Clonal Organization of Proliferating Spermatogonial Stem Cells in Adult Males of Two Species of Non-Human Primates, *Macaca mulatta* and *Callithrix jacchus*

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ABSTRACT

The present study examines the existence of clonogenetic patterns in the proliferation and differentiation of spermatogonial stem cells in two species of non-human primates, the marmoset and the rhesus monkey. We developed a novel approach to detect proliferating spermatogonial clones in whole mounts of seminiferous tubules. Dual fluorescence labeling of bromodeoxyuridine and acrosin in conjunction with confocal microscopy allows the description of the clonogenic and spatial arrangement of proliferating spermatogonia at specific stages of the seminiferous epithelial cycle. Cross-sections of paraffin-embedded tissue were labeled by the same approach. For both monkey species we demonstrate the presence of proliferating spermatogonial clones of variable size at specific stages of the cycle of the seminiferous epithelium. Detailed analysis of the rhesus monkey reveals proliferating *A* pale spermatogonia at stages VII and IX of the cycle of the seminiferous epithelium, and of proliferating *B* spermatogonia at stages II, IV, VI, and XII. Proliferating *A* pale spermatogonia at stages VII and IX of the cycle are organized in pairs or quadruplets. *B* spermatogonia appear as quadruplets or eight-cell clones, and *B* spermatogonia as 8- or 16-cell clones. We conclude that spermatogenesis in the rhesus monkey is initiated by two divisions of duplets or quadruplets of *A* pale spermatogonia: a first division at stage VII, after which the clones of *A* pale spermatogonia separate, and a second division at stage IX, which leads to clones of *B* spermatogonia as well as pairs and quadruplets of *A* pale spermatogonia replenishing the seminiferous epithelium to maintain the original size of the *A* spermatogonial population.

INTRODUCTION

In the testis of adult mammalian species, spermatogonial stem cells maintain their numbers by self-renewal and give rise to differentiating germ cells. Most of the diploid germ cells are differentiating spermatogonia undergoing several rounds of mitotic divisions before entering meiotic prophase. In non-human primates seven different types of spermatogonia have been identified [1–6]: the reserve stem cell *A* dark spermatogonium; the renewing stem cell *A* pale spermatogonium; the intermediate *A* transition spermatogonium; and four generations of *B* spermatogonia, *B*1, *B*2, *B*3, and *B*4. It has been unequivocally demonstrated that *B* spermatogonia derive from *A* pale spermatogonia [1]. However, the complex kinetics and the circumstances of the unequal division of *A* pale spermatogonia replenishing their own numbers and giving rise to *B* spermatogonia are poorly understood.

Previous studies have focused on the cycle of the seminiferous epithelium and on the proliferation of spermatogonial stem cells in different species of non-human primates and other mammalian species, leading to several models for spermatogonial expansion and differentiation [1–3, 5, 7–14]. Some of these studies examined whole mount preparations of seminiferous tubules to obtain qualitative data on spermatogonial organization. Most previous studies applied morphometric approaches on (serial) cross-sections of seminiferous tubules, producing valuable data on spermatogonial counts and labeling indices. However, none of the previous studies has so far presented an in-depth evaluation of the proposed [1, 2, 13] clonal organization of spermatogonial stem cells at different stages of spermatogenesis, and some models of the expansion of spermatogonial stem cells contradict each other.

In the adult testis, proliferating spermatogonia and preleptotene spermatocytes in S-phase of meiosis can be detected by nuclear incorporation of bromodeoxyuridine (BrdU) [15]. We first encountered clones of proliferating spermatogonia in the marmoset (*Callithrix jacchus*) by using a whole mount approach. As the seminiferous epithelium in this species does not present longitudinally separated stages of spermatogenesis, it is difficult to correlate spermatogonial clones with specific stages of spermatogenesis [16]. We therefore continued our studies using the rhesus monkey (*Macaca mulatta*), which shows a longitudinal separation of 12 different stages of the cycle of the seminiferous epithelium.

