

# Identification of the starting point for spermatogenesis and characterization of the testicular stem cell in adult male rhesus monkeys

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**BACKGROUND:** Spermatogonial expansion in man and non-human primates has been studied for decades. Controversy persists about the cell type representing the testicular stem cell and the exact kinetics of spermatogonial proliferation. We recently determined the starting point of spermatogenesis and proposed a model for clonal expansion of spermatogonia in adult macaques. Here we want to confirm the initiation event, study and compare the details of the kinetics of spermatogonial expansion *in vivo* and *in vitro*, and characterize a population of A spermatogonia acting as testicular stem cells. **METHODS and RESULTS:** We localized BrdU-positive spermatogonia in whole mounts and sections of adult rhesus monkey testes. Culture of testicular tissue was used to determine the expansion and differentiation of premeiotic germ cells. We confirm that A<sub>pale</sub> spermatogonia divide equally at stage VII and produce two types of progeny after mitosis at stage IX of the seminiferous cycle following defined clonal patterns. Small numbers of proliferating single A spermatogonia exist which present a population of label-retaining cells. **CONCLUSIONS:** In the rhesus monkey the population of A<sub>pale</sub> spermatogonia cycle continuously and initiate spermatogenesis by a self-renewing division at stage VII of the seminiferous epithelial cycle. Rarely dividing single A spermatogonia exist which potentially are the male germline stem cells in the primate testis.

*Key words:* *Macaca mulatta*/non-human primates/proliferation/rhesus monkey/spermatogonial stem cells

## Introduction

In the human and non-human primate testis two types of spermatogonia can be distinguished in the light microscope. From 1950 to 1973 Clermont and coworkers presented extensive studies on the stages of germ cell associations in different species of old-world monkeys (*Cercopithecidae*) and man, classified the two spermatogonial subtypes as reserve stem cells (A<sub>dark</sub>) and proliferating stem cells (A<sub>pale</sub>), and postulated a series of models on spermatogonial expansion (Clermont and Leblond, 1959; Heller and Clermont, 1963, 1964; Clermont, 1966a,b, 1969, 1972; Clermont and Antar, 1973. See also Roosen-Runge and Barlow, 1953; Vilar *et al.*, 1970; Schulze and Rehder, 1984; Fouquet and Dadoune, 1986; Schulze *et al.*, 1986; Zhengwei *et al.*, 1997). Despite these efforts, no unequivocal definition of the germline stem cell in the human and non-human primate testis has been made. In addition, two models for spermatogonial expansion were proposed. However, which model would be true remained open until we recently developed a new approach to study spermatogonial expansion by confocal microscopy (Ehmcke *et al.*, 2005). The three dimensional tracing of BrdU-labeled cells allowed us to demonstrate the stage-

specific pattern of spermatogonial expansion in the rhesus monkey testis. We concluded that spermatogenesis in the rhesus monkey is initiated by a first division of pairs or quadruplets of A<sub>pale</sub> spermatogonia at stage VII of the spermatogenic cycle of the seminiferous epithelium. This first division is followed by a second division of A<sub>pale</sub> spermatogonia at stage IX. A well defined pattern of clonal splitting and clonal expansion leads to generation of 12 B<sub>1</sub> spermatogonia and four A<sub>pale</sub> spermatogonia from an initial clone of four A<sub>pale</sub> spermatogonia. This pattern of expansion is best supported by the second model proposed previously for *Macaca arctoides* (Clermont and Antar, 1973).

In the present study we extended our novel bio-imaging approach with additional scientific tools including organ culture and detection of label-retaining cells to functionally define several features of spermatogonial stem cells and their differentiating progeny in the primate testis. The aims of the present study are (i) to unequivocally determine that the starting point of spermatogenesis in the monkey testis is a division of A<sub>pale</sub> spermatogonia at stage VII of the seminiferous epithelial cycle, (ii) to substantiate the proposed model for the kinetics of spermatogonial expansion (Ehmcke *et al.*,

**Table I.** Animal use

Monkey number (Plum Boro Primate Facility)	Age, years and months	Body weight (kg) at time of orchidectomy; 1. testis/2. testis	Testis weight (g) at time of orchidectomy; 1. testis/2. testis	Time between BrdU injection and orchidectomy; 1. testis/2. testis	Animal use
2509 <sup>a</sup>	12y 0m	13.5/13.0	28.9/29.1	3h/11d	Sections + labeling indices
2518 <sup>a</sup>	6y 2m	9.8/9.4	20.9/23.3	3h/11d	Sections + labeling indices
2556 <sup>a</sup>	6y 1m	9.5/8.9	23.7/26.0	3h/11d	Sections + labeling indices
2699 <sup>a</sup>	9y	9.9/9.5	18.0/16.9	3h/11d	Sections + labeling indices
3048 <sup>b</sup>	7y	12.4/11.9	61.5/66.7	40d	Detection of label-retaining cells + controls
3060 <sup>b</sup>	11y	11.5/9.9	43.2/48.8	No BrdU	<i>In vitro</i> BrdU pulse-chase

<sup>a</sup>Tissue provided by Drs T.M.Plant and G.R.Marshall, University of Pittsburgh School of Medicine.

<sup>b</sup>Tissue provided by Dr D.R.Simorangkir, University of Pittsburgh School of Medicine.

2005) and (iii) to determine whether A<sub>dark</sub> spermatogonia represent a population of true stem cells with a very low rate of proliferative activity. Our studies are highly relevant to extend our very limited knowledge of human spermatogonial stem cells since the human testis contains the same two subtypes of spermatogonia. It is quite likely that human spermatogonial expansion follows similar patterns as observed in non-human primate testes.

