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Focus on Stem Cells

A revised model for spermatogonial expansion in man: lessons from non-human primates

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Abstract

We have recently described a revised scheme for spermatogonial expansion in non-human primates. We proposed that A_{pale}-spermatogonia act as self-renewing progenitors and premeiotic germ cells are organized and divide as small clones. Here, we are revisiting the model described for man and propose a modified scheme for spermatogonial expansion. Our revised model shows high similarity to the scheme proposed for non-human primates and is in accordance with all previous and present data.

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Spermatogonia are a unique type of adult stem cells

Stem cells are defined from a functional point of view. They have the capability to both self-renew and generate differentiating progeny. Adult stem cells are required for the maintenance of tissue homeostasis (Potten & Loeffler 1990, van der Kooy & Weiss 2000, Watt & Hogan 2000). It is intriguing that adult stem cells are rare in most organs, revealing that it is not their task to generate large quantities of differentiated cells through efficient proliferation. Stem cells are maintained as regenerative reserve with very low mitotic activity under steady-state conditions. The differentiation status of the stem cells is the lowest of all cells of a given organ. Adult stem cells usually give rise to organ-specific progenitor cells, whose task is to generate appropriate types and numbers of cell lineages to maintain short-term tissue homeostasis and thus normal organ functions. The number of different lineages can be numerous as in the bone marrow, can be reduced to very few as in the skin or neuronal tissues, or can be limited to one as in the male germ line.

Adult stem cells are characterized by intrinsic genetic programming. However, their actions are strictly controlled by the microenvironment creating a usually complex stem cell niche. It is obvious from research on many adult stem cells in bone marrow, skin, and testis that the stem cell microenvironment plays a major role for the survival and activation of stem cells (Watt & Hogan 2000, Li & Xie 2005). The niche for spermatogonial stem cells is formed by Sertoli cells, peritubular cells, adjacent germ cells, and components of the basement membrane (Chiarini-Garcia *et al.* 2001, 2003, Chiarini-Garcia & Russell 2002). The population size of Sertoli cells determines the population size of spermatogonial stem cells, showing that the number of Sertoli cells is critical for generation of stem cell niches, and the niches are created prior to the final expansion and functional differentiation of spermatogonial stem cells (Ryu *et al.* 2003, Shinohara *et al.* 2003). In addition, the repopulation pattern of spermatogonia after transplantation supports the presence of a germ cell niche (Ohta *et al.* 2000*a*, 2000*b*). It is intriguing that stem cell niches share common features, structures, and signals among all adult stem cell types and across all species from flies to humans. However, these niches also show a great variation in regard to anatomical and functional arrangements (Li & Xie 2005).

Spermatogonial stem cells represent only a small proportion of spermatogonia, as the latter term defines all diploid germ cells in the mature testis (de Rooij & Russell 2000). In rodents, the spermatogonial stem cells are recognized as a small population of rarely dividing cells, organized as single cells, which self-renew and give rise to differentiating spermatogonia (Huckins 1971, de Rooij *et al.* 1985, Lok *et al.* 2003). Spermatogonia initially form pairs or chains of up to 16 cells known as A_{paired}- or $A_{aligned}$-spermatogonia. Although these aligned cells are synchronized with the spermatogenic wave, they already express several differentiation markers (see de Rooij & Russell 2000). The recognition

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of the rodent stem cells and the pairs, quadruplets, and small chains of its pre-spermatogenic progeny rely on the analysis of whole mounts of seminiferous tubules (Huckins 1978, Huckins & Oakberg 1978). In rodents, spermatogonia enter the cycle of the seminiferous epithelium as A_1 spermatogonia and progress through the highly synchronized steps of spermatogenesis. Since they undergo six more divisions before entering meiotic prophase, the clonal expansion of germ cells is intense and in theory leads to 8192 spermatids arising from one pair of A_{paired} spermatogonia in mice (Ehmcke *et al.* 2006). This enormous premeiotic amplification renders a progenitor step obsolete, and it appears that upstream of the spermatogonial stem cell, no self-renewing progenitor cell is present in the rodent testis. The differences between rodents and primates in regard to a more or less significant amplification of premeiotic germ cells and the incorporation of a progenitor cell to generate sufficient numbers of differentiating progeny in the absence of many amplification steps can be depicted as a schematic model shown in Fig. 1.

