Sertoli cell differentiation in rhesus monkey (*Macaca mulatta*) is an early event in puberty and precedes attainment of the adult complement of undifferentiated spermatogonia

D R Simorangkir^{1,†}, S Ramaswamy¹, G R Marshall³, R Roslund¹ and T M Plant^{1,2}

¹Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213, USA, ²Magee-Womens Research Institute, University of Pittsburgh, 204 Craft Avenue, Room B311, Pittsburgh, Pennsylvania 15213, USA and ³Department of Natural Sciences, Chatham University, Pittsburgh, Pennsylvania 15232, USA

Correspondence should be addressed to T M Plant at Magee-Womens Research Institute, University of Pittsburgh; Email: planttm@mwri.magee.edu

[†]D R Simorangkir is now at St. Matthew's University School of Medicine, Grand Cayman, BWI

Abstract

In primates, the time course of Sertoli cell proliferation and differentiation during puberty and its relationship with the expansion of undifferentiated type A spermatogonia that occurs at this critical stage of development are poorly defined. Mid and late juvenile and early and late pubertal male rhesus monkeys were studied. Testes were immersion fixed, embedded in paraffin, and sectioned at 5 µm. Sertoli cell number per testis, S-phase labeling (BrdU), and growth fraction (Ki67 labeling) were determined and correlated with corresponding parameters for undifferentiated type A spermatogonia (A dark and A pale). Dual fluorescence labeling was used in addition to histochemistry to monitor spermatogonial differentiation during the peripubertal period using GFR α -1 and cKIT as markers. While the adult complement of Sertoli cells/testis was attained in early pubertal monkeys after only a few weeks of exposure to the elevated gonadotropin secretion characteristic of this developmental stage, the number of undifferentiated type A spermatogonia several months later in mid pubertal monkeys was only 50% of that in adult testes. Both A dark and A pale spermatogonia exhibited high S-phase BrdU labeling at all stages of juvenile and pubertal development. Spermatogonial differentiation, as reflected histochemically and by relative changes in GFR α -1 and cKIT expression, was not observed until after the initiation of puberty. In the rhesus monkey and maybe in other higher primates including human, the pubertal proliferation of undifferentiated spermatogonia is insidious and proceeds in the wake of a surge in Sertoli cell proliferation following termination of the juvenile stage of development.

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Introduction

The number of Sertoli cells within the adult testis is considered to be the major determinant of spermatogonial stem cell niches and therefore of spermatogenic ceiling (Sharpe 1994, Griswold 1995, Sharpe *et al.* 2003). While the control of Sertoli cell proliferation and differentiation has been extensively studied in rodents (Sharpe 1994, Griswold 1995, 1998), this aspect of testicular development has only been scantily investigated in higher primates (Nistal *et al.* 1982, Cortes *et al.* 1987, Sharpe *et al.* 2003, Plant *et al.* 2005). In monkey and human, the postnatal pattern of Sertoli cell proliferation may be divided into three major developmental phases (Sharpe *et al.* 2003, Plant *et al.* 2005). The first is seen during infancy when transiently elevated gonadotropin secretion results in a relatively high rate of

Sertoli cell proliferation (Simorangkir *et al.* 2003). The second encompasses the remainder of the prepubertal phase of development, when Sertoli cell proliferation continues, albeit at a reduced rate (Marshall & Plant 1996, Simorangkir *et al.* 2003), as a result of the relative hypogonadotropic state that characterizes this stage of primate development (Plant & Witchel 2006). The third and final stage of Sertoli cell proliferation is again robust and occurs during puberty (Marshall & Plant 1996, Sharpe *et al.* 2003, Plant *et al.* 2005) in association with the re-augmentation of LH and FSH secretion that is triggered at this phase of development (Plant & Witchel 2006). During puberty, Sertoli cell differentiation is completed and the adult complement of this somatic cell type of the testis is established.

The spermatogonial stem cell in primates is considered to reside within the population of

undifferentiated type A spermatogonia that, on the basis of nuclear staining, may be separated into two distinct populations, dark type A (Ad) and pale type A (Ap; Clermont 1972, Plant 2010). As in the case of the Sertoli cell, the adult complement of type A spermatogonia in the primate testis is obtained as a result of a protracted phase of proliferation that spans the infantile, juvenile, and pubertal phase of postnatal development (Plant *et al.* 2005, Simorangkir *et al.* 2005). The proliferation of undifferentiated type A spermatogonia, however, appears less dependent on gonadotropin stimulation than that of Sertoli cells, as reflected by relatively high S-phase labeling during juvenile development when LH and FSH secretion are low (Simorangkir *et al.* 2005).