## Materials and methods

### Animals

Six adult (age 6.1 to 12 years, body weight 8.9 to 13.5 kg, testicular weight 18.03 to 61.5 g) male rhesus monkeys (*Macaca mulatta*) were included in this study (Table I). These monkeys had either been involved in a previously published study (Simorangkir *et al.*, 2004) or had taken part in an as yet unpublished experiment designed and conducted by Drs T.M.Plant and G.R.Marshall (University of Pittsburgh School of Medicine) to examine the kinetics of the cycle of the seminiferous epithelium in the rhesus monkey. The animals were maintained in the University of Pittsburgh Plum Boro Primate Research Facility in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. Two of the monkeys had taken part in endocrinological studies in the past, but had been allowed to recover for a period of more than 6 months prior to the present study. The two monkeys whose testicular tissue was used for the *in vitro*-experiment served as control animals in an ongoing study examining the effect of FSH and LH on spermatogenesis. For this purpose, endocrine gonadotropin release was blocked by treatment with a GnRH antagonist (acyline; for details on establishing a chronic hypogonadotropic state in rhesus monkeys using acyline see Ramaswamy *et al.*, 2003) and the restoration of physiologically normal FSH and LH blood levels was attempted using exogenous recombinant human FSH and recombinant human LH (Simorangkir *et al.*, 2004). In both monkeys, blood testosterone levels and testicular volumes were monitored weekly. The blood testosterone levels, the testicular volumes, the diameters of the seminiferous tubules as well as thickness and histological appearance of the seminiferous epithelium indicate that the given LH dose was appropriate to establish normal levels of androgens and that the dose of FSH was rather stimulatory than suppressive. As a consequence the testis weights are in the upper normal range for rhesus monkeys. Histological analysis of the testes revealed normal spermatogenesis. All experimental procedures described in this study were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

### Surgical procedures

Four rhesus monkeys received an intravenous (iv) bolus injection of bromodeoxyuridine (BrdU; 33 mg/kg body weight; Sigma, St Louis, MO) 3 h prior to castration or hemi-castration. One rhesus monkey received an iv bolus injection of BrdU 40 days prior to the removal of the testes. For surgery, the monkeys were first sedated with ketamine hydrochloride (50–100 mg im, Ketaject, Phoenix Scientific Inc., St Joseph, MO) and then anesthetized with isoflurane in oxygen (1–2.5%; Abbott Laboratories, North Chicago, IL). All surgical procedures were performed under aseptic conditions. Postsurgically, all animals received one daily intramuscular injection of penicillin (300 000 U, Bicillin L-A, Wyeth Laboratories, Philadelphia, PA) and an analgesic (1 mg/kg body weight, Meperidine hydrochloride, Demerol, Abbott Laboratories, North Chicago, IL) for 4 days. The second testis of the hemi-castrated animals was removed 11 days after the removal of the first testis. The procedure for the second surgery was similar to the first, but the animals did not receive an iv bolus injection of BrdU prior to this second surgery.

### Tissue preparation

For preparation of tissue sections testicular tissue samples were fixed overnight at room temperature (RT) in fresh Bouin's fixative, washed with 70% ethanol, dehydrated and embedded in paraffin. Five-micrometer serial sections were cut.

For preparation of tissue culture, testicular tissue from one adult rhesus monkey (which had not received an iv bolus injection of BrdU) was teased in sterile PBS to obtain fragments of seminiferous tubules between several millimeters and 2 cm in length. The fragments were incubated for 2 h at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM; 4.5 g/l glucose; Mediatech, Herndon, VA) supplemented with nonessential amino acids (NAA; Cambrex Bio Science, Walkerville, MD; dilution following manufacturer's instructions), glutamine (365 mg/l; Sigma, St Louis, MO), antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml; Mediatech, Herndon, VA) and BrdU (100 µM; Sigma, St Louis, MO) to allow cells in S-phase to incorporate BrdU. Some of these fragments were then fixed immediately in fresh Bouin's fixative and were transferred into 70% ethanol for storage until further processing. Other fragments were subjected to organ culture. Seminiferous tubules were placed on top of 25 mm tissue culture inserts (polycarbonate membrane, 8 µm pores; Nalge Nunc, Naperville, IL) floating upside down in 35 mm cell culture wells (6-well plates; Greiner Bio-One, Frickenhausen, Germany) filled with DMEM (1.0 g/l glucose; Mediatech, Herndon, VA) supplemented with nonessential amino acids (NAA; Cambrex Bio Science, Walkerville, MD; dilution following manufacturer's instructions), glutamine and antibiotics. The tissue, thus placed at the interphase between cell culture medium and surrounding atmosphere, was cultured at 35 °C in 5% CO<sub>2</sub> in air and satu-

rated humidity for 48 and 72 h. Finally, the tissue fragments were fixed overnight at 4°C in fresh Bouin's fixative and stored in 70% ethanol.

Testicular tissue retrieved from the macaque which had received a bolus injection of BrdU 40 days before castration was teased in cold PBS, fixed overnight at 4°C in fresh Bouin's fixative and stored in 70% ethanol.

#### Immunohistochemical staining procedure

The staining procedures of sections and whole mounts were quite similar and have been described previously (Ehmcke *et al.*, 2005). In brief, tissue sections were deparaffinized, rehydrated, incubated in 1M hydrochloric acid for 10 min at RT, washed in distilled water, incubated for 5 min at RT with Trypsin solution [0.1% in Tris-buffered saline (TBS); Sigma, St Louis, MO], washed with distilled water followed by TBS, incubated for 30 min at RT with blocking solution [5% goat serum and 0.1% bovine serum albumine (BSA) in TBS; goat serum and BSA from Sigma, St Louis, MO] and incubated overnight (ON) at 4°C with the primary antibody (monoclonal anti-BrdU, clone BU-33; either from Sigma, St Louis, MO or from Biomedica, Foster City, CA; diluted 1:50 in TBS containing 0.1% BSA). Then the sections were washed with TBS, incubated for 1 h at RT with the secondary antibody (goat anti-mouse, biotinylated; Sigma, St Louis, MO; diluted 1:100 in TBS containing 0.1% BSA), washed with TBS, incubated for 1 h at RT with a mix of a second primary antibody and a streptavidin-conjugated fluorescent dye (monoclonal anti-Acrosin, clone Acr-C5F10; Biosonda, Miami, FL, and fluorescent dye AlexaFluor 488, streptavidin-conjugated; Molecular Probes, Eugene, OR; both diluted 1:100 in TBS containing 0.1% BSA), washed in TBS, incubated for 1 h at RT with a secondary antibody (goat anti-mouse, fluorescent dye AlexaFluor 546-conjugated; Molecular Probes, Eugene, OR; diluted 1:100 in TBS containing 0.1% BSA), washed with TBS and mounted using VectaShield Mounting Medium (Vector, Burlingame, CA) containing 4,6-diamidino-2-phenylindole (DAPI; 1.5 µg/ml). When whole mounts were stained the incubation times for hydrochloric acid and trypsin were prolonged (15 min at RT for both). Whole mounts were mounted on microscope slides using VectaShield Mounting medium without DAPI (Vector, Burlingame, CA).