Spermatogonial stem cells are integral constituents of the male germ line. They are required for the maintenance of a pool of diploid germ cells, which enter spermatogenesis and guarantee a constant high sperm output. Therefore, in contrast to other adult stem cells in the organism, spermatogonia inherit several unique features: (1) spermatogonial stem cells are at least theoretically immortal. It appears that mouse spermatogonia have indeed a very long lifespan, since serial transplantation leads to both enormous expansion of the spermatogonial stem cell pool and induction of spermatogenesis for several generations (Kanatsu-Shinohara *et al.* 2003, Ogawa *et al.* 2003). This indicates that the aging mechanisms in spermatogonia might be different from other adult stem cells. (2) It is of foremost importance that spermatogonia maintain a high degree of DNA integrity. From an evolutionary point of view, the male genome relies on almost error-free proliferation of germ cells and generation of excellent male gametes. Thus, spermatogonial stem cells hold a key position in driving a balanced evolutionary modification of the genome. This is a specifically demanding task, as spermatogonial stem cells are reserve cells, which can recolonize the seminiferous epithelium following DNA damage. It is striking that recovery of spermatogenesis from radiodepletion leads to healthy gametes after a transient period of poor DNA integrity, indicating a highly efficient repair or cell selection mechanisms in spermatogonial stem cells (Stahl *et al.* 2004). (3) Spermatogonial stem cells generate differentiating progeny with sperm as the only type of final differentiated phenotype. (4) They are the only stem cells in the adult organism which give rise to cells undergoing meiosis.

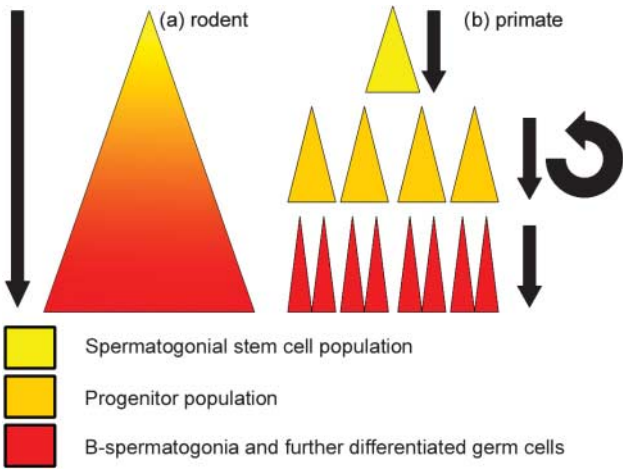

Figure 1 Schematic of the kinetics of germ cell amplification in (a) rodents and (b) primates. Note that in rodents, the starting cell type of spermatogenesis, the A_s-spermatogonia, proliferates regularly, followed by 11 mitotic steps and meiosis, leading to a total of 8192 sperm. In primates, the spermatogonial stem cells (A_{dark}) proliferate only irregularly. The amplification of the germ cell number is achieved by self-replenishing divisions of the progenitor cells (A_{pale}). In the rhesus monkey (five mitotic steps and meiosis), 128 sperm can theoretically be derived from each A_{pale}-spermatogonium, and in the human (two mitotic steps and meiosis), 16 sperm can be derived.