The purpose of this study was to examine the time course of Sertoli cell proliferation and differentiation during puberty in the rhesus monkey and to determine whether the pubertal expansion of undifferentiated type A spermatogonia occurs concomitantly with the increase in Sertoli cell number or follows in the wake of Sertoli cell proliferation.

Results

Testis weight and gross morphology

Mean combined testicular weights at each of the four stages of development are shown in Table 1, together with values for seminiferous cord/tubule diameter. Testis from mid juvenile and late juvenile monkeys comprised seminiferous cords surrounded by connective tissue and grouped into several lobules (not shown). In two of the early pubertal animals rudimentary lumen were observed on occasion, and by the mid pubertal stage of development, mature lumen was observed in many of the seminiferous tubules (Fig. 1). In late juvenile animals, the seminiferous cords contained numerous Sertoli cells with spherical or ovoid nuclei and Ad and Ap spermatogonia (Fig. 2). Some Sertoli cell nuclei in the early pubertal testes were pleomorphic, and in the mid pubertal group, these somatic cells were fully mature (Fig. 2). As described previously (Simorangkir *et al.* 2005), Ad appeared as small cells with a spherical and darkly stained (hematoxylin) nucleus containing dense and homogeneous chromatin, while Ap spermatogonia

were larger cells with a lightly stained nucleus and coarse granular chromatin (Fig. 2). The morphology of undifferentiated spermatogonia, as assessed by histochemistry, was indistinguishable in testis from juvenile and pubertal animals.

Occasional differentiating type B1 spermatogonia were observed in testes from two of the three late juveniles. Not surprisingly, the seminiferous epithelium at later stages of development comprised more mature germ cells. All early pubertal testes had germ cells as mature as primary spermatocytes, and one animal in this group had a few round spermatids in about 10% of the cross sections evaluated. Mid pubertal testes had germ cells as mature as step 14 spermatids, and ~95% of the cross sections at this stage of development had identifiable stages of the cycle of the seminiferous epithelium.

Sertoli cell number and labeling indices

The mean number of Sertoli cells per testis in the early pubertal group was $1802 \pm 221 \times 10^6$, and this was nearly fourfold greater and significantly different from that of the late juvenile group (Fig. 3). However, there was no further increase in Sertoli cell number between the early pubertal and the mid pubertal stages of development. While BrdU-labeled Sertoli cells were found in testis from late juveniles, S-phase-labeled Sertoli cells were not found in the two pubertal groups (Figs 3 and 4). Ki67 labeling of Sertoli cells was observed in testes of late juvenile monkeys (Fig. 3), minimal in testes from early pubertal animals and absent in the mid pubertal group (Fig. 3). Both BrdU and Ki67 labeling of Sertoli cells was significantly greater in the late juvenile group than in the two more mature groups (Fig. 3). In the mid juvenile group, the mean Ki67-labeling index was $1.2 \pm 0.1\%$.

Interestingly, the mean number of Sertoli cells per tubular cross section in the early pubertal group (25.4 ± 1.7) was significantly greater than that of either the late juvenile (14.1 ± 2.1) or mid pubertal group (18.8 ± 1.9). This was not the case for total A spermatogonia (late juvenile, 0.9 ± 0.4 ; early pubertal, 1.1 ± 0.1 ; and mid pubertal, 1.2 ± 0.3).

Table 1 Age, combined testes weight, seminiferous cord/tubule diameter, and body weight of mid juvenile, late juvenile, early pubertal, and mid pubertal monkeys (mean $\pm$ s.d.).

Group	Age at castration (months)	Combined testis weight (g)	Seminiferous cord/tubule diameter (μ m)	Body weight at castration (kg)
Mid juvenile ($n=3$)	13.8 ± 0.43	0.90^a	NA	2.4 ± 0.15
Late juvenile ($n=3$)	34.2 ± 1.68	1.73 ± 0.93	50.67 ± 6.21	4.2 ± 0.68
Early pubertal ($n=3$)	42.1 ± 4.73	5.07 ± 1.03	75.52 ± 3.14	5.5 ± 0.40
Mid pubertal ($n=3$)	49.0 ± 3.46	17.8 ± 1.32	130.34 ± 8.47	7.4 ± 1.02

NA, not available.