Intense haematoxylin staining of the nuclei is necessary to enable unequivocal morphological identification of the two spermatogonial subtypes. To achieve this goal, tissue sections were deparaffinized, rehydrated, incubated in 1M hydrochloric acid for 15 min at RT, washed in distilled water, stained with hematoxylin solution (Sigma, St Louis, MO), incubated in tap water followed by distilled water,

incubated with Trypsin solution (10 min; 0.1 % in TBS; see above) and washed with distilled water followed by TBS. The next steps of the immunohistochemical staining was performed following the haematoxylin staining as described above, starting with the incubation in blocking solution.

#### Tissue analysis

Sections and wholemounts were analyzed using a Nikon Eclipse E800 fluorescence microscope (Nikon, Melville, NY) with attached digital camera (Olympus, Melville, NY) and Nikon CI confocal scanning system. All images were acquired digitally using Magna-Fire Software (Optronics, Goleta, CA).

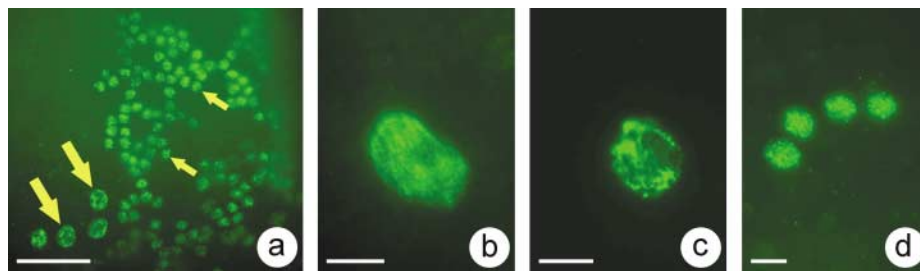
#### Determination of labeling indices

A total of 18 sections derived from the testis of four adult male rhesus monkeys (five sections each for monkeys #2509, #2518 and #2556, three sections for monkey #2699) were used for the determination of the labeling indices of  $A_{\text{pale}}$  and  $A_{\text{dark}}$  spermatogonia. A total of randomly selected 332 fields of view (78 for monkey #2509, 71 for monkey #2518, 130 for monkey #2556 and 53 for monkey #2699) were evaluated at  $\times 40$  magnification in brightfield mode. All  $A_{\text{dark}}$  spermatogonia (regardless of the stage of the cycle of the seminiferous epithelium) and all  $A_{\text{pale}}$  spermatogonia (only in stage VII of the cycle of the seminiferous epithelium) encountered in each evaluated field of view were viewed at  $\times 100$  magnification in both brightfield mode (to confirm cell type) and in fluorescent mode (to determine BrdU labeling). The percentage of labeled cells (labeling index) was calculated.

## Results

### BrdU-positive cells at stage VII of the seminiferous epithelial cycle after acute labeling

Whole mounts of seminiferous tubules at stage VII contain large numbers of BrdU-positive pre-leptotene spermatocytes. Small chains or groups of considerably larger cells are observed in close proximity to or in a second plain of focus below or above the cohorts of pre-leptotene spermatocytes (Figure 1A). The larger cells are located closer to the basement membrane of the seminiferous tubule when compared with the pre-leptotene spermatocytes. We used tissue sections to determine the subtype of BrdU-positive spermatogonia closely associated with pre-leptotene spermatocytes at stage



**Figure 1.** Micrographs showing immunofluorescent detection of BrdU in whole mounts of rhesus monkey testes. (A) BrdU-positive  $A_{\text{pale}}$  spermatogonia (big arrows) and pre-leptotene spermatocytes (small arrows) are both present in stage VII of spermatogenesis 2 h after BrdU injection. The different nuclear size and the more central location of the spermatocytes (different plain of focus) allow an easy distinction of these cell types. BrdU-positive  $A_{\text{pale}}$  spermatogonia are usually forming 4-cell clones as seen in the micrograph. Scale = 50 µm. (B and C) High power micrograph of isolated BrdU-positive spermatogonia 2 h after BrdU injection; scale for (B) = 10 µm, scale for (C) = 5 µm. Single positive cells are rare. They appear occasionally at different stages of spermatogenesis [the cell depicted in (B) was encountered at stage III, the cell in (C) at stage I]. (D) 4-cell clone of BrdU-positive spermatogonia 40 days after BrdU injection. Scale = 10 µm.

VII of the seminiferous epithelial cycle. We can clearly identify that the BrdU-positive spermatogonia at stage VII are almost exclusively  $A_{\text{pale}}$  spermatogonia (Figure 2A–C). To verify a high proliferative activity of  $A_{\text{pale}}$  spermatogonia at stage VII, we determined the ratio of BrdU-positive and negative  $A_{\text{pale}}$  spermatogonia. Of 218  $A_{\text{pale}}$  spermatogonia encountered at stage VII, >27% are BrdU-positive (Table II).

#### **Single BrdU-positive cells in the seminiferous epithelium after acute labeling**

Single BrdU-positive cells can very occasionally be encountered in the seminiferous epithelium with no apparent relation to seminiferous cycle. These rare cells can be found at stages of the cycle, where no other BrdU-positive spermatogonia can be routinely encountered (stages II, IV, VI, VII, IX and XII; see also Ehmcke *et al.*, 2005), such as stages I and III (Figure 1B and C).