Spermatogonial subtypes in primates

In non-human primates, the expansion of spermatogonial stem cells has extensively been studied in the past (Clermont & Leblond 1959, Clermont 1969, 1972, Clermont & Antar 1973, de Rooij *et al.* 1986, van Alphen *et al.* 1988*a*, 1988*b*, de Rooij *et al.* 1989, Ohmura *et al.* 2003). Clermont proposed the most commonly accepted scheme, stating that two classes of A-spermatogonia are present in the monkey (Clermont & Leblond 1959, Clermont 1969): the A_{dark}-spermatogonia, which he originally defined as 'reserve stem cell'. These cells are recognized by their dark hematoxylin staining in tissue sections as well as by other morphological criteria. He has considered these cells as reserve stem cells, since they show low proliferative activity during normal spermatogenic activity. However, they become proliferatively active during pubertal expansion (Simorangkir *et al.* 2005) of germ cells and following depletion of spermatogonia due to irradiation or toxic exposure (van Alphen *et al.* 1988*b*, 1989). In contrast, A_{pale}-spermatogonia show a less dense nuclear staining and distinctly different morphological criteria. They proliferate continuously during each spermatogenic cycle. Clermont has therefore considered them as 'renewing stem cells'.

Spermatogonia have to produce two different kinds of progeny. Much effort has been spent over several decades to clarify the kinetics of this process. More than 60 years ago, Rolshoven (1941) 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 have addressed this question (Clermont & Leblond 1959, Clermont

1969, 1972, Clermont & Antar 1973, de Rooij *et al.* 1989, Plant & Marshall 2001), but none of these has proven the existence of an unequal mitosis. For non-human primates, it can be stated that, even as the A_{pale}-spermatogonia population gives rise to both new A_{pale}- and B_1-spermatogonia, no single A_{pale}-spermatogonium has been found to result in one A_{pale}- and one B_1-spermatogonium after mitosis. Any spermatogonial division always produces two daughter cells of the same type.

The model of two classes of A-spermatogonia has been widely accepted for many studies using non-human primates. In many of those studies, the A_{dark}/A_{pale} distinction proved to be very helpful to describe changes among spermatogonial populations during development or after experimental interventions, but often the validity of the model was questioned and additional spermatogonial subtypes were proposed. Some authors suggest the existence of a population of $A_{transition}$-spermatogonia. These show histological characteristics which allow to assume that these spermatogonia occupy an intermediate position between the A_{dark}- and the A_{pale}-spermatogonia (Clermont & Leblond 1959, Clermont 1969, Clermont & Antar 1973, Kluin *et al.* 1983, Fouquet & Dadoune 1986, van Alphen *et al.* 1988*a*, Zhengwei *et al.* 1997). The distinction between A_{dark}- and A_{pale}-spermatogonia relies on adequate fixation, embedding, and staining techniques, and on the morphological recognition of delicate histological details. Although most of the spermatogonia can be easily assigned to the one or the other class, in our experience around 25–50% of the spermatogonia in adult primate tissue and an even larger proportion of cells in immature primate testes do not show unequivocal morphological characteristics of either the one or the other subtype. The distinction of the spermatogonial subtypes is open to methodological and individual variations, rendering it highly difficult to compare different studies. However, in the absence of better markers for spermatogonial subtype distinction, it is impossible to propose a revised and fully valid scheme. For the near future, the distinction of these two classes of spermatogonia might be the best, although by far not ideal model for analysis of spermatogonial changes.

A profound knowledge on the exact mechanisms of spermatogonial expansion appears to be as relevant as the exact and reliable classification of spermatogonial subtypes. This raises the question whether the distinction of two A-spermatogonial subtypes represents a valid model for A-spermatogonial function in the primate testis. Is it possible that the distinction of only two subtypes has misled the research community to reduce a more complex spermatogonial physiology to a simple two-class scheme? Is the assumption correct that A_{pale}-spermatogonia do only divide once to give rise to B-spermatogonia? Our present understanding of stem cells in rapidly dividing tissues fits to the concept of two different cell types involved in this process: the slow cycling self-renewing stem cell and the rapidly dividing transient amplifying population of self-renewing progenitors. In this regard, Clermont's original definition of A_{dark}-spermatogonia as a reserve stem cell appears to be accurate and the cells showing an A_{dark}-phenotype might indeed be the true stem cells. The definition of A_{pale}-spermatogonia as renewing stem cells, however, might be misleading as they might not be true stem cells but amplifying progenitors, which inherit the ability of self-renewal for thus far unknown numbers of cell cycles.

