Interplay of spermatogonial subpopulations during initial stages of spermatogenesis in adult primates

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Abstract

The spermatogonial compartment maintains spermatogenesis throughout the reproductive lifespan. Single-cell RNA sequencing has revealed the presence of several spermatogonial clusters characterized by specific molecular signatures. However, it is unknown whether the presence of such clusters can be confirmed in terms of protein expression and whether protein expression in the subsets overlaps. To investigate this, we analyzed the expression profile of spermatogonial markers during the seminiferous epithelial cycle in cynomolgus monkeys and compared the results to human data. We found that in cynomolgus monkeys, as in humans, undifferentiated spermatogonia are largely quiescent, and the few engaged in the cell cycle were immunoreactive to GFRA1 antibodies. Moreover, we showed that PIWIL4^{+} spermatogonia, considered the most primitive undifferentiated spermatogonia in scRNA-seq studies, are quiescent in primates. We also described a novel subset of early differentiating spermatogonia, detectable from stage III to stage VII of the seminiferous epithelial cycle, that were transitioning from undifferentiated to differentiating spermatogonia, suggesting that the first generation of differentiating spermatogonia arises early during the epithelial cycle. Our study makes key advances in the current understanding of male germline premeiotic expansion in primates.

Key words: spermatogonial stem cells, spermatogonia, spermatogonial differentiation, primates, cynomolgus monkey, human, spermatogenesis, seminiferous epithelial cycle, PIWIL4, GFRA1, KIT.

Introduction

The daily production of millions of male gametes relies on the biological activity of the spermatogonial stem cells (SSCs), a rare cell population of the basal compartment of the seminiferous tubules. The SSCs can both self-renew and give rise to committed progenitors that, after a limited number of divisions, give rise to differentiating spermatogonia that eventually enter meiosis. The balance between SSC self-renewal and differentiation is essential to maintain the male germline throughout life.

Based on their morphological appearance, primitive spermatogonia have been called *undifferentiated spermatogonia* (undiff-SPG) whereas more advanced spermatogonia are collectively called *differentiating spermatogonia* (diff-SPG) (de Rooij and Russell, 2000). In all mammals, the kinetics of spermatogonial expansion in undiff-SPG versus diff-SPG are inherently different (Boitani et al., 2016). Undiff-SPG divide asynchronously during the seminiferous epithelial cycle and can be detected in all stages of the cycle of the seminiferous epithelium. By contrast, the successive generations of diff-SPG divide in a synchronized fashion during specific stages of the epithelial cycle. Consequently, each generation of diff-SPG can be found during specific stages of spermatogenesis (de Rooij and Russell, 2000). Diff-SPG are produced from undiff-SPG only once during each cycle of the seminiferous epithelium, that the transition is a cyclic event, occurring during specific stages and requiring retinoic acid, at least in mice (Endo et al., 2015). In humans, the first generation of diff-SPG is derived from undiff-SPG without cell division (Di Persio et al., 2017), whereas in monkeys, the first generation of diff-SPG is thought to be generated by division of undifferentiated progenitors (Apale) at stage VIII-IX (Clermont, 1972). Given that spermatogenesis is a highly conserved process in primates, the seemingly large difference between these two modes suggests that a unifying model of spermatogonial amplification in primates has not yet been identified.

