonia with the phenotype GFRA1$^+$, PLZF$^+$, NGN3$^+$, KIT$^-$ could be either stem or progenitor (shaded as transition cells in Fig. 3E). Lastly, KIT expression begins in longer chain progenitors (i.e. $A_{aligned\ 8–16}$) in rodents and continues in differentiating types A1–4 spermatogonia (Schrans-Stassen *et al.* 1999). Some A_{pale} in the adult rhesus testis exhibit a transition phenotype (GFRA1$^+$, PLZF$^+$, NGN3$^+$, KIT$^+$, like longer chain rodent $A_{aligned}$; 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_{dark} and at least 50% of A_{pale} spermatogonia, and that

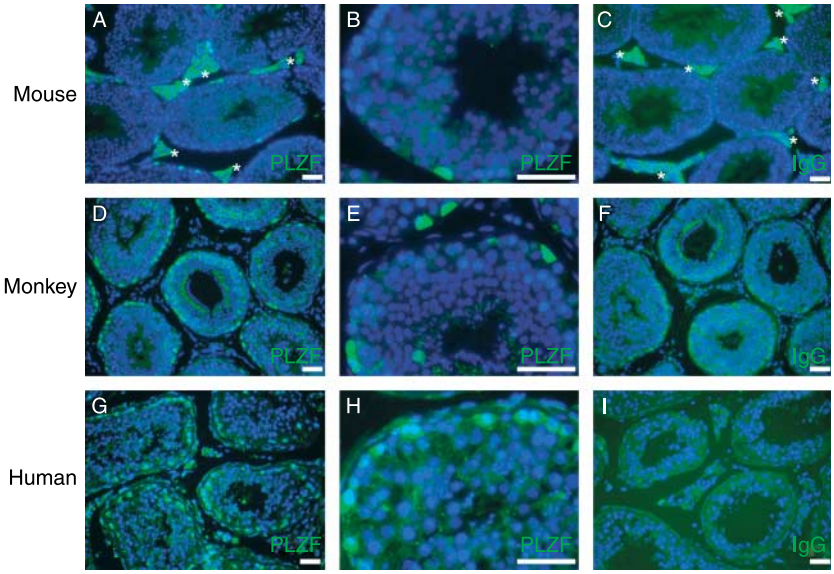

Figure 4 Comparative immunohistochemical analysis reveals species-specific staining profiles for the stem/progenitor marker PLZF. To begin translating knowledge of rodent and monkey SSCs to humans, we have initiated comparative marker analysis using immunohistochemistry for the transcription factor PLZF 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).

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).

Future directions

The considerable degree of phenotypic similarity between A_{dark} and A_{pale} spermatogonia in the adult rhesus testis raises questions about the distinct functional classification of A_{dark} and A_{pale} 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. G_0 versus G_1/S/G_2/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 A_{pale} spermatogonia, suggesting that the initiation of NGN3 coincides with monkey spermatogenic differentiation. Future studies

will begin to dissect this transition in A_{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 (Al) to stimulate thinking about how rhesus spermatogenic lineage development might compare/contrast with the rodent (Fig. 5).

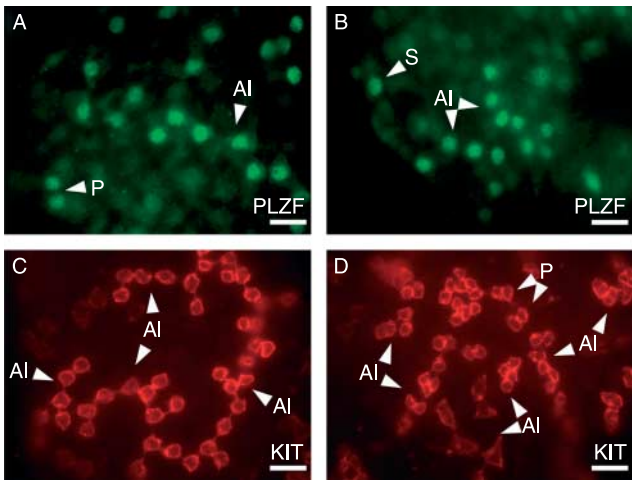

Figure 5 Clonal organization of undifferentiated and differentiating spermatogonia in adult rhesus seminiferous tubules. Determining the clonal arrangement of undifferentiated and differentiating spermatogonia may be possible using whole mount immunohistochemistry in intact seminiferous tubules. In separate 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 (Al). Scale bars = 25 μm. (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_{single} spermatogonia in mice and among human A_{dark} and A_{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_{paired} and $A_{aligned}$) 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