A_{pale}-spermatogonia are self-renewing progenitors in the monkey testis

The clonal organization of spermatogonia at different stages of the spermatogenic cycle has been long proposed (Clermont & Leblond 1959, Clermont 1969, van Alphen *et al.* 1988*b*), and recent investigations have given new insight into the expansion of clones of spermatogonia in the rhesus monkey (*Macaca mulatta*; Ehmcke *et al.* 2005*a*, 2005*b*). This species and related members of the old-world primate family Cercopithecidae are excellent models for studying the kinetics of spermatogonial proliferation, as all species of this family studied so far show a clear spatial separation of 12 different stages of the seminiferous epithelium, thus allowing to relate any given clone of spermatogonial stem cells to a given stage of the spermatogenic cycle.

The commonly used staging of non-human primate seminiferous epithelium has first been established by Clermont *et al.* in the rhesus monkey (*Macaca mulatta*; Clermont & Leblond (1959)), the vervet monkey (*Cercopithecus aethiops*; Clermont 1969), and the stump-tailed macaque (*Macaca arctoides*; Clermont & Antar 1973) based on periodic acid-Schiff's reagent-stained sections with hematoxylin counterstaining. Clermont & Leblond (1959) used arbitrary and subjective morphological characteristics of the spermatogonia, the acrosomal cap of the round spermatids, and the presence or absence of elongated spermatids on the luminal surface of the seminiferous epithelium as key factors for determining 12 stages of the rhesus monkey seminiferous epithelium. The dissection of the continuous germ cell development into 12 consecutive stages is a scientific tool to describe consecutive changes occurring during germ cell development. The correlation of spermatogonial mitoses with specific stages allows determination of the kinetics and dynamics of premeiotic germ cell expansion. Recently, we established a novel staining method on whole mounts and sections of rhesus monkey testicular tissue (Ehmcke *et al.* 2005*a*, 2005*b*). When this method was applied to histological tissue sections, it allowed the identification of proliferating spermatogonia at each stage of the seminiferous epithelium. The same staining technique applied on whole mounts of seminiferous tubules revealed the

number and the clonal organization of proliferating spermatogonia at each stage of adult rhesus monkey seminiferous epithelium.

Based on both the spermatogonial counts at different stages of spermatogenesis and the positioning of spermatogonia of the different types to each other, Clermont & Leblond (1959) postulated a model on spermatogonial expansion and differentiation. According to this first model, the A_{dark}-spermatogonia (referred to as A_1-spermatogonia in the cited study) divide at stage X of the spermatogenic cycle to form A_{pale}-spermatogonia (referred to as A_2-spermatogonia in the cited study). The latter divide one full cycle later, again at stage X, to form 2 B_1-spermatogonia. Further divisions – to form 4 B_2-, 8 B_3-spermatogonia, and 16 spermatocytes – are at stages II, IV, and VI of the spermatogenetic cycle respectively. If the A_{pale}-spermatogonia divided only once, to become B_1-spermatogonia, there would be no self-replenishing division of the A_{pale}-spermatogonia, and all the A_{pale}-spermatogonia which become B_1-spermatogonia would have to be replaced by the A_{dark}-population. However, this model had to be modified, as no regular divisions of A_{dark}-spermatogonia were encountered in adult non-human primate (Clermont & Antar 1973, Fouquet & Dadoune 1986, Ehmcke *et al.* 2005*b*, Simorangkir *et al.* 2005). After studying spermatogenesis of another non-human primate, *Cercopithecus aethiops*, Clermont (1969, 1972) revised the early model given for the rhesus monkey. In his second model, he described the A_{dark}-spermatogonia as mitotically quiescent reserve spermatogonia. Furthermore, Clermont described a fourth division of the B-spermatogonia, stating now that the B_1-spermatogonia divide in stage XII, the B_2-spermatogonia in stage II, the B_3-spermatogonia in stage IV, and the B_4-spermatogonia in stage VI. Clermont again described only a single mitosis of the A_{pale}-spermatogonia in *Cercopithecus aethiops*, taking place at stage IX–X of the spermatogenic cycle. Based on the differing result obtained from *Macaca mulatta* and *Cercopithecus aethiops*, Clermont (1969, 1972) proposed species-specific kinetics of spermatogonial expansion. Later, studying the spermatogenesis of a third species of non-human primates (*Macaca arctoides*), Clermont & Antar (1973) first described the occurrence of two mitotic divisions of A_{pale}-spermatogonia during each spermatogenic cycle, occurring at stages VII and IX. In their discussion, they stated that in the rhesus monkey – which they had studied more than a decade earlier – they may have missed an A_{pale}-division in stage VII of the cycle. In fact, the data presented by Clermont & Leblond (1959) on spermatogonial counts at different stages of the spermatogenic cycle seem to indicate that there is a sharp increase in the A_{pale}-spermatogonial population at what the authors refer to as a late stage VIII (the number of A_{pale}-spermatogonia at late stage VIII is about 50% higher than at all other stages). This would make an A_{pale}-spermatogonial self-replenishing division at stage VII highly probable.