^aBased on the weight of one testis from two animals.

Figure 1 Photomicrographs obtained from PAS–hematoxylin-stained sections from testes of late juvenile (left-hand panels), early pubertal (center panels), and mid pubertal (right-hand panels) monkeys. Top and bottom sections represent the least and most mature testis, respectively, in each of the three groups. Note, in the mid pubertal testis, many tubules are characterized by the presence of lumen and identifiable stages of the seminiferous epithelial cycle. Scale bar, 50 μ m.

Ad and Ap number and labeling indices

The number of Ad and Ap spermatogonia per testis in the late juveniles was $20.5 \pm 24.4 \times 10^6$ and $23.9 \pm 18.1 \times 10^6$, respectively, and by mid puberty, the number of these undifferentiated spermatogonia had increased approximately two- to three-fold (Fig. 5). The increase in the Ap spermatogonia number during this period of pubertal development was statistically significant. The amplification in the number of Ad and Ap spermatogonia during this phase of pubertal development was associated with robust BrdU labeling (3–14%; Fig. 5). Examples of BrdU-labeled Ad spermatogonia in testis from late juvenile, early pubertal, and mid pubertal monkeys are shown in Fig. 4.

cKIT expression in spermatogonia

cKIT-expressing germ cells were rarely observed in testes from mid juvenile and late juvenile animals, accounting on an average for only one in ~ 35 spermatogonia expressing GFR α -1 and/or cKIT (Fig. 6). With the initiation of puberty, however, the relative proportion of cKIT-positive spermatogonia increased dramatically and in the testes of mid pubertal animals, two of every three cells expressing one or both of these markers were cKIT positive (Fig. 6).

Discussion

A major finding of this study on the rhesus monkey was that the pubertal proliferation and terminal differentiation of Sertoli cells is an early, and relatively rapidly completed, event during this critical stage of postnatal development in primates. The testis of the adult rhesus monkey typically contains 1500–2000 million Sertoli cells (Marshall & Plant 1996), and in this study, this number was observed in the early pubertal group. Moreover, in two of the three monkeys in this developmental group, the juvenile testes had been exposed to only 4–6 weeks of elevated gonadotropin secretion, as reflected by the time course of circulating testosterone concentrations. The attainment of an adult complement of Sertoli cells in the early pubertal monkeys was consistent with the mature morphology and absence of S-phase and Ki67 labeling of these somatic cells at this stage of development. The appearance of spermatocytes and rudimentary lumen in testis from early pubertal monkeys is indicative of establishment of the blood–testis barrier (Gondos & Berndtson 1993) and, therefore, further supports an early cessation of Sertoli cell proliferation at puberty in this species. The rapid attainment of an adult number of Sertoli cells during spontaneous pubertal development in the monkey is in line with results reported for this

Figure 2 Photomicrographs obtained from PAS–hematoxylin-stained sections from testes of late juvenile (left-hand panel), early pubertal (center panel), and mid pubertal (right-hand panel) monkeys showing morphology of Sertoli cell nuclei (yellow arrow), Ap spermatogonia (red arrow), and Ad spermatogonia (black arrow) at each of the three developmental stages. Scale bar, 10 μ m.

Figure 3 Mean number ($\pm$ s.d.) of Sertoli cells/testis (black histogram), BrdU Sertoli cell (SC)-labeling index (white histogram), and Ki67 Sertoli cell-labeling index (gray histogram) for late juvenile (LJ), early pubertal (EP), and mid pubertal (MP) monkeys. $n=3$ for each developmental stage. *Significantly different from early and mid pubertal groups.

species when testicular puberty was induced prematurely by stimulation with either exogenous or endogenous gonadotropin, the latter generated by a pulsatile GnRH infusion. Treatment of juvenile monkeys, ~ 18 months of age, with recombinant human gonadotropin for 11 days resulted in a 50% increase in Sertoli cell number (Ramawamy *et al.* 2000), and initiation of precocious puberty in similar aged animals with 10 weeks of pulsatile GnRH treatment produced a fivefold increase in Sertoli cell number (Marshall & Plant 1996). Together, these findings provide support for the view that the proliferation and differentiation of the Sertoli cell during spontaneous puberty is driven by the increase in gonadotropin secretion at this stage of development (Plant & Witchel 2006), as reflected in this study by the time course of circulating testosterone. The relative role and temporal actions of LH and FSH in this regard, however, remains to be fully documented for higher primates.