Criteria for the staging of the macaque seminiferous epithelium had first been established by Clermont and Leblond [1], Clermont [2], and Clermont and Antar [3] based on periodic acid Schiff reagent staining of the acrosome and hematoxylin counterstaining of nuclei. The immunofluorescent localization of acrosin allowed us to accurately determine the size and shape of the acrosome. We used changes of the acrosomal immunostaining to accurately determine the stages of spermatogenesis [1–3]. It was our first aim to correlate our novel staining method on sections and whole mounts of rhesus monkey testicular tissue to the originally described 12 stages of the seminiferous epithelial cycle. We describe well-defined categories enabling us to identify these 12 stages of the spermatogenic cycle both in...
whole mounts of seminiferous tubules and on testicular sections using our dual- and triple-label immunohistochemical approach.

We then aimed to analyze and describe the initiating division(s) and the clonogenic expansion of spermatagonia to propose a well-defined model of premeiotic germ cell development in the rhesus monkey.

**MATERIALS AND METHODS**

**Animals**

Two adult (one age 2.7 yr, body weight 375 g, and the other 4.1 yr and 350 g, with a testicular weight between 0.3 and 0.5 g) male marmosets (C. jacchus) were included in this study. The animals where maintained at the University of Pittsburgh Plum Borough Primate Research Facility in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. One of the monkeys had taken part in previous endocrinological studies, but had been allowed to recover for a period of more than 6 mo before the present study. Three additional monkeys 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 [17]), and the restoration of physiologically normal FSH and LH blood levels was attempted using the German Federal Law on the Care and Use of Laboratory Animals. One of the monkeys had been allowed to recover for a period of more than 6 mo before the present study. Three additional monkeys 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 [17]), and the restoration of physiologically normal FSH and LH blood levels was attempted using acyline.

**Surgical Procedures**

The marmosets received an intraperitoneal (i.p.) bolus injection of BrdU (100 mg/kg body weight; Sigma-Aldrich, Munich, Germany) 3 h before castration. Four rhesus monkeys received an intravenous (i.v.) bolus of BrdU (33 mg/kg body weight; Sigma, St. Louis, MO) 3 h before castration or hemi-castration. One rhesus monkey received an i.v. bolus injection of BrdU 40 days before the removal of the testis. For surgery the monkeys were first sedated with ketamine hydrochloride: 10 mg/kg body weight for the marmosets (Park-Advise, Munich, Germany) and 10 mg/kg body weight i.m. for the rhesus monkeys (Ketaject, Phoenix Scientific Inc., St. Joseph, MO). The marmosets were then killed by exsanguination and the testes were removed postmortem. The rhesus monkeys were anesthetized with isoflurane in oxygen (1%-2.5%; Abbott Laboratories, North Chicago, IL). All surgical procedures were performed under aseptic conditions. Postsurgically rhesus monkeys 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) for 4 days.

**Tissue Preparation**

For sectioning, testicular tissue samples were fixed overnight at 4°C in Bouin fixative, washed in 70% ethanol, dehydrated, and routinely embedded in paraffin. Five-micrometer serial sections were cut.

For whole mount staining, fresh testes were decapsulated and tissue samples were treated in sterile PBS to obtain fragments of seminiferous tubules, each between several millimeters and 2 cm in length. At this stage, some fragments of testicular tissue retrieved from the macroae that had received an i.v. bolus of BrdU 40 days before castration was incubated for 2 h at 37°C in Dulbecco modified eagle medium (DMEM; 4.5 g glucose/L; Mediatech, Herndon, VA) supplemented with nonessential amino acids (Cambrex Bio Science, Walkerville, MD; dilution following manufacturer’s instructions); Glutamine (365 mM/L; Sigma); antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml; Mediatech, Herndon, VA); and BrdU (100 µM; Sigma) to allow cells in S-phase to incorporate BrdU. All testicular tubules were fixed overnight at 4°C in Bouin fixative and washed and stored in 70% ethanol.