We therefore assumed that the A_{pale}-spermatogonial division in stage VII of the spermatogenic epithelial cycle has indeed been missed in both *Macaca rhesus* and *Cercopithecus aethiops* due to technical reasons. The corresponding studies used tissue sections, and analytical strategies relied on approaches using either counts of colchicine-blocked mitotic metaphases or incorporation of tritiated thymidine and subsequent qualitative and quantitative analysis of radiographs for detection of cells in S-phase of the cell cycle (Clermont 1969, Clermont & Antar 1973). In the first study, which evaluated the number of mitotic figures in different stages of the spermatogenic cycle in the rhesus monkey, the mitoses of A_{pale}-spermatogonia at stage VII of the cycle could have been missed due to their very low number (see also discussion in Clermont & Antar 1973). In the second study, in which tritiated thymidine was employed to detect cells in S-phase at different stages of the seminiferous cycle of *Cercopithecus aethiops*, it is not unlikely that the radiation emitted from the small number of proliferating A_{pale}-spermatogonia going through S-phase at stage VII has been masked by the high radiation emission from a much larger number of preleptotene spermatocytes going through S-phase prior to meiosis at exactly the same stage. In addition, Fouquet & Dadoune (1986), and Zhengwei *et al.* (1997) presented data on spermatogonial renewal and spermatogenesis in a fourth species of non-human primates, the crab-eating macaque (*Macaca fascicularis*). The former demonstrated two peaks of mitoses of A_{pale}-spermatogonia in this species during one spermatogenic cycle. They state that spermatogonial differentiation in this species is identical as in *Macaca arctoides*, as described by Clermont & Antar (1973).

Taking into account these previous data, we demonstrated with our new detection approach in whole mounts that at stage VII of the spermatogenic cycle of the rhesus monkey, small clones of A_{pale}-spermatogonia are in S-phase of the cell cycle in addition to more abundant preleptotene spermatocytes which are in prophase of meiosis (Ehmcke *et al.* 2005*a*, 2005*b*). This was an unequivocal proof that in the rhesus monkey, a first division of A_{pale}-spermatogonia occurs at stage VII of the spermatogenic cycle; hence, premeiotic germ cell expansion in this species proceeds in exactly the same way as in most other old-world monkeys of the family Cercopithecidae studied so far.