During recent years, the development of single-cell RNA sequencing (scRNA-seq) technology has advanced our understanding of the spermatogonial compartment. scRNAseq analysis of adult testes of different mammalian species has revealed the presence of several spermatogonial clusters and subclusters characterized by specific molecular signatures and differentially expressed genes, further stratifying the undiff-SPG and diff-SPG. Computational analyses have unveiled complex transitions among subclusters occurring during renewal, differentiation and meiotic commitment (Di Persio et al., 2021; Guo et al., 2018; Hermann et al., 2018; Lau et al., 2020; Sohni et al., 2019). In these studies, the different clusters were identified based on the expression of well-established SPG markers such as MAGEA4, UCHL1, GFRA1, UTF1 and KIT (Boitani et al., 2016). In primates, the MAGEA4 protein is a general marker for all spermatogonia, whereas UCHL1, GFRA1 and UTF1 are heterogeneously expressed by undiff-SPG, while KIT is a marker for diff-SPG. The relative expression pattern of UCHL1, GFRA1, UTF1 and KIT is conserved in rodent spermatogonia, suggesting their important functions in spermatogonial development in mammals (Boitani et al., 2016). Interestingly, the transcriptome alignment of SPG clusters in mice, humans and macaques revealed six common SPG molecular stages, showing a conserved transcriptional profile across species with several markers commonly expressed in the three species, such as PIWIL4, ID4, GFRA1, LIN28, and others (Shami et al., 2020). Among them, PIWIL4, a piRNA-binding protein belonging to the Argonaute family of proteins, is a specific marker for the undiff-SPG state in the three species, suggesting a relevant function in mammalian SSCs (Shami et al., 2020; Wang et

al., 2018). In rodents, the PIWIL4 ortholog of MIWI2, which is expressed in a subset of NGN3-expressing undifferentiated spermatogonia, is essential for efficient testicular regeneration after injury (Carrieri et al., 2017). In humans, infertile cryptozoospermic patients show an increased number of PIWIL4-expressing spermatogonia compared to fertile men (Di Persio et al., 2021).

Whether the different SPG clusters identified by scRNA-seq in primates harbor distinctive physiological functions is unclear (Tan and Wilkinson, 2020). Some of the undiff-SPG clusters may represent hierarchically arranged cell generations, whereas other SPG clusters represent equipotent cellular states whose transcriptional profiles vary in relation to cell cycle, microenvironment, or the epithelial cycle. More importantly, it is still unknown whether all the different SPG clusters can also be identified at the protein level and whether any overlap exists between different SPG subsets in terms of protein expression. In human testis, the rare mitotic undiff-SPG are part of the GFRA1^{high}/UTF1^{neg} SPG subset, suggesting that the expression of GFRA1 marks the mitotic SSCs (Di Persio et al., 2017). However, as indicated via pseudotime trajectory analysis, GFRA1 expression may instead define an SPG state more advanced along the developmental trajectory (Guo et al., 2018; Sohni et al., 2019; Wang et al., 2018).

To gain more insight into spermatogonial expansion in primates, we evaluated the protein expression of selected SPG/cell cycle markers using a co-localization approach. Expression analysis was performed by immunofluorescence on intact seminiferous tubules and confocal microscopy analysis ("whole mount"). By this approach, the topographical arrangement of the spermatogonial clones on the basal lamina of the seminiferous tubules can be studied, providing a further layer of three-dimensional information that is lost in histological sections. We used the cynomolgus monkey (*Macaca fascicularis*) as a model and compared the results with those in humans. In cynomolgus monkey, the 12 stages of the cycle of the seminiferous epithelium are separated along the tubules and occupy large areas of the basal lamina allowing one to correlate spermatogonial clones to the stages of the seminiferous epithelial cycle (Wistuba et al., 2003). By contrast, in humans the 12 epithelial stages occupy only small areas of the tubule basal lamina and adjacent areas are in randomly different epithelial stages hindering the assignment of SPG clones to specific stages of the cycle (Muciaccia et al., 2013). Therefore, the use of cynomolgus monkeys has the advantage of allowing us to analyze the kinetics of spermatogonial proliferation and differentiation during the cycle of the seminiferous epithelium.

In this study, by using marker combinations, we quantified the proportion of undiff-SPG and diff-SPG in cynomolgus monkeys, then we focused on the undiff-SPG that include SSCs and transient-amplifying progenitors. Using well-established and novel markers we defined the distribution and the proliferative activity of the undiff-SPG subsets throughout the cycle of the seminiferous epithelium in cynomolgus monkey testis. We next focused on the key transition between undiff-SPG and diff-SPG and analyzed the progression of the diff-SPG during the cycle of the seminiferous epithelium. Finally, we compared results obtained in cynomolgus monkeys with those in humans, allowing us to propose a novel unifying model for spermatogonial amplification in primates.