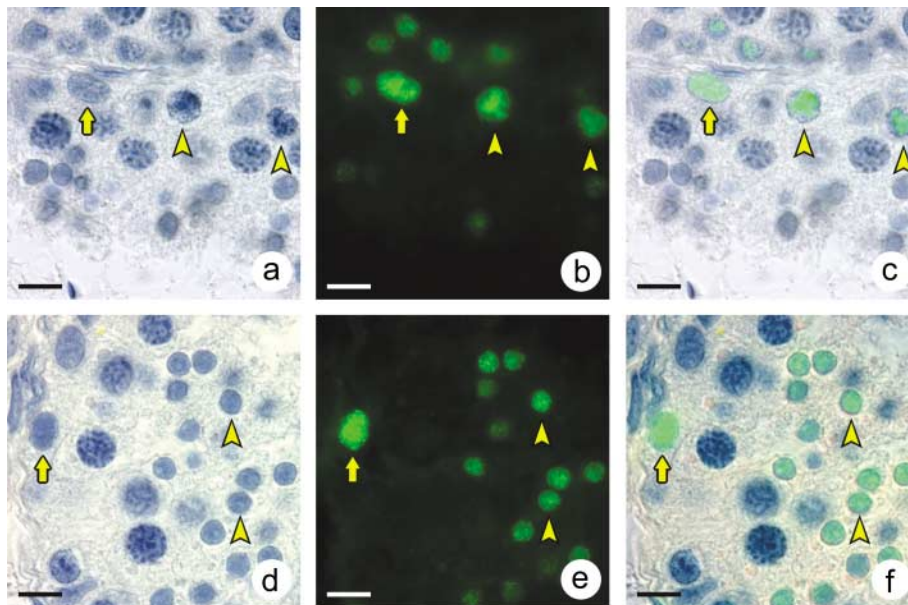
#### **Detection of label-retaining cells 40 days after BrdU incorporation**

Whole mounts of seminiferous tubules of the monkey, which had received an iv bolus injection of BrdU 40 days prior to castration, were stained immunohistochemically for BrdU and Acrosin as described above. Since 40 days after labeling, all differentiating germ cells should have been eliminated

from the testis as mature sperm, we expect to see only those cells that remain as positive progeny, which had not differentiated and have rarely divided in the meantime. In other stem cell systems these label-retaining cells have been recognized as adult stem cells (Cotsarelis *et al.*, 1990). In the tissue of this monkey we observe occasionally BrdU-positive single cells and very rarely small clones of BrdU-positive cells (Figure 1D).

#### **BrdU label in $A_{\text{dark}}$ spermatogonia after acute labeling**

We used tissue sections to determine whether any BrdU-positive  $A_{\text{dark}}$  spermatogonia were present in the seminiferous tubules and to analyze whether they are preferentially observed at specific stages of the seminiferous epithelial cycle. We noted that very few  $A_{\text{dark}}$  spermatogonia show an intense label for BrdU and that these cells were distributed among different stages of the seminiferous epithelial cycle (Figure 2D–F). When we determined the ratio of BrdU-labeled  $A_{\text{dark}}$  spermatogonia across all stages of the seminiferous epithelial cycle, <1% of these cells were intensively BrdU-positive representing the intense staining of cells in S-phase of the cell cycle (Table III). Surprisingly we encountered a large number (19%, Table III) of  $A_{\text{dark}}$  spermatogonia showing a weak, but clearly visible granular nuclear label for BrdU which was not observed in control sections after omission of the primary antibody or when the tissue had not been



**Figure 2.** High power micrographs of crosssections stained both with hematoxylin and immunohistochemically for the detection of BrdU (green label). Seminiferous epithelium of an adult rhesus monkey 3 h after injection of BrdU, stage VII of spermatogenesis. Scales = 10  $\mu\text{m}$ . (A) Brightfield micrograph of the hematoxylin staining showing an  $A_{\text{pale}}$  spermatogonium (arrow), pre-leptotene spermatocytes (arrowheads), pachytene spermatocytes and round spermatids. (B) Fluorescence micrograph of the same section as presented in Figure 2A, showing the distinct BrdU-labeling of several cells in the seminiferous epithelium. The arrow marks the BrdU-labeling detected in the  $A_{\text{pale}}$  spermatogonium pointed out in Figure 2A. The arrowheads mark the BrdU-labeling detected in the pre-leptotene spermatocytes pointed out in (A). (C) Digital overlay of (A) and (B). The  $A_{\text{pale}}$  spermatogonium (arrow) and two pre-leptotene spermatocytes (arrowheads) are clearly BrdU-positive. (D) Brightfield micrograph of the hematoxylin staining showing an  $A_{\text{dark}}$  spermatogonium (arrow), pachytene spermatocytes and round spermatids (arrowheads). (E) Fluorescence micrograph of the same section as presented in (D), showing the distinct BrdU-labeling of cells in the seminiferous epithelium. The arrow marks the BrdU-labeling detected in the  $A_{\text{dark}}$  spermatogonium pointed out in (D). The arrowheads mark the BrdU-labeling detected in the round spermatids pointed out in Figure 2D (F) Digital overlay of (D) and (E). The  $A_{\text{dark}}$  spermatogonium (arrow) and the round spermatids (arrowheads) are BrdU-positive.



**Table II.** Labeling index of  $A_{\text{pale}}$ -spermatogonia in stage VII

Animal number	Total $A_{\text{pale}}$	Labeled $A_{\text{pale}}$	LI (%) $A_{\text{pale}}$
1	94	36	38.30
2	61	9	14.75
3	26	7	34.62
4	37	6	16.22
Total/average	218	60	27.52

exposed to BrdU (Figure 3A and B). A similar weak and granular staining pattern for BrdU was observed in elongating spermatids at stages VII and VIII of the cycle of the seminiferous epithelium (Figure 2D–F). Apart from  $A_{\text{dark}}$  spermatogonia and elongating step 7 and 8 spermatids, no other cell type showed this type of weak granular BrdU label.