**Conventional Staining Procedure**

Sections were deparaffinized, rehydrated, incubated for 15 min at room temperature (RT) in fresh periodic acid solution (1% in distilled water), washed with distilled water, incubated for 15 min in Schiff reagent, washed with distilled water, stained with hematoxylin solution (all reagents from Sigma), washed in tap water followed by distilled water, transferred through rising ethanol concentrations into xylene, and mounted with permanent mounting medium.

Whole mounts were rehydrated, stained with hematoxylin solution, washed with tap water followed by distilled water, and—to avoid shrinkage occurring during dehydration—mounted with VectaShield aqueous mounting medium (Vector, Burlingame, CA).

**Immunohistochemical Staining Procedure**

Sections of testicular tissue 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 (0.1% in Tris buffered saline [TBS]; Sigma); washed with distilled water followed by TBS; incubated for 30 min at RT with blocking solution (5% goat serum and 0.1% bovine serum albumin [BSA] in TBS; goat serum and BSA from Sigma); and incubated overnight at 4°C with the primary antibody (monoclonal anti-BrdU, clone BU-33 [either from Sigma or Biomaed, 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], diluted 1:100 in TBS containing 0.1% BSA); washed with TBS; incubated for 1 h at RT with a mix of a secondary primary antibody and a streptavidin-conjugated fluorescent dye (monoclonal anti-acrosin, clone Acr-CSF10 [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], diluted 1:100 in TBS containing 0.1% BSA) washed with TBS and mounted using VectaShield Mounting Medium (Vector) containing 4,6-Diamidino-2-phenylindole (DAPI; 1.5 µg/ml).

For immunohistochemical staining of whole mounts, a similar protocol as described for sections was applied, differing in longer incubation times for hydrochloric acid and trypsin solution (15 min at RT for both in whole mount staining). For the visualization of the BrdU label in some seminiferous tubules of the marmoset, a peroxidase-conjugated secondary antibody (Goat anti-Mouse, peroxidase-conjugated; Sigma-Aldrich) and DAB-staining (3,3’-diaminobenzidine, peroxidase substrate; Sigma-Aldrich) were used to produce an insoluble brown precipitate in BrdU-positive nuclei. All whole mounts were mounted on microscope slides using VectaShield mounting medium without DAPI (Vector).

**Tissue Analysis**

Sections and whole mounts 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 MagnaFire Software (Optronics, Goleta, CA).

**Determination of Stages of the Cycle of the Seminiferous Epithelium on Sections and Whole Mounts of Seminiferous Tubules After Immunohistochemistry**

When the tissue had been exposed to hydrochloric acid and trypsin to allow access of the antibody to BrdU incorporated into the nuclear DNA, the nuclear counterstaining with DAPI was only intense in the heads of elongated spermatids from step 13 onward (stages 1 to VI of the cycle of the seminiferous epithelium as defined by Clermont and Leblond [1]). This allowed us to distinguish the following types of spermatids: 1) round spermatids from step 1 to step 7 (stage I to stage VII of the cycle); 2) DAPI-negative elongating spermatids from step 8 to step 12 (corresponds to stage VIII to stage XII of the cycle); 3) DAPI-positive elongated spermatids (from step 13 onward).

The localization of BrdU as a proliferation marker, across as a spermatid label, and DAPI as DNA-staining of the most mature spermatids enabled us to identify proliferating cells and to correlate their presence
with accurately determined stages of spermatogenesis depending on acrosomal structure and the presence of one or two types of spermatids. Therefore, we first evaluated criteria to correlate our staining patterns in tissue sections with the morphological criteria used for the determination of stages by Clermont and Leblond [1]. In whole mounts of tissue, the DAPI staining was obsolete as the presence of elongated spermatids in the seminiferous tubules could easily be determined using conventional phase contrast microscopy.