BrdU labeling of Sertoli cells in the late juvenile animals (0.55%) was somewhat greater than that reported by us for younger juveniles (0.25%; Simorangkir *et al.* 2003), comparable in age to those comprising

the mid juvenile group of this study. This difference may indicate that the onset of puberty had already been initiated in the late juvenile animals, although the endocrine index (weekly circulating testosterone) was insufficiently sensitive to reflect the developmental event. This view is supported by the finding that in two of the late juvenile monkeys the very occasional differentiating B1 spermatogonia was observed, and in one of these two animals, numerous Leydig cells were found (Verhagen & Plant, unpublished observations, 2010). The finding that the foregoing mitotic index for Sertoli cells was similar to the Ki67-labeling index suggests that the cell cycle of the proliferating population of Sertoli cells during juvenile development is of short duration and that only a small proportion of this cell type is likely in the growth fraction at any one time.

In contrast to the dynamics of Sertoli cell division at puberty, the proliferation of undifferentiated spermatogonia, both Ad and Ap, was relatively insidious throughout this phase of development, and as a result, the number of these germ cells reported for the adult monkey (250×10^6 for Ad and for Ap) by our laboratory (Marshall & Plant 1996) had not been reached by the time the mid juvenile group were castrated. The protracted proliferation of undifferentiated spermatogonia through puberty was associated with an S-phase-labeling index, in both Ad and Ap, that was greatest in the early pubertal animals. It is tempting to speculate that the high S-phase labeling in undifferentiated spermatogonia in early pubertal monkeys is a consequence of an increase in spermatogonial niches, resulting from the pubertal surge in Sertoli cell proliferation and reflected by the significant increase in the number of Sertoli cells per cross section at this stage of development. The failure to observe an early pubertal peak in Sertoli cell number per cross section in boys (Paniagua & Nistal 1984) probably reflects the fact that this metric was related to age rather than stages of sexual maturation. The S-phase-labeling index for Ap spermatogonia of the late juvenile group ($\sim 3\%$) was similar to that reported for this type of undifferentiated spermatogonia in mid juvenile animals (Simorangkir *et al.* 2005). In the case of

Figure 4 Photomicrographs of representative testis section from late juvenile (left panel), early pubertal (center panel), and mid pubertal (right panel), in which S-phase-labeled cells have been identified immunohistochemically (brown) and counterstained with PAS-hematoxylin. BrdU-labeled Sertoli cells (SC) were observed in only testis from late juvenile, while Ad and Ap spermatogonia were observed at all three stages of development. Left inset in each panel shows BrdU-labeled Ad (left) and Ap (right) spermatogonia at a higher magnification. Right inset in late juvenile shows BrdU-labeled SC. Scale bar, 50 and 5 μ m (insets).

Figure 5 Mean number ($\pm$ s.d.) of Ad spermatogonia/testis (top panel, black histogram) and Ap spermatogonia/testis (bottom panel, black histogram), together with their BrdU-labeling indices (gray histogram) for the late juvenile (LJ), early pubertal (EP), and mid pubertal (MP) groups. #Significantly different from LJ and MP; *significantly different from LJ. $n=3$ for each group.

Ad spermatogonia, S-phase labeling observed for the late juveniles in this study was noticeably less than that reported earlier for mid juveniles (Simorangkir *et al.* 2005), and we are unable to offer an explanation for this. The full time course of the pubertal increase in undifferentiated spermatogonial number remains to be determined because a late pubertal group was not included in this study.

Considerable controversy surrounds the issue of the mitotic behavior of Ad dark spermatogonia (Ehmcke & Schlatt 2006, Hermann *et al.* 2010, Plant 2010). In the adult, classic studies on the monkey testis conducted in the 1960s led Clermont (1972) to propose that these cells very rarely divide and he therefore tentatively termed them reserve stem cells. This view, in general, has prevailed (de Rooij & Russell 2000, Simorangkir *et al.* 2009, Plant 2010), although recent studies of molecular markers expressed by Ad and Ap in the adult monkey (Hermann *et al.* 2009) testis have led some investigators to begin to question the classic view of Clermont (Hermann *et al.* 2010). In the prepubertal testis, however,

extensive S-phase BrdU labeling of Ad spermatogonia was reported earlier by this laboratory (Simorangkir *et al.* 2005): a finding confirmed in this study. Thus, it seems reasonable to conclude that robust proliferation of Ad spermatogonia in the prepubertal testis accounts for the ~500-fold amplification in the population of Ad spermatogonia in the monkey testis that occurs from birth until attainment of adulthood (Marshall & Plant 1996). The signals in the adult testis that are responsible for taking Ad spermatogonia out of the cell cycle remain to be established.