The A_{pale}-spermatogonia at stage VIII/IX result from the A_{pale}-division at stage VII. Therefore, they should occur at least as pairs. Clermont & Leblond (1959) described A_{pale}-spermatogonia in the rhesus monkey as always to be organized in pairs or quadruplets, and Ehmcke *et al.* (2005*a*, 2005*b*) demonstrated that proliferating A_{pale}-spermatogonia at stages VII and IX are present as pairs or quadruplets, which are of clonal origin and interconnected by cytoplasmic bridges (see also Gondos & Zemjanis 1970, Dym & Fawcett 1971,

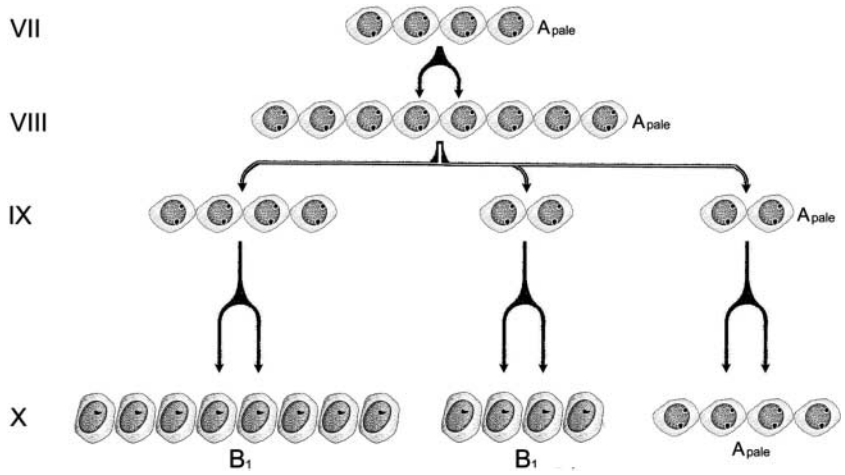

Figure 2 Schematic of the kinetics of proliferation of A-spermatogonia in the rhesus monkey, as proposed by Ehmcke *et al.* (2005*a*, 2005*b*). Note the clonal pattern of spermatogonial organization and the self-renewal of the A_{pale}-spermatogonia.

Huckins 1978). We therefore proposed a new clonal model of spermatogonial expansion in the monkey testis (Fig. 2). A total number of 16 A_{pale}-spermatogonia proliferate at stage VII/VIII of the cycle, resulting in a doubling of the A_{pale}-population at stage VIII/IX. This initial division of A_{pale}-spermatogonia occurs from several pairs and quadruplets which entered S-phase at stage VII. After this initial division, the four- to eight-cell chains split into halves. The doubling of A_{pale}-spermatogonia leads not to the growth of clones but to the doubling of the number of two- and four-cell clones. Thereafter, one-third of these clones remain mitotically quiescent to replenish the A_{pale}-population. The remaining cells continue to proliferate and differentiate into B_1-spermatogonia, which enter mitosis in stage XII of the spermatogenic cycle, resulting in the formation of larger (8–16 cells) clones of B_2-spermatogonia. This model of clonal splitting is in accordance with the ratio of A- and B-spermatogonia described by Clermont & Leblond (1959), Clermont & Antar (1973) and Clermont (1969), and it also takes into account that no unequal divisions are observed. During subsequent divisions, the clones of B-spermatogonia remain connected, thus forming larger clones after each B-spermatogonial division. Theoretically, 16-cell clones of B_2-, 32-cell clones of B_3-, 64-cell clones of B_4-spermatogonia, and thus 128-cell clones of spermatocytes can be expected following this model. This calculation was supported by results presented by Ehmcke *et al.* (2005*a*, 2005*b*), at least up to stage II of the cycle, where 8- and 16-cell clones of B_2-spermatogonia can be detected, supporting the hypothesis of a clonal expansion of the spermatogonia.