Results

Classification of the spermatogonial compartments in the cynomolgus monkey based on marker combinations

To characterize the ratio between undifferentiated and differentiating SPG in the cynomolgus monkey, we quantified the relative proportion of MAGEA4$^+$/UCHL1$^+$ and MAGEA4$^+$/KIT$^+$ SPG (Fig. 1A, B). MAGEA4 is a general marker of SPG, UCHL1 is a marker of undiff-SPG (i.e., Adark and Apale), while KIT is a marker of diff-SPG (i.e., B spermatogonia) (Aubry et al., 2001; Schrans-Stassen et al., 1999; Tokunaga et al., 1999). Analyses revealed that in cynomolgus monkey testicular tissue, less than 30% of SPG were UCHL1$^+$ (undiff-SPG) and about 70% of SPG were KIT$^+$ (diff-SPG) (Fig.1C). The analysis of the fluorescence intensity values at the single-cell level showed that during spermatogonial progression, MAGEA4 protein levels decreased while KIT levels increasd (Fig. S1). Notably, in all samples, around 3% of SPG was immunoreactive for UCHL1 and KIT antibodies (Fig. 1C). Using the fluorescence intensity values for MAGEA4, UCHL1 and KIT of single SPG, we performed a principal component analysis (PCA) followed by cluster analysis to gain insight into SPG cluster distribution based on these markers (Fig. 1D, S2). In line with the quantitative data, this analysis revealed the presence of three distinct SPG populations: MAGEA4$^+$/UCHL1$^+$/KIT$^-$ (cluster 1); MAGEA4$^+$/UCHL1$^+$/KIT$^+$ (cluster 2); and MAGEA4$^+$/UCHL1/ KIT$^+$ (cluster 3) (Fig. 1E). Importantly, the centroids of the three samples in all the PCAs were located in close proximity indicating low variation among the different biological replicates (Fig. S2).

These data indicate that alongside undiff-SPG (cluster 1) and diff-SPG (cluster 3) there is a small SPG population (3% of SPG) expressing intermediate levels of MAGEA4, UCHL1 and KIT (cluster 2) when compared to the other clusters.

Distribution of undifferentiated spermatogonia subsets during the seminiferous epithelial cycle of cynomolgus monkey

In humans, undiff-SPG are highly phenotypically heterogeneous, as shown by marker analysis revealing the presence of different subsets (Di Persio et al., 2017; Sohni et al., 2019). To assess whether this protein-level heterogeneity is also present in nonhuman primates, we analyzed the expression of known human SPG markers such as PIWIL4, UTF1 and GFRA1 (Fig. 2A, D, G). In cynomolgus monkeys, we found that a large proportion (about 67%) of UTF1$^+$ SPG was immunoreactive to the PIWIL4 antibody (constituting UTF1$^+$/PIWIL4$^+$ SPG), 30% was UTF1$^+$/PIWIL4$^-$ SPG, and no proportion (0%) was UTF1$^-$/PIWIL4$^+$ SPG (Fig 2A, B). Conversely, GFRA1$^+$ and PIWIL4$^+$ SPG had a small amount of overlap, with less than 10% of SPG immunoreactive to both antibodies (Fig. 2D, E). Finally, results showed that around 70% of SPG were UTF1$^+$/GFRA1$^-$, around 30% of UTF1$^+$ SPG also co-expressed GFRA1, and we found no UTF1$^-$/GFRA1$^+$ SPG (Fig. 2G, H). Fluorescence intensity analysis showed that GFRA1$^+$ SPG expressed low levels of UTF1 compared to UTF1$^+$/GFRA1$^-$ SPG (Fig. S3).