The discordancy in the time course of Sertoli cell proliferation vs that of undifferentiated spermatogonia likely reflects the relatively greater dependency of Sertoli cell proliferation on gonadotropin secretion (Marshall & Plant 1996, Plant *et al.* 2005). Accordingly, with the reaugmentation of robust LH and FSH release at the initiation of puberty, a surge of Sertoli cell proliferation is stimulated, but proliferation of undifferentiated spermatogonia being relatively gonadotropin independent (Marshall *et al.* 2005, Simorangkir *et al.* 2005) continues at a rate similar to that for the juvenile.

The absence of histochemically identified differentiating B spermatogonia in testes from the mid juvenile group is consistent with our earlier observations (Marshall & Plant 1996, Simorangkir *et al.* 2005) and with the present finding of a very low ratio of cKIT⁺ to GFR α -1⁺ germ cells in testes at this stage of development. Although, the occasional B spermatogonia was observed in two of the three late juvenile animals in this study, the ratio of these molecular markers of spermatogonial differentiation at this stage of development was similar to that in the mid juvenile group. In any event, the present findings illustrate the imprecision that can arise when using circulating testosterone and testicular volume as markers of the initiation of the onset of puberty in the male monkeys. Before leaving the question of spermatogonial differentiation in the prepubertal primate testis, it should be noted that, in contrast to the monkey, significant numbers of differentiating spermatogonia have been described in boys at ages equivalent to our mid to late juvenile monkeys (Paniagua & Nistal 1984). It is likely that this difference reflects the relative gonadotropin drive to the prepubertal testis in juvenile boys and male monkeys.

In summary, the present investigation has demonstrated that, in the rhesus monkey, the pubertal proliferation of undifferentiated spermatogonia is insidious and proceeds in the wake of a surge in Sertoli cell number following termination of the juvenile stage of development. The entire time course of the postnatal increase in the numbers of Sertoli cells and undifferentiated type A spermatogonia in the monkey testis from birth to adulthood is shown in Fig. 7. The data shown in this figure have been generated by this laboratory over the last 15–20 years (Marshall & Plant 1996, Simorangkir *et al.* 2003, 2005; and this study).

Figure 6 cKIT expression increases markedly in the testes of early and mid pubertal monkeys. Left panel: confocal immunofluorescence projections showing GFR α -1 (left-hand panels, green fluorescence, Alexa 488) or cKIT (middle panels, red fluorescence, Cy3) expressing germ cells and their merged images (right-hand panels) in testes of a mid juvenile (MJ), late juvenile, (LJ) early pubertal (EP), and mid pubertal (MP) monkeys. Arrows indicate dual labeled spermatogonia. Scale bar, 20 μ m. Right panel: the ratio of cKIT $^{+}$ to GFR α -1 $^{+}$ spermatogonia (mean $\pm$ s.d.) in testes from MJ, LJ, EP, and MP monkeys. *Significantly different from all other groups.

Materials and Methods

Animals

Nine male rhesus monkeys (*Macaca mulatta*), born either in the USA (Indian rhesus) or in China and purchased from Three Springs Scientific (Perkasie, PA, USA), were employed. At the beginning of the experiment, the animals ranged from 28 to 32 months of age. The monkeys were maintained under a controlled photoperiod (lights-on 0700–1900 h) at an ambient temperature of 21 $^{\circ}$ C. The experiments were conducted in accordance with NIH Guidelines for the Care and Use of Experimental Animals and were approved by the University of Pittsburgh Institutional Animal Committee on Use and Care.