Evaluation of Spermatogonial Clones

In accordance with the definitions used to describe clones of undifferentiated spermatogonia in rodents [18, 19] and cohorts of monkey spermatogonia [1–3], we defined cohorts of neighbor BrdU-positive spermatogonia as clones whenever the distance between two BrdU-positive nuclei was less than 1.5 times the diameter of the nucleus. As in the rat [18, 19] and as we show here, spermatogonia located at this distance are connected by cytoplasmic bridges. As additional criteria the clones had to show the same or a very similar intensity and pattern of BrdU labeling as this indicates a synchrony of their cell cycle and the distance between two similarly stained nuclei was not more than approximately 1.5 times the diameter of such a nucleus. These criteria were chosen because a similar BrdU staining pattern in various cells indicates that the timing of S-phase in these cells is synchronized. In hematoxylin-stained whole mounts, cytoplasmic bridges (indicating a clonal origin of several cells) between spermatogonia can only be detected in cells not further separated than approximately 1.5 times their nuclear diameter. Using these criteria, the number of clones of BrdU-positive spermatogonia and the number of spermatogonia per clone was determined for stages VII, IX, and XII of the seminiferous epithelium using seminiferous tubules of two adult male rhesus monkeys. The total number of BrdU-labeled spermatogonia was counted for a certain number of sites (a site being an area of a seminiferous tubule fragment showing a given stage of the cycle of the seminiferous epithelium) per stage, and the number of cells of each spermatogonial clone was determined.

RESULTS

Detection of Proliferating Cells in Whole Mounts of Adult Marmoset Seminiferous Tubules

Whole mounts derived from testes removed 3 h after an i.v. bolus injection of BrdU show BrdU-labeled cells forming long chains oriented in longitudinal direction along the tubule (Fig. 1a). These cells are located adjacent to the basement membrane of the tubule. In other areas fields of scattered BrdU-positive cells were observed (Fig. 1, b and c). The sizes of the nuclei differed distinctly between 8 and 12 μm.

Stages of Spermatogenesis and Spermatogonial Clones As Visualized in Whole Mounts of Adult Rhesus Monkey Seminiferous Tubules

The whole mounts consisted of intact seminiferous tubules with diameters of 200–300 μm. In the whole mounts, BrdU-labeled germ cells were easily visualized by our immunohistochemical staining (Fig. 2a). The positive cells were attached to or located close to the basement membrane of the structurally intact seminiferous tubules. The three-dimensional organization becomes obvious using confocal microscopy. Whereas the BrdU-positive germ cells are al-
FIG. 3. Micrographs showing different stages of rhesus monkey spermatogenesis. The micrographs are representative examples of the six stages of the seminiferous epithelial cycle when BrdU-positive premeiotic germ cells are present. (The number of the stage shown in each row appears in the upper left corner of each micrograph in the left column.) Column I: Haematoxylin/PAS-stained paraffin-embedded tissue, 5-μm sections. Bar = 50 μm. Column II: Triple-immunofluorescent detection of BrdU (green/yellow), acrosin (red), and DNA (blue) in paraffin-embedded tissue, 5-μm sections. Bar = 15 μm. Column III: Immunofluorescent labeling of BrdU (green/yellow) and acrosin (red) in whole mounts of seminiferous tubules. Bar = 50 μm. a–c) Stage II of rhesus monkey spermatogenesis (a). Strongly DAPI-stained elongated spermatids are present. The acrosomal droplet in round
ways observed in the periphery of the seminiferous tubules, the acrosomal staining of round and elongating spermatids was located close to the lumen of the seminiferous tubules (Fig. 2b). Depending on their variable duration, each stage of the cycle of the seminiferous epithelium extended longitudinally over a distance of 200–800 μm of the seminiferous tubule.