Spermatogonial expansion in the human testis

Six stages of the cycle of the seminiferous epithelium were described by Clermont (1963). He also proposed a scheme for spermatogonial expansion in the human testis 40 years ago (Clermont 1966*a*, 1966*b*). Figure 3a

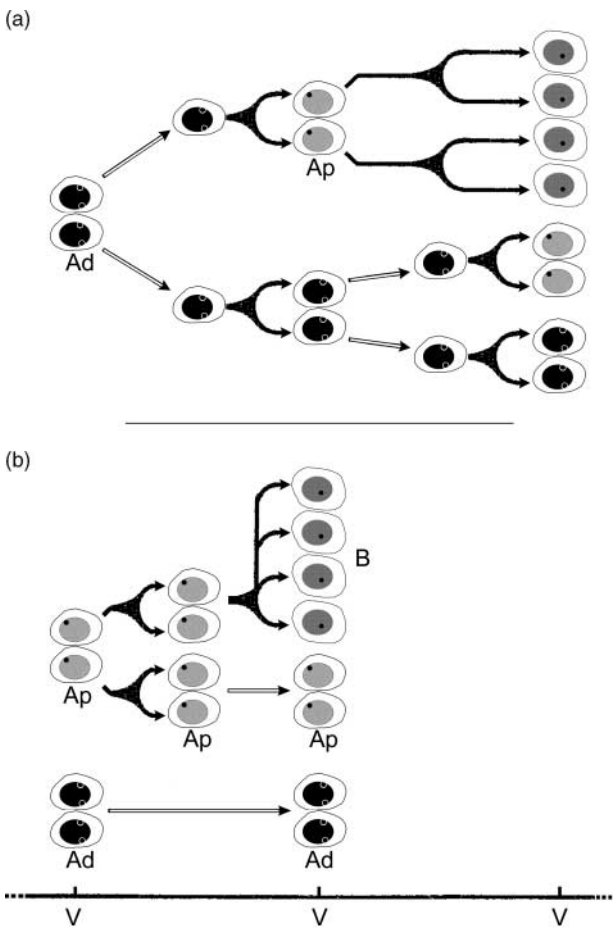

Figure 3 Kinetics of the proliferation of A-spermatogonia in the human. (3a) Schematic of the proliferation model presented by Clermont (1966*a*, 1966*b*). Note that in this model, A_{pale}-spermatogonia do not self-renew; their pool is being replenished by proliferating A_{dark}-spermatogonia. (3b) Schematic of our model of the proliferation and renewal of A-spermatogonia. Note the relative mitotic inactivity of the A_{dark}-spermatogonia and the self-renewal of the A_{pale}-spermatogonia.

shows a revised version of the Clermont scheme taking into account his claim that the regular, synchronized spermatogenic cycle in the human testis is initiated from the pairs of A_{pale}-spermatogonia. This finding corresponds to our recent findings in non-human primates. One of the most significant differences to non-human primates in his model is the claim that spermatogonial expansion in men is limited to only one division of A_{pale}-spermatogonia and that A_{dark}-spermatogonia undergo regular mitotic divisions.

Several studies have proposed alternate types of A-spermatogonia in the human testis and electron microscopic studies were unable to confirm the hematoxylin-based distinction of only two types of A-spermatogonia in the human testis (Roosen-Runge & Barlow 1953, Mancini *et al.* 1960, Schulze 1978). We have revisited the proposed model and started an attempt to transfer our recent findings from the rhesus monkey into the human situation. This has enabled us to propose a modified scheme of spermatogonial expansion in man (Fig. 3b).

In the following, we summarize the most significant facts known for human spermatogonial expansion, judge the validity of these findings, and put these in context with our newly proposed model.