Experimental protocol

The nine juveniles were randomly assigned to three groups, namely, late juvenile, early pubertal, and mid pubertal. Testicular development in these nine animals was tracked by monitoring testicular volume and location (inguinal vs scrotal) and morning ($\sim$ 0830 h) and nighttime (Fig. 8; $\sim$ 1900 h) circulating testosterone concentrations on a weekly basis. The initiation of the onset of puberty in the male rhesus monkey may be recognized by an increase in nighttime circulating testosterone concentrations, which, in our laboratory, typically occur at $\sim$ 36 months of age (Plant 1985). Animals were classified as early pubertal when combined testicular volume reached 5 ml and maintenance of nighttime testosterone concentration in excess of 1 ng/ml and for several weeks (Fig. 1, Fig. 8). Mid pubertal animals were classified as such by combined testicular volumes of at least 15 ml and maintenance of nighttime testosterone concentrations in excess of 1 ng/ml for >10 weeks (Fig. 1, Fig. 8). The late juvenile animals were 28–30 months of age with testicular volumes <5 ml and showing no evidence of increased circulating testosterone concentrations (Fig. 1, Fig. 8).

Late juvenile monkeys were castrated between 33 and 36 months of age, while early pubertal and mid pubertal animals

were castrated between 38 and 47, 45 and 51 months of age respectively. An i.v. bolus of BrdU (33 mg/kg body weight as a 2% solution in PBS; Sigma Chemical Co.) was injected 3 h before the removal of the testes.

In addition, testes from three additional juvenile monkeys (13–15 months of age) that were castrated for an unrelated study (Ramaswamy *et al.* 2010) were also employed. Testes from these animals, which were also labeled with BrdU, were fixed in Bouin's or 4% paraformaldehyde and comprised a mid juvenile group.

The total number of Sertoli cells and Ad and Ap spermatogonia per testis were enumerated for the late juvenile, early pubertal, and mid pubertal groups. S-phase

Figure 7 The temporal relationship throughout postnatal development between the attainment of adult complements of Sertoli cell number (closed histograms) and the undifferentiated type A spermatogonia number (gray histograms) in the testis of the rhesus monkey. Equal numbers of Ad and Ap per testis are typically observed throughout postnatal development. N, neonate (1–2 days old); I, infant (4–5 months old); MJ, mid juvenile (38–47 months old); LJ, late juvenile (33–36 months old); EP, early pubertal (45–51 months old); MP, mid pubertal (45–51 months old) and AD, adult (>6 years of age). Data for N, I, MJ, and AD from Marshall & Plant (1996) and Simorangkir *et al.* (2005, 2009).

Figure 8 Combined testicular volume (TV, top panels) and nighttime plasma concentrations of testosterone (T, bottom panels) for each of the late juvenile (left-hand panels), early pubertal (center panels), and mid pubertal (right-hand panels) monkeys between 28 and 51 months of age. $n=3$ for each developmental stage.

(BrdU)-labeling indices were calculated for Sertoli cells and for Ad and Ap spermatogonia at these three developmental stages.

At all four stages of development, Sertoli cell growth fraction was determined using Ki67 as a marker of proliferating cells (Whitfield *et al.* 2006). For this purpose, GATA4 was used as a marker of Sertoli cells (LaVoie 2003). Spermatogonial differentiation was assessed using GFR α -1 and cKIT as markers of undifferentiated and differentiating spermatogonia (Aponte *et al.* 2005, Hermann *et al.* 2009) respectively.

Blood sampling

Single daily blood samples (~ 1 ml) were drawn into heparinized syringes once per week at 0830 and 1900 h, after monkeys had been sedated with ketamine hydrochloride (10–20 mg/kg body weight i.m., Ketaject; Phoenix Scientific, Inc., St. Joseph, MO, USA). Plasma was separated and stored at -20°C until required for assays.

Hormonal measurements

Circulating testosterone concentrations were measured using a RIA (Coat-A-Count, TKTT5; Diagnostic Products Corp., Los Angeles, CA, USA) as described previously (Ramaswamy 2005). The sensitivity of this assay was 0.4 ng/ml, and the intra- and interassay coefficients of variation were <7.1 and $<10.2\%$ respectively.

Testicular volume measurement

Testis width and length were measured by one of the authors (S Ramaswamy) with a caliper while monkeys were sedated for weekly blood sample collection. The location of testes was noted at this time. Combined testis volume was calculated as described previously (Ramaswamy *et al.* 2000).