Labeled spermatogonia in all stages of the seminiferous epithelium appear as chains of closely arranged germ cells of identical nuclear size, shape, structure, and staining intensity. In these chains the distance from one nucleus to the other was not wider than the diameter of the nucleus (Fig. 2b). The BrdU-staining intensity and nuclear morphology was similar in all spermatogonia of a single chain but differed considerably between chains even when they were closely arranged, indicating a high degree of cell cycle synchrony in a given chain. Hematoxylin-stained whole mounts revealed that the juxtaposed cells in these chains were connected by cytoplasmic bridges, thus creating a cytoplasmic continuum (Fig. 2c). As the spermatogonia within these chains are closely positioned to one another, they are connected via cytoplasmic bridges, and have high synchrony of their cell cycle, we postulate a clonal origin of these chains of spermatogonia. Therefore we will refer to the chains of spermatogonia in the following as “spermatogonial clones.”

BrdU-labeled Cells at Different Stages of the Cycle of Spermatogenesis 3 h Post±BrdU Injection

In whole mounts of rhesus monkey seminiferous tubules, which show a longitudinal arrangement of the stages of the cycle of the seminiferous epithelium, the dual label for BrdU and acrosin enabled us to identify cohorts of proliferating spermatogonia and to accurately determine the stage

spermatids is extremely small and barely visible. The BrdU-positive B2 spermatogonia are often seen as single cells or pairs in cross-sections (b). Whole mount showing B2 spermatogonia organized as an eight-cell clone (c). d–f Stage IV of rhesus monkey spermatogenesis (d). Strongly DAPI-stained elongated spermatids are present. The acrosomal droplet in round spermatids is now clearly visible. The BrdU-positive B3 spermatogonia are often seen as single cells or pairs in cross-sections (e). Whole mount showing B3 spermatogonia organized as 16-cell-clone (f). g–i Stage VI of rhesus monkey spermatogenesis (g). Strongly DAPI-stained elongated spermatids are lined up close to the lumen of the tubules. The acrosome is an irregular droplet with protrusions beginning to form a cap on round spermatids. Several BrdU-positive B4 spermatogonia are seen in each tubule cross-section along the basement membrane (h). B4 spermatogonial clones can be detected forming large networks (i). j–l Stage VII of rhesus monkey spermatogenesis (j). No elongated spermatids are present. The acrosome is forming a cap on round spermatids. Numerous BrdU-positive preleptotene spermatocytes (small arrows) are seen in each tubule cross-section as a layer of germ cells slightly detached from the basement membrane. In some areas, BrdU-positive A pale spermatogonia are observed adjacent to the basal lamina (k, big arrows). The large number of small preleptotene spermatocytes form a dense network of BrdU-positive cells in whole mounts (small arrows). The larger BrdU-positive A pale spermatogonia (big arrows) can typically be seen as four-cell clones in whole mount preparations (l). m–o Stage IX of rhesus monkey spermatogenesis (m). No elongated spermatids are present. The acrosome is forming an irregular cap on the early elongating spermatids. BrdU-positive A wall spermatogonia are observed adjacent to the basal lamina (n). BrdU-positive A pale spermatogonia are typically forming four-cell clones in whole mount preparations (o). p–r Stage XII of rhesus monkey spermatogenesis (p). No elongated spermatids are present. The acrosome forms a V-shaped irregular cap on elongating spermatids. BrdU-positive B1 spermatogonia are seen adjacent to the basal lamina (q). B1 spermatogonia are mostly encountered as four- or eight-cell clones in whole mounts (r). In which these cells are located. We had to define criteria for our staining method in cross-sections and whole mounts to allow a correlation of all stages of spermatogenesis showing BrdU-positive spermatogonia with the conventionally used morphological criteria for the stages of the cycle of the seminiferous epithelium of the rhesus monkey (Fig. 3) [1]. Our criteria are as follows.

Stage II. In stage II of spermatogenesis (Fig. 3a), few labeled B1 spermatogonia are observed in sections (Fig. 3b). Acrosin is virtually undetectable. Elongated spermatids are present at the luminal surface of the epithelium. In whole mounts, the labeled B2 spermatogonia are clearly organized as 8- or 16-cell clones, and acrosin staining is not visible (Fig. 3c).