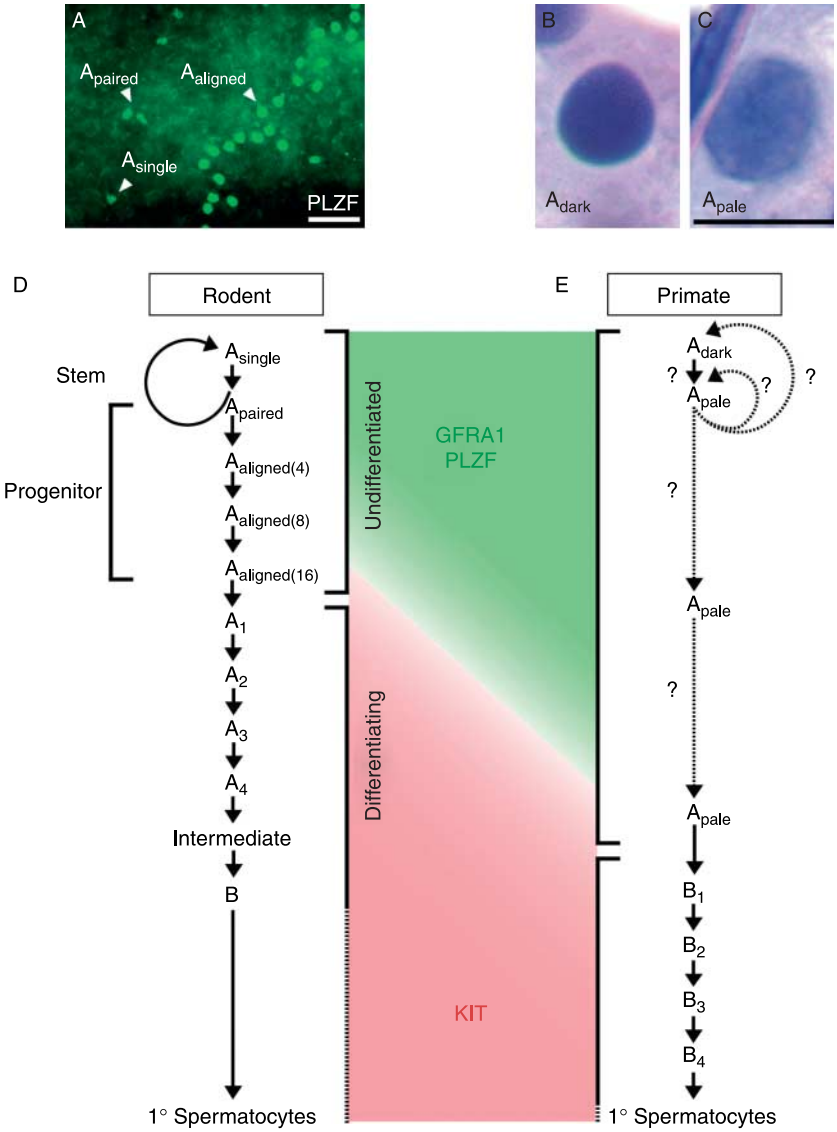

Figure 6 Current conceptual models of rodent and primate spermatogenesis. (A) Immunohistochemical staining for PLZF (green) was performed using whole mount preparations of adult rat seminiferous tubules, and clones of PLZF$^+$ spermatogonia were identified as A_{single}, A_{paired}, or $A_{aligned}$ using the 25 μm criteria (Huckins 1971, de Rooij & Russell 2000). Scale bar = 50 μm. Sections of rhesus macaque testes were stained using the periodic acid-Schiff method and counterstained with Gills hematoxylin (Simorangkir *et al.* 2003) to reveal nuclear morphology and identify (B) A_{dark} and (C) A_{pale} spermatogonia. Scale bars = 10 μm. (D) Rodent undifferentiated spermatogonia are noted (bracket) including stem spermatogonia (SSCs) comprised $A_{singles}$ and some A_{paired} spermatogonia that will complete cytokinesis to produce new $A_{singles}$ and maintain the stem cell pool. Transit-amplifying progenitors include some A_{paired} and $A_{aligned}$ spermatogonia (chains of 4–16 cells). Whole mount and transplantation analyses provided phenotypes for cells in these categories: stem (A_{single} and some A_{paired}; GFRA1$^+$, PLZF$^+$, and KIT$^-$), progenitor (some A_{paired} and $A_{aligned}$; GFRA1$^+$, PLZF$^+$, and KIT$^{+/-}$) and differentiating (A1–4, intermediate, and B; GFRA1$^-$, PLZF$^-$, and KIT$^+$). (E) In primate testes, undifferentiated (type-A) spermatogonia are designated A_{dark} and A_{pale} based on nuclear architecture and staining intensity with hematoxylin. Recent progress has provided information about the molecular phenotype of A_{dark} and A_{pale} spermatogonia that allow alignment with rodent spermatogonia exhibiting a similar phenotype. The number of A_{pale} generations is still not clear and may be resolved by future whole mount marker and clone size analysis (dotted arrows with question marks).

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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