### ***BrdU-labeled spermatogonia at stage VII 11 days after BrdU injection***

The duration of the seminiferous cycle of macaques is 10–11 days (de Rooij *et al.*, 1986). As a consequence, and as we had shown in previous studies, the majority of BrdU-positive germ cells develop into the next generation of differentiating germ cells when the period between BrdU-incorporation and fixation of the tissue is 11 days (Rosiepen *et al.*, 1997). If any  $A_{\text{pale}}$  spermatogonia had divided but not differentiated at a given stage of the cycle, we expect to localize these BrdU-positive cells 10–11 days after BrdU exposure in the same stage of the seminiferous epithelial cycle. We used tissue sections to determine the types of BrdU-positive germ cells present in the seminiferous epithelium at stage VII of the epithelial cycle 11 days after BrdU injection. We detected large cohorts of intense BrdU-positive pachytene spermatocytes

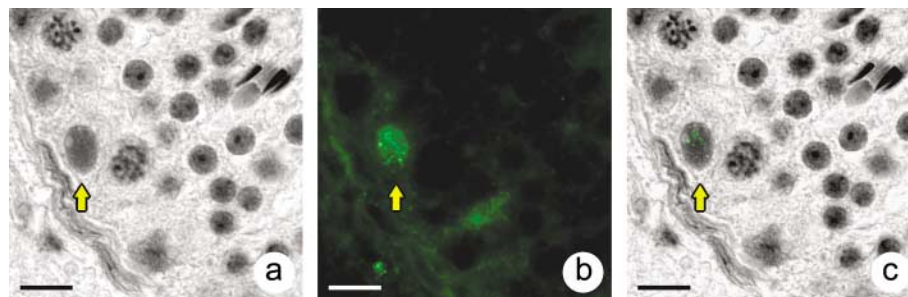
located within the seminiferous epithelium (Figure 4A and B). These cells had been pre-leptotene spermatocytes in prophase of meiosis at the time of BrdU incorporation and had undergone no divisions during the last seminiferous cycle. In addition, we detected weakly labeled pre-leptotene spermatocytes. These cells had been A spermatogonia when they incorporated the BrdU label and had gone through a number of premeiotic divisions as differentiating A and B spermatogonia leading to significant dilution of the label intensity. However, some intensively BrdU-labeled cells with large nuclei were also observed. These cells are located close to the basement membrane and their nuclear size and shape indicates that they are  $A_{\text{pale}}$  spermatogonia which had gone through S-phase once but then did not differentiate into more mature germ cells (Figure 4A and B).

### ***Tracing of BrdU-labeled cells in whole mounts during organ culture for 48 and 72 h***

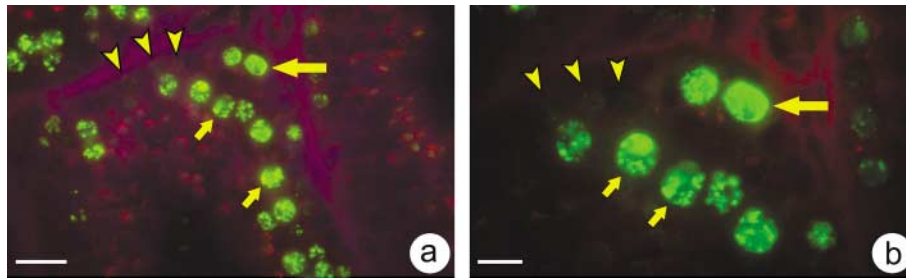
Organ culture of tissue fragments allows observation of the differentiation of pulse chased BrdU-positive cells over several days. We were able to detect BrdU-positive cells in fragments of seminiferous tubules which had been cultured at the interphase of air and culture medium for up to 3 days. During culture, no obvious damage to the integrity of the tubules was observed. The fragments of seminiferous tubules were exposed to BrdU for the first 2 h and then washed and cultured in BrdU-free culture medium to follow the differentiation of BrdU-labeled cells from the original stage VII which is easily identifiable by the large number of pre-leptotene spermatocytes. After 48 h of organ culture, the BrdU label is now detected in large numbers of leptotene spermatocytes. Few considerably larger A spermatogonia are

**Table III.** Labeling index of  $A_{\text{dark}}$ -spermatogonia

Animal number	Total $A_{\text{dark}}$	Labeled $A_{\text{dark}}$ (weak label only)	Labeled $A_{\text{dark}}$ (strong label only)	LI (%) $A_{\text{dark}}$ (weak label only)	LI (%) $A_{\text{dark}}$ (strong label only)
1	91	8	0	8.79	0.00
2	56	9	1	16.07	1.76
3	75	30	1	40.00	1.33
4	42	3	0	7.14	0.00
Total/average	261	48	2	18.39	0.77



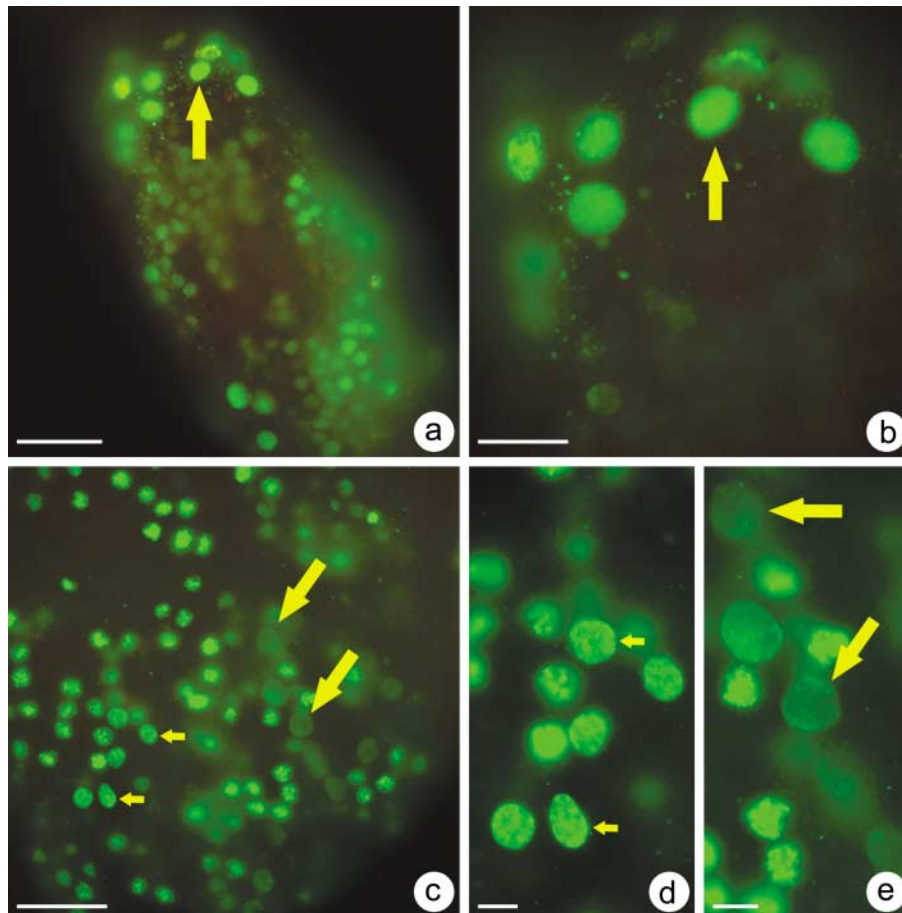
**Figure 3.** High power micrographs of cross-sections stained both with hematoxylin and immunohistochemically for the detection of BrdU (green label). Seminiferous epithelium of an adult rhesus monkey 3 h after injection of BrdU. Scales = 10  $\mu\text{m}$ . (A) Brightfield micrograph of the hematoxylin staining showing an  $A_{\text{dark}}$  spermatogonium (arrow), spermatocytes, round and elongated spermatids. (B) Fluorescence micrograph of the same section as presented in (A). The arrow marks the BrdU-labeling detected in the  $A_{\text{dark}}$  spermatogonium pointed out in (A). (C) Digital overlay of (A) and (B). The  $A_{\text{dark}}$  spermatogonium (arrow) shows a weak BrdU label of granular appearance.