Stage IV. In stage IV of spermatogenesis (Fig. 3, day), numerous labeled B2 spermatogonia are present in sections (Fig. 3e). The acrosome of the round spermatids visualized by acrosin staining appears as a small pale cap with one brighter spot. Clusters of elongated spermatids are embedded at the luminal surface of the seminiferous epithelium. In whole mounts, a loose network of labeled B3 spermatogonia is visible. Most of those spermatogonia are apparently organized in clones of 16 or 32 cells. One 16-cell clone is depicted in Figure 3f. The acrosome appears as a small bright dot.

Stage VI. In stage VI of spermatogenesis (Fig. 3g), many labeled B3 spermatogonia are present (Fig. 3h). Acrosomal caps as visualized by acrosin extend over one third of the round spermatids and show bright tips. The caps are randomly oriented and do not uniformly point to the outer wall of the seminiferous tubule. Elongated spermatids are present at the luminal surface of the seminiferous epithelium. In whole mounts, the labeled B3 spermatogonia appear as a dense network (Fig. 3i). Distinction between individual clones is no longer possible because of the close proximity of each clone to adjacent clones. The acrosome appears as bright spots.

Stage VII. In stage VII of spermatogenesis (Fig. 3j), two types of BrdU-labeled cells appear in the sections, few A pale
spermatogonia in mitotic S-phase and a large number of preleptotene spermatocytes in S-phase before meiosis (Fig. 3k). The acrosin staining reveals an acrosomal cap that extends over half of each round spermatid, with the tip of the acrosome being uniformly oriented toward the outer wall of the seminiferous tubule. Elongated spermatids are absent. In whole mounts, the labeled A pale spermatogonia appear to be organized as two- or four-cell clones (Fig. 3l). Labeled preleptotene spermatocytes are present throughout most of the seminiferous epithelium at this stage of spermatogenesis. Acrosin staining shows the cap-shaped acrosome of the round spermatids.

Stage IX. In stage IX of spermatogenesis (Fig. 3m), few labeled A pale spermatogonia are present in the sections (Fig. 3n). The acrosin staining depicts the V-shaped acrosomal caps of elongating spermatids. In whole mounts, the labeled A pale spermatogonia are organized in two- or four-cell clones (Fig. 3o).

Stage XII. In stage XII of spermatogenesis (Fig. 3p), few labeled B1 spermatogonia are present (Fig. 3q). The acrosin staining clearly visualizes the narrow V-shape of the elongating spermatids. Elongated spermatids are absent. In whole mounts, the labeled B1 spermatogonia appear to be arranged as four- or eight-cell clones (Fig. 3r).

**Spermatogonial Clones**

The number of BrdU-labeled spermatogonia, the number of BrdU-positive spermatogonial clones, and the number of spermatogonia per clone were determined quantitatively for stages VII, IX, and XII of the seminiferous epithelium in whole mount preparations of two adult male rhesus monkeys during the initial phase of spermatogenesis. Whereas a dark spermatogonia were repeatedly shown to have a very low proliferation rate in healthy adult non-human primates and are therefore considered proliferatively inactive reserve stem cells [3, 5]. The cycling A spermatogonium is the A pale. Controversy persists to date whether one or two divisions of A pale spermatogonia occur in macaque testes during the initial phase of spermatogenesis. Whereas a single division of A pale spermatogonia for the rhesus monkey and the vervet monkey was postulated to occur at stages IX–X, two divisions have been described for the stump-tailed macaque occurring at stages VII and IX [1, 3, 8]. The

**DISCUSSION**

Spermatogenesis in non-human primates has been extensively studied in the past [1–6]. Several types of spermatogonia have been described and a series of models explaining the kinetics of spermatogonial expansion have been postulated [8]. However, some controversy persists on this topic, and none of the previous investigations has presented an in-depth evaluation of the long-proposed [1, 2, 13] clonal organization of spermatogonial stem cells at different stages of spermatogenesis.