Clermont (1966*a*, 1966*b*) has based his model on the numeric relationship between A_{dark}/A_{pale}/B-spermatogonia and preleptotene spermatocytes to be 1:1:2:4. These relationships are, however, estimated exclusively at spermatogenic stages when these cell types are present. The ratio of A- to B-spermatogonia was encountered exclusively at stages I–II and the ratio of A-spermatogonia to preleptotene spermatocytes at stages III and IV of the seminiferous epithelial cycle. It is important to note that the dissection of the longitudinal process of spermatogenesis into small spermatogenic stages is not as efficient and useful in man when compared with other species. The spermatogenic cycle in the human lasts 16 days (Heller & Clermont 1963). Only six stages of quite long duration are used to dissect this extended process. Stages I and II were combined by Clermont (1966*a*, 1966*b*) to determine the A- and B-spermatogonial counts. When taken together, these two stages make up around 50% of the spermatogenic cycle, which in time corresponds to around 8 days. This long time period easily allows more than one cell division to occur. Clermont's approach and the model derived from his counts, however, did not account (and could not account) for the fact that eventually more than one division occurred during these 8 days. His cell counts are only proportional values and do not reflect any true cell counts obtained at small distinct periods which could have shown absolute changes in cell numbers (Clermont 1966*a*, 1966*b*). In consequence, he could not have detected a potential second division of A_{pale}-spermatogonia occurring during stages I–II. Any model, however, has to fit the ratio of 1:2 between A_{pale}- and B-spermatogonia encountered over the first 8 days of spermatogenic progression. We propose here that Clermont has missed a second division of A_{pale}-spermatogonia occurring in stages I–II of the spermatogenic cycle. We propose that similar to the monkey, a pair or quadruplet of A_{pale}-spermatogonia enters a first division. These clones of cells split and undergo a second division. This second division not only leads to pairs, quadruplets, or eight-cell clones of B-spermatogonia, but also generates a pair or quadruplet of A_{pale}-spermatogonia to replenish the pool of A_{pale}-spermatogonia. If the two A_{pale}-divisions occur with similar kinetics, they provoke a transient increase of A-spermatogonia in stages I–II. Therefore, his counting resulted in a 1:2 ratio of A_{pale}- to B-spermatogonia, which truly might be a 1:3 ratio as outlined in Fig. 2b. With this assumption, our revised model fulfills the prediction made by Clermont (1966*a*, 1966*b*). However, our model also accommodates many other facts which had so far been unresolved. In Clermont's model, A_{dark}-spermatogonia must undergo regular mitotic divisions. Although this has not been conclusively studied in man, A_{dark}-spermatogonia in adult monkeys proliferate rarely. It needs to be shown that this holds true for man, but the likelihood that the A_{dark}-spermatogonia behave very differently is in our view unlikely. The model also allows that A_{dark}-, A_{pale}-, and B-spermatogonia do not form mixed pairs or chains, but always appear in identical arrangements. In our model, no unequal division of any of these premeiotic germ cell types occurs. The decision to enter the differentiation pathway or remain a progenitor is determined by clonal splitting. That no unequal division is needed fits to the observations described by Clermont (1966*a*, 1966*b*). Our model also takes into account the fact that clusters of dividing spermatogonia are mainly four cells, with many also being two-, six-, and eight-cell clones (Clermont 1966*a*). The fact that the regular cycle of spermatogenesis is always initiated from a pair and that clonal splitting occurs favors the occurrence of four cells as a dividing cluster (Ehmcke *et al.* 2005*a*, 2005*b*). However, larger dimensions of mitotic clusters up to 64 (Roosen-Runge & Barlow 1953, Clermont 1966*a*) can occur by synchronous initiation of spermatogenesis in adjacent pairs or quadruplets of A_{pale}-spermatogonia. A synchronous initiation of spermatogonial divisions leading to large clusters of synchronously dividing cells was also observed in the monkey testis (Ehmcke *et al.* 2005*a*, 2005*b*).

In conclusion, taking into accounts our recent findings in the monkey and previously published data, we propose a new scheme for spermatogonial proliferation in the human testis. Spermatogenesis starts with a division of a pair of A_{pale}-spermatogonia at stage I of spermatogenesis. The quadruplets split into pairs after this first division and while three of these pairs differentiate into B-spermatogonia, one remains an

A_{pale}-spermatogonial and replenishes the A_{pale}-spermatogonial pool. A_{dark}-spermatogonia divide only rarely. While several details of this scheme have to be confirmed, we anticipate that most known facts on human spermatogenesis are accommodated with this model. Further support for this concept comes from non-human primates, which show a very similar premeiotic expansion of spermatogonia.

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