Castration and processing of testicular tissue

The testes were removed via a midline incision, after sedation with ketamine hydrochloride (~ 20 mg/kg body weight, i.m.) and anesthesia with 1.5–2.5% isoflurane in oxygen. Following

castration, testes were weighed and cut into several fragments for immersion fixation overnight in Bouin's solution or 10% neutral buffered formalin or 4% paraformaldehyde and processed for paraffin embedding. Postoperatively, each monkey received antibiotic and analgesic treatments as described previously (Ramaswamy *et al.* 2010).

Morphometric analysis

Bouin's fixed testicular tissue was sectioned at $5\ \mu\text{m}$ and stained either with hematoxylin only or with periodic acid-Schiff (PAS)–hematoxylin (Sigma Chemical Co.). Because of the need to relate the data from this study to those observed earlier for neonate, infant, mid juvenile, and adult stages of postnatal development, the same classical method of cell enumeration that had been employed previously was again selected, rather than switching to the more contemporary optical dissector. In the late juvenile group, Sertoli cell numbers were enumerated by counting the number of Sertoli cell nuclei in seminiferous cords with circular profiles, a method similar to that used by this laboratory to estimate Sertoli cell number in the neonate, infant, and mid juvenile testes (Simorangkir *et al.* 2003). The nuclei of Sertoli cells in the monkey testis before the onset of puberty are spherical/ovoid, a requirement for quantification (Abercrombie 1946). In the early and mid pubertal groups, Sertoli cell number was estimated by counting their nucleoli, instead of their nuclei, also as described (Simorangkir *et al.* 2009). Differentiating Sertoli cells possess an irregular shaped nucleus but contain only a single spherical and prominent nucleolus. The volume fraction, absolute volume, diameter, and length of the seminiferous cords/tubules were measured and calculated as described (Marshall & Plant 1996). The total number of Sertoli cells per testis was then determined by multiplying cell number per cross section (after a correction using Abercrombie's formula; Abercrombie 1946) with the total length of cord or tubule divided by the section thickness ($5\ \mu\text{m}$). A total 60–202 circular profiles per animal in each developmental group were examined. Circular profiles were identified across the entire section by moving the microscope stage systematically in order to avoid counting the same profile more than once. This principle was applied for both cell number enumeration and estimation of labeling indices (below).

Enumeration of Ad and Ap spermatogonia was performed according to Marshall & Plant (1996). Ad and Ap spermatogonial nuclei were counted from 200 to 229 circular profiles of seminiferous cord cross section per animal. These numbers were also corrected using Abercrombie's formula (Abercrombie 1946). Total number of Ad dark and Ap pale spermatogonia per testis was obtained as described earlier for Sertoli cell number.

Sertoli cell and spermatogonial number were not determined in the testes from mid juvenile monkeys.

Immunohistochemistry

BrdU labeling

BrdU incorporated into DNA was immunohistochemically identified using the method described by our laboratory (Simorangkir *et al.* 2009). The anti-BrdU antibody (mouse IgG MAB; Roche Diagnostic Corp.) was used at a dilution of 1:33 in 50 mM PBS containing 0.05% Triton and 1% normal horse serum. After several rinses, sections were incubated at room temperature with biotinylated horse antimouse antiserum (Vector Laboratories, Inc., Burlingame CA, USA, diluted 1:200 in PBS) for 1 h. After rinsing, sections were treated with avidin–HRP complex (Vectastain ABC Elite Kit, Vector Laboratories, Inc.) for 1 h at room temperature. HRP was then visualized with 3,3'-diaminobenzidine (SigmaFast DAB/Cobalt; Sigma Chemical Co.). A dark brown precipitate indicated the presence of BrdU in the cells. Sections were then counterstained using PAS–hematoxylin.

Ki67 and GATA4 dual fluorescent labeling

Formalin- or 4% paraformaldehyde-fixed testicular sections (5 μ m thick) were deparaffinized in xylene and rehydrated through a series of decreasing concentrations of ethyl alcohol and washed in PBS (0.1 M, pH 7.2). The sections were then subjected to antigen retrieval for 1 h at 97.5 °C in sodium citrate buffer (tri-sodium citrate solution, pH 6.0, with 0.05% Tween 20), allowed to cool at room temperature for 30 min, and washed in PBST buffer (PBS with 0.1% Triton X-100 and 1% Tween-20, pH 7.2). This was followed by incubations in blocking buffer (PBS with 5% normal donkey serum, 3% BSA and 1% Triton X-100 (PBST)) for 30 min and then in a cocktail of primary antibodies composed of goat antimouse GATA4