Using a new approach, we first encountered clones of proliferating spermatogonia in the marmoset (C. jacchus). The seminiferous epithelium in this species resembles the arrangement of the stages of spermatogenesis in the human seminiferous epithelium as several stages are found per tubular cross-section and the stages are not longitudinally separated along the seminiferous tubules [20]. The complex organization made it difficult to establish a strong correlation between spermatogonial clones and specific stages of spermatogenesis. More accessible models for studying the clonal expansion in non-human primates are members of the old-world primate family Cercopithecidae, since all species of macaques studied so far show a longitudinal separation of the stages of spermatogenesis along the seminiferous tubules. The most commonly used criteria for staging (12 stages of the seminiferous epithelium) were established by Clermont and Leblond [1] using the rhesus monkey (M. mulatta), Clermont [2] using the vervet monkey (Cercopithecus aethiops), and Clermont and Antar [3] using the stump-tailed macaque (Macaca arctoides). Other research groups have used identical criteria for the crab-eating macaque (Macaca fascicularis) [6].

A pale Spermatogonial Self-replenishing Division

A pale spermatogonia were repeatedly shown to have a very low proliferation rate in healthy adult non-human primates and are therefore considered proliferatively inactive reserve stem cells [3, 5]. The cycling A spermatogonium is the A pale. Controversy persists to date whether one or two divisions of A pale spermatogonia occur in macaque testes during the initial phase of spermatogenesis. Whereas a single division of A pale spermatogonia for the rhesus monkey and the vervet monkey was postulated to occur at stages IX–X, two divisions have been described for the stump-tailed macaque occurring at stages VII and IX [1, 3, 8]. The
first division of Apale spermatogonia at stage VII is a self-replenishing division of Apale spermatogonia as their numbers increase but no B spermatogonia are formed. Here we present unequivocal evidence for the existence of two divisions of Apale spermatogonia also in the rhesus monkey occurring at stages VII and IX of spermatogenesis. Several reasons might explain why the first division of Apale spermatogonia has not been detected in previous studies. These earlier studies used tissue sections, and their analytical strategies relied on approaches using either 1) counts of colchicine-blocked mitotic metaphases [1] or 2) incorporation of tritiated thymidine and subsequent qualitative and quantitative analysis of radiographs for detection of cells in the S-phase of the cell cycle [2, 3]. For the former, the mitoses of Apale spermatogonia at stage VII of the cycle of the rhesus monkey have apparently been missed due to their very low numbers (see discussion in [2]). For the latter, studying spermatogenesis in C. aethiops, it is not unlikely that the radioactivity emitted from the small number of Apale spermatogonia going through S-phase at stage VII is masked by the high radiation emission from a much larger number of preleptotene spermatocytes going through S-phase before meiosis at the same stage of spermatogenesis. Indeed we obtained the first and most obvious evidence of a first division of Apale spermatogonia at stage VII by analysis of whole mounts. We observed that at stage VII of the cycle of the semiferous epithelium of the rhesus monkey, in addition to BrdU-labeled preleptotene spermatocytes, duplexes and quadruplets of BrdU-positive cells are present with significantly bigger nuclei than the preleptotene spermatocytes. Comparing the size and location of those BrdU-labeled cells with the position and appearance of Apale spermatogonia in conventionally stained sections and whole mounts, we confirmed that these BrdU-positive cells are Apale spermatogonia. Our data reveal that spermatogenesis in the rhesus monkey is initiated with a division of Apale spermatogonia at stage VII of spermatogenesis as in other old world monkeys of the family Cercopithecidae, thus rendering the concept of interspecific differences obsolete.