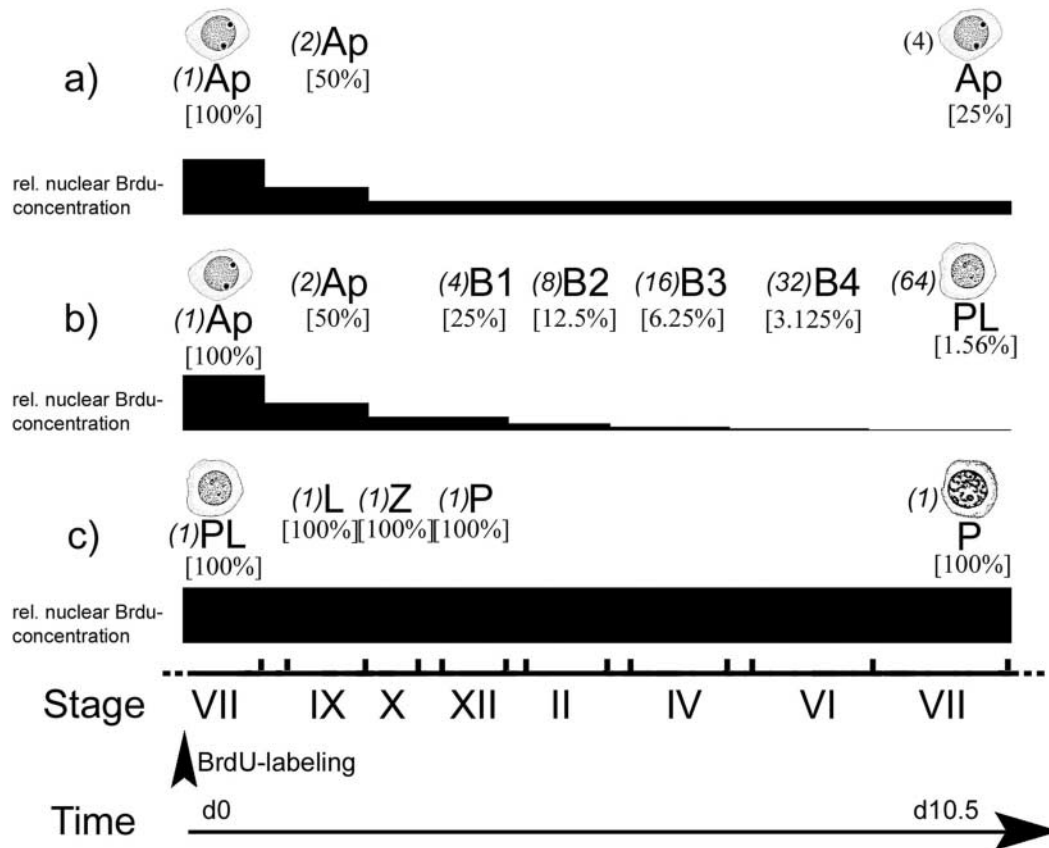
**Figure 4.** (A and B) Micrographs showing stage VII of the cycle of the seminiferous epithelium of a rhesus monkey that had received an injection of BrdU 11 days prior to dissection of the testis. Scale for (A) = 25  $\mu\text{m}$ , scale for b = 10  $\mu\text{m}$ . Triple-immunofluorescent labeling of BrdU (green/yellow), Acrosin (red) and DNA (DAPI/blue). The most abundant BrdU-positive cells are pachytene spermatocytes (small arrows). A strongly BrdU-positive  $A_{\text{pale}}$  spermatogonium (big arrow) is located on the basement membrane below the pachytene spermatocytes. In addition, pre-leptotene spermatocytes can be seen carrying a very weak BrdU label (arrowheads).

also observed (Figure 5A and B). Interestingly, the labeling in the nuclei of the spermatogonia—which have a nuclear diameter of  $\sim 10\ \mu\text{m}$ —is now much more homogeneously distributed throughout the nucleus when compared to the pattern observed after acute labeling (compare with Figure 1).

After 72 h of organ culture, the large cohorts of originally BrdU-positive pre-leptotene spermatocytes have differentiated into zygotene spermatocytes. In close proximity to these large cohorts we can now detect spatially separated cohorts of two different types of spermatogonia (Figure 5C).