antibody (sc-1237, 1:400; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse monoclonal antihuman Ki67 antibody (Clone MIB-1, 1:200; Dako North America, Inc., Carpinteria, CA, USA) for 90 min at room temperature in a humidified box. Control sections without the primary antibody were incubated with blocking buffer only. Sections were then rinsed in PBST, incubated in a mixture of secondary fluorescent antibodies, namely Cy3-conjugated AffiniPure donkey antigoat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), and Alexa Fluor 488 donkey antimouse IgG (Invitrogen Corporation), both at 1:200 dilution in blocking buffer, for 45 min at room temperature in the dark. Finally, sections were washed in PBST, briefly cleared in xylene, and coverslipped with Fluoromount-G mounting medium (Southern Biotech, Birmingham, AL, USA).

Confocal imaging of dual fluorescence (GATA4 and Ki67)-labeled Sertoli cells was performed as described previously (Ramaswamy *et al.* 2008), using an Olympus FV1000 confocal microscope equipped with a four-laser system with transmitted light, differential interference contrast (DIC), and complete integrated image analysis software system (Olympus America, Inc., Melville, NY, USA). Optical images along the z-axis were collected at 1 μ m intervals. Composite digital images were then converted to TIFF format, imported into Adobe Photoshop (Adobe Photoshop CS3 extended, version 10.0.1; Adobe Systems, Inc.), and color balance was adjusted for presentation (Fig. 9). Selective omission of primary antibodies led to loss of the respective immunohistochemical signal (Supplementary Figure 1, see section on supplementary data given at the end of this article).

GFR α -1 and cKIT dual fluorescence labeling

Formalin- or 4% paraformaldehyde-fixed testicular sections (5 μ m thick) were processed for GFR α -1 and cKIT immunofluorescence labeling essentially as described earlier. The cocktail of primary antibodies was composed of a mouse MAB against recombinant human GFR α -1 (1:200; R&D Systems, Inc., Minneapolis, MN, USA) and a rabbit polyclonal antihuman cKIT antibody (1:400; Dako North America, Inc., Carpinteria, CA, USA). Alexa Fluor 488 donkey antimouse IgG (Invitrogen Corporation) and Cy3-conjugated AffiniPure donkey antirabbit IgG (Jackson ImmunoResearch Laboratories, Inc.), both at 1:200 dilution, were used as the secondary antibodies. Confocal imaging of dual fluorescence of GFR α -1

Figure 9 Confocal immunofluorescence projections (1 μ m optical sections) showing co-localization of Ki67 (green fluorescence, Alexa 488) and GATA4 (red fluorescence, Cy3) in Sertoli cells from a mid juvenile testis. From left to right, immunopositive Ki67 (left-hand panel), GATA4 (center panel), and their merged image (right-hand panel). White arrows, Ki67-positive Sertoli cells. Broken circles in center panel; site of Ki67-positive spermatogonia. Scale bar, 10 μ m.

and cKIT was performed as described earlier. Selective omission of primary antibodies led to loss of the respective immunohistochemical signal (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Labeling indices

S-phase

This labeling index was determined for individual animals by dividing the number of BrdU-labeled cells by the respective total number of cells determined in circular profiles as described previously (Simorangkir *et al.* 2003, 2009). As many as 7000 Sertoli cells and 1200 undifferentiated A spermatogonia were evaluated for each monkey.

Sertoli cell growth fraction

The Sertoli cell growth fraction was estimated for individual animals by dividing the number of Sertoli cells in the cell cycle (Ki67 and GATA4 positive) by the total number of Sertoli cells (GATA4 positive) determined in circular profiles. Approximately, 3500 Sertoli cells per animal were examined.

cKIT-positive spermatogonia

Approximately, 1000 spermatogonia positive for GFR α -1 and/or cKIT and located on the basement membrane were counted in circular cord/tubule profiles for each animal in each of the four groups and the ratio of cKIT/GFR α -1 was then calculated.

Statistical analysis

All numerical data are expressed as mean $\pm$ s.d. For all parameters, the significance of differences between the means across peripubertal development was compared using one-way ANOVA followed by the Newman–Keuls multiple comparison test (Prism 5; GraphPad Software, Inc., La Jolla, CA, USA). As percentages are binominally distributed, the labeling indices were rendered to a normal distribution using arcsine transformation (Zar 1974). Significance was assigned at $P \leq 0.05$.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0411>.

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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