Spermatogonial Division Modalities and Clonal Size

Spermatogonia in the mammalian testis must 1) maintain their own population and 2) give rise to differentiating cells. Much effort has been spent over several decades to describe the kinetics of this process. More than 60 yr ago, Rolshoven [21] proposed the concept of a so-called differential mitosis. He stated that an unequal spermatogonial mitosis gives rise to one spermatogonium and one spermatocyte. Many subsequent studies [1–3, 8, 14, 22] have addressed this question, but in none of these studies the existence of an unequal mitosis has been proven. For nonhuman primates it can be concluded that although the Apale spermatogonial population gives rise to both new Apale spermatogonia and to B1 spermatogonia, no individual mitotic division of an Apale spermatogonion will result in a different kind of progeny, becoming a mixed pair of an Apale and B1 spermatogonium. Every spermatogonial division always produces two identical daughter cells [1–3, 8].

Looking more into the details of the generation of different types of daughter cells, it becomes obvious that the first division of Apale spermatogonia occurs already from pairs of cells. These pairs have been described to be of clonal origin as they are connected by cytoplasmic bridges [18, 19, 23, 24]. Clermont and Leblond [1] described Apale spermatogonia in the rhesus monkey to be at all stages organized in pairs or quadruplets, which strongly supports the fact that the starting point of spermatogenesis is the synchronized division of a pair or quadruplet of Apale spermatogonia. Using the whole mount approach, we demonstrate here that 1) the total number of labeled spermatogonia doubles from stages VII to IX and again from stages IX to stage XII; and 2) proliferating Apale spermatogonia at stages VII and IX of spermatogenesis are always encountered as at least as pairs, but more frequently as quadruplets; and c) B1 spermatogonia are encountered forming clones of four or eight cells. Although no detailed analysis of the sizes of B2+, B3+, and B4 spermatogonia could be made due to the close juxtaposition of several clones and the resulting difficulties in differing between one clone and the next, the clones appeared to become larger after each mitotic division. Based on our findings (Fig. 4), we propose the following model (Fig. 5): Apale spermatogonia at stage VII of spermatogenesis are organized as functional units of pairs or quadruplets. Most if not all Apale spermatogonia proliferate at stage VII/VIII, resulting in a doubling of the Apale population at stage VIII/IX. The quadruplets or eight-cell clones of Apale spermatogonia resulting from the first mitosis are unstable and separate into pairs or quadruplets of Apale spermatogonia. This explains the doubling of spermatogonial numbers and clones while having a stable number of Apale spermatogonia in each clone. At stage IX the separated clones enter a new mitosis, giving rise to either eight B1 spermatogonia, or separate further into two pairs, giving rise to one quadruplet of Apale spermatogonia and one quadruplet of B1 spermatogonia (Fig. 5). Whereas the four-cell clone of Apale spermatogonia remains mitotically quiescent until the next stage VII, replenishing the original Apale population for this cycle of spermatogenesis, the B1 clones enter the subsequent differentiation steps and mitotically divide into B1 spermatogonia, thus forming always bigger clones after each further B spermatogonial division. Theoretically, 16-cell clones of B2+, 32-cell clones of B3+, 64-cell clones of B4 spermatogonia, and thus 128-cell clones of spermatocytes can be expected following our model, which is in accordance with our qualitative observations. Often the starting point appears not to be a four-cell clone, but a pair of Apale spermatogonia at stage VII of the cycle. Then the numbers of the differentiating germ cells are only half of those stated in the first example. Our data reveal a mixture of two- and four-cell clones at stage VII.

Our model (Fig. 5) is identical with the second of the two proposed models by Clermont and Antar [3]. They presented these two models because from their own data they could not unequivocally determine the correct kinetics of premeiotic germ cell expansion. In the light of our data, we conclude that their model 2 is correct as it is supported by our data presented in Figure 5. In addition to their previous findings, we add the clonal modalities of spermatogonial expansion, thus rendering the model more acceptable. Although our proposed model is in accordance with our novel findings as well as the previously published data [3], further studies are needed to unequivocally confirm the modalities of the clonal expansion in the monkey testis.

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