**Figure 5.** Micrographs showing immunofluorescent detection of BrdU in whole mounts of rhesus monkey testicular tissue, which has been labeled with BrdU *in vitro* for 2 h and has then been cultured for an additional 48 to 72 h. (A) In addition to numerous BrdU-positive leptotene spermatocytes, BrdU-positive  $A_{\text{pale}}$  spermatogonia (arrow) (both have been labeled with BrdU 48 h ago in stage VII of spermatogenesis) are detectable in stage IX of the cycle of the seminiferous epithelium. Scale = 50  $\mu\text{m}$ . (B) High power magnification of the cells shown in (A). Scale = 20  $\mu\text{m}$ . (C) Remaining BrdU-positive  $A_{\text{pale}}$  spermatogonia (small arrows),  $B_1$ -spermatogonia (big arrows) and zygotene spermatocytes (all of which have been labeled with BrdU 72 h ago in stage VII of spermatogenesis) are detectable in stage X. Scale = 50  $\mu\text{m}$ . (D) High power magnification of the BrdU-positive  $A_{\text{pale}}$  spermatogonia (arrow) shown in (C). Scale = 10  $\mu\text{m}$ . (E) High power magnification of the BrdU-positive  $B_1$  spermatogonia (arrow) shown in (C). Scale = 10  $\mu\text{m}$ .



**Figure 6.** Graph showing the presumed progressive dilution of a BrdU label incorporated by all cell types in S-phase at stage VII of the seminiferous epithelial cycle during the following cycle (= 10.5 days) in adult rhesus monkey tissue. (A) BrdU incorporated into an  $A_{pale}$  spermatogonium, which does not go into differentiation, is diluted through two mitotic divisions to 25%. (B) BrdU incorporated into an  $A_{pale}$  spermatogonium, which goes into differentiation, is diluted through six mitotic divisions to <2%. (C) BrdU incorporated into a pre-leptotene spermatocyte in S-phase of meiosis is not diluted. Number in italic: cell number; number in square brackets: relative percentage of nuclear BrdU as compared to the amount incorporated during BrdU exposure (100%); Ap:  $A_{pale}$  spermatogonium; Ad:  $A_{dark}$  spermatogonium; B1, B2, B3 and B4: differentiating spermatogonia; L: leptotene spermatocyte; P: pachytene spermatocyte; PL: pre-leptotene spermatocyte; Z: zygotene spermatocyte.

An intense BrdU label can be detected in the nuclei of smaller spermatogonia with a nuclear diameter of  $\sim 10 \mu\text{m}$  (Figure 5D) and a weaker BrdU label can be detected in the nuclei of bigger spermatogonia with a nuclear diameter of  $\sim 15 \mu\text{m}$  (Figure 5E). The size and shape of these nuclei indicate that we do now encounter two different types of BrdU-positive cells:  $A_{pale}$  and  $B_1$  spermatogonia (Clermont, 1969).

## Discussion

In a previous study, we have applied incorporation and immunohistochemical detection of BrdU on sections as well as on whole mounts of seminiferous tubules to confirm the exact starting point of spermatogenesis in the rhesus monkey as A spermatogonia at stage VII of the seminiferous epithelial cycle (Ehmcke *et al.*, 2005). Here we unequivocally characterize these spermatogonia as  $A_{pale}$  spermatogonia using a novel staining approach on sections of testicular tissue, thus confirming that  $A_{pale}$  spermatogonia in macaques undergo a first equal division at stage VII of the cycle of the seminiferous epithelium. These findings support our earlier

hypothesis that spermatogonial expansion follows an identical mode in all macaque species (Ehmcke *et al.*, 2005).

In addition, in the present study we demonstrate the presence of BrdU-positive spermatogonia at stage VII of the cycle of the seminiferous epithelium not only after acute labeling (Ehmcke *et al.*, 2005) but also 11 days after BrdU injection. It is important to consider that the intensity of the BrdU label diminishes by 50% with each mitotic division, as unlabeled nucleotides are incorporated into the newly synthesized DNA. In Figure 6 we present a calculation of the progressive dilution of the BrdU label. The startpoint is set at stage VII and the presumptive changes over one full spermatogenic epithelial cycle (10.5 days, de Rooij *et al.*, 1986) are indicated. Following this calculation, the presence of strongly BrdU-positive ( $A_{pale}$ ) spermatogonia (25% of the incorporated BrdU is still present, Figure 6A) in stage VII of the seminiferous epithelium 11 days after an iv bolus injection of BrdU revealed that at least some of the  $A_{pale}$  spermatogonia, which proliferated at stage VII in the previous cycle, did not undergo differentiation. We conclude that the persistence of non-differentiating  $A_{pale}$  spermatogonia is the mechanism to maintain a stable  $A_{pale}$  spermatogonial population under steady-state conditions. The same cross-sections contained

weakly labeled pre-leptotene spermatocytes (dilution of the original BrdU label to  $<2\%$ , Figure 6B). These cells derived from the differentiating  $A_{\text{pale}}$  spermatogonia of the previous cycle. The label is significantly diluted since these cells had gone through five subsequent mitotic divisions. In addition, we found a strong BrdU label in all pachytene spermatocytes which had developed without further divisions from pre-leptotene spermatocytes of the previous cycle and therefore maintain 100% of the incorporated BrdU label (Figure 6C).

To further clarify the kinetics of  $A_{\text{pale}}$  spermatogonial expansion, we performed *in vitro* BrdU-chase experiments. Our tissue culture model did not lead to obvious degenerative changes during the culture period up to 72 h. The supply with nutrients and oxygen seems to be optimized. Any germ cell observed in the seminiferous epithelium at a particular stage should advance to the subsequent stages of germ cell development during the culture period. When we take into consideration the calculated spermatogenic progression by de Rooij *et al.* (1986), a stage VII  $A_{\text{pale}}$  spermatogonium should divide twice and undergo differentiation into two unequal types of spermatogonia during the following 72 h. We observed BrdU-positive spermatogonia in seminiferous tubules cultured for 48 h together with leptotene spermatocytes. After 72 h of incubation we encountered two types of spermatogonia—with different nuclear sizes and different BrdU staining intensity—together with zygotene spermatocytes at stage X of the cycle. These spermatogonia are most likely  $A_{\text{pale}}$  and  $B_1$  spermatogonia, which are to be expected at stage X of the cycle of the seminiferous epithelium. The encountered cellular compositions are in accordance with our expectations. Our findings support our earlier model for spermatogonial expansion in macaques (Ehmcke *et al.*, 2005) and also indicate that spermatogenesis can be studied *in vitro*, since spermatogenesis in the cultured tissue fragments apparently advances with the same kinetics as it would *in vivo*. In addition, the detection of the two types of spermatogonia in spatially separated groups of either the one or the other spermatogonial type supports our earlier hypothesis (Ehmcke *et al.*, 2005) and the findings of others (Clermont and Antan, 1973) that  $A_{\text{pale}}$  and  $B_1$  spermatogonia do not result from unequal divisions of  $A_{\text{pale}}$  spermatogonia.

We can only speculate which (if any) of the two A-spermatogonial subtypes in the monkey testis corresponds to the single undifferentiated spermatogonium acting as male germline stem cell in the rodent (Huckins, 1971).  $A_{\text{dark}}$  spermatogonia only very occasionally proliferate in healthy adult rhesus monkeys (Clermont and Antan, 1973; Fouquet and Dadoune, 1986). Therefore,  $A_{\text{dark}}$  spermatogonia are considered to be quiescent or reserve stem cells which only proliferate after the significant loss of  $A_{\text{pale}}$  spermatogonia due to X-ray irradiation or cytotoxic exposure. Then, the  $A_{\text{pale}}$  population is restored from the  $A_{\text{dark}}$  pool (Dym and Clermont, 1970; van Alphen *et al.*, 1988a,b). When such repopulation occurs, long chains of  $A_{\text{dark}}$  and  $A_{\text{pale}}$  spermatogonia are observed, but  $\sim 20\text{--}40\%$  of both types of A-spermatogonia are single cells (van Alphen *et al.*, 1988b). Unfortunately, no analysis of the proliferation indices has been performed in the radiation study. Here we show that

under normal conditions proliferating single spermatogonia are extremely rare. Taken together, this might indicate that the monkey testis contains a significant number of single A-spermatogonia which very rarely divide. The latter is true for the  $A_{\text{dark}}$  spermatogonial population. However, we do not know whether  $A_{\text{dark}}$  spermatogonia would more intensively proliferate during recolonization or whether they would be replenished from the  $A_{\text{pale}}$  pool as was proposed by van Alphen *et al.* (1988b). In conclusion, which cell acts as the primate male germline stem cell can not be unequivocally determined from the previous radiation study as well as from our study. The questions which need to be answered are (i) are label-retaining cells exclusively found among the population of one A-spermatogonial subtype, (ii) to what extent are the pools of  $A_{\text{pale}}$  and  $A_{\text{dark}}$  spermatogonia separate populations and how intense is the transition between both subtypes under normal conditions as well as during testis growth or recolonization after germ cell depletion. We can, however, confirm that  $A_{\text{pale}}$  spermatogonia are the only cells which cycle regularly, replenish their own numbers and give rise to B-spermatogonia. Due to their effective self-renewal, only very few  $A_{\text{pale}}$  spermatogonia would need to be generated by stem cells.

In the present study we also detect a very weak and granular BrdU label in  $A_{\text{dark}}$  spermatogonia and in round-to-elongating spermatids at stages VII and VIII of the cycle of the seminiferous epithelium. This label is BrdU-specific because it is absent in tissue derived from animals which have not received a BrdU injection, and in immunohistochemical controls after omission of the primary antibody. This finding was surprising as we did not expect significant numbers of  $A_{\text{dark}}$  spermatogonia entering mitotic S-phase, and as round spermatids (haploid germ cells) by definition can not enter the S-phase of the cell cycle. Therefore, the weak BrdU label must be the result of a different BrdU incorporation process.

It is well known that during the condensation of DNA in elongating spermatids, histones are removed from the supercoiled DNA. As part of this process, nuclease activity induces multiple single-strand breaks to release tension of the coiled DNA in order to enable stretching (McPherson and Longo, 1993). Nuclear transient proteins attach to the single-strand breaks and help stabilize the DNA until the breaks are repaired by an as yet unidentified ligase (Caron *et al.*, 2001; Brewer *et al.*, 2002; Zhao *et al.*, 2004). Extensive chromatin remodeling is not an exception, but the rule during spermiogenesis (Kierszenbaum, 2001; Boissonneault, 2002; Marcon and Boissonneault, 2004). During chromatin remodeling, BrdU incorporation into the DNA of the elongating spermatids occurs, however, at a much lower level than during mitotic S-phase. A highly sensitive BrdU-detection system as we used here is able to detect such small amounts of incorporated BrdU as was shown previously for repair processes of the lesioned mouse retina (Menu Dit Huart *et al.*, 2004). We therefore conclude that our weak granular label in elongating spermatids represents BrdU-incorporation processes occurring during chromatin remodeling in spermiogenesis.



About 18% of the  $A_{\text{dark}}$  spermatogonia showed the same pattern of weak granular BrdU label. We certainly were surprised by the high number of weakly positive cells which were highly specific for  $A_{\text{dark}}$  spermatogonia but were never seen in  $A_{\text{pale}}$  spermatogonia. At this time, we cannot present a decisive explanation for the weak granular BrdU label. As these cells seem to proliferate only very occasionally, the labeling may be caused by DNA-repair processes enabling  $A_{\text{dark}}$  spermatogonia to preserve their DNA integrity over extended periods of quiescence.

In conclusion, we have presented further evidence supporting our earlier conclusion (Ehmcke *et al.*, 2005) that rhesus monkey spermatogenesis is initiated by a first mitotic division of  $A_{\text{pale}}$  spermatogonia at stage VII of the seminiferous epithelial cycle. Under *in vivo* and *in vitro* conditions the  $A_{\text{pale}}$  spermatogonial population apparently generates both  $A_{\text{pale}}$  and  $B_1$ -spermatogonia after two mitotic divisions at stages VII and IX of the cycle of the seminiferous epithelium. Single proliferating spermatogonia are very rare and proliferate independently of the seminiferous epithelial cycle.

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