Subsequently we analyzed the distribution of the identified SPG subsets during the cycle of the seminiferous epithelium. To this end, intact tubules were concomitantly stained for SPG markers and for ACROSIN to detect the stages of the seminiferous epithelium, as described in the Material and Methods section (Fig. 2C, F, I). The different SPG subsets were always detected in all groups of stages with nonsignificant differences in their stage-distribution (Fig. 2 C, F, I). Interestingly, the relative proportion of SPG subsets for each marker combination was generally maintained, with some exceptions. For instance, even though the PIWIL4$^+$/GFRA1$^-$ SPG were generally more abundant compared to PIWIL4$^-$/GFRA1$^+$ SPG,

their numbers were almost equal at stages VIII-IX and XII-I (Fig. 2E, F). Again, even though UTF1^{+}/GFRA1^{-}SPG were more abundant than UTF1^{+} /GFRA1^{+} SPG, in the second half of the cycle (i.e.; from stage VIII onward) their abundances were similar (Fig. 2 H, I).

These data suggest that the heterogeneity of undiff-SPG is not directly correlated to the stages of the seminiferous epithelial cycle.

Proliferative activity of undifferentiated spermatogonia in cynomolgus monkey testis

The molecular phenotype of proliferating SPG in nonhuman primates remains largely unknown. To uncover the proliferative activity of SPG subsets, we analyzed the MKI67 immunoreactivity in the different SPG subsets, over the cycle of the seminiferous epithelium with a particular focus on undiff-SPG (Fig. 3). Interestingly, PIWIL4^{+} SPG never stained positive for MKI67 (Fig. 3A), while among other undiff-SPG subsets, MKI67 immunoreactivity was detected only in UCHL1^{+}/GFRA1^{+} SPG but never in UCHL1^{+}/GFRA1^{-} SPG (Fig. 3B, C). The proportion of GFRA1^{+} SPG engaged in the cell cycle was around 35% (Fig. S4A) and these cells were localized in all epithelial stages with an increase at stages VI-XI (Fig. 3D). Finally, among UTF1^{+} SPG, only those co-expressing GFRA1 were engaged in the cell cycle (Fig. S4B).

Next, we investigated the cell cycle kinetics of GFRA1^{+} SPG by treating the seminiferous tubules *in vitro* for two hours with an EdU (5-ethynyl-2´-deoxyuridine) pulse to detect the S phase cell cycle progression (Pereira et al., 2017) (Fig. 3E). Around 14 % of GFRA1+ SPG were labelled by EdU and they were found in all stages, with two peaks at stage IV and IX (Fig. 3F, Fig. S4C). To clarify the topographical arrangement of proliferating SPG, we counted how many cells made up EdU$^{+/}$GFRA1^{+} clones; spermatogonia belonging to the same clone were identified using the criterion of the intranuclear distance to assign cells to clones (Huckins, 1971). GFRA1^{+} SPG in the S phase were mostly arranged as single-cell clones and clone pairs with very few 4-cell clones. They showed a large nuclear size, around 12 µm (Fig. S4C). Early in the cycle (stage II-III) they were arranged as single-cell clones, whereas from stage IV to IX, they were arranged as single-cell clones and clone pairs; 4-cell clones were found only in the second half of the cycle, from stage VII to stage XII (Fig. 3G, H).

Altogether these data indicate that among undiff-SPG, only those immunoreactive to GFRA1 antibodies and not PIWIL4 antibodies, are engaged in the cell cycle. The fraction of proliferating GFRA1^{+} SPG is distributed in all epithelial stages, and they proliferate mostly as single cells and pairs of SPG.

Identifying of the early diff-SPG in cynomolgus monkey

To elucidate the transition between undifferentiated and differentiating SPG, we analyzed the different B spermatogonia generations during the seminiferous epithelial cycle using KIT to detect differentiating SPG and MAGEA4 to detect all SPG (Fig. 4A, S5A). As expected from the previous analysis (Fig. 1), the qualitative analysis of data pointed to the presence of three different populations: MAGEA4^{high}/KIT^{-} SPG, (Fig. 4A, inset i); MAGEA4^{medium}/KIT$^{med/low}$ SPG (Fig. 4A, inset ii) and MAGEA4^{low} /KIT^{high} SPG (Fig. 4A, inset iii). Interestingly, we noted that MAGEA4^{medium}/KIT$^{med/low}$ SPG were characterized by larger nuclei compared to the other SPG. The PCA analysis confirmed the presence of the three different SPG clusters (Fig. 4B; S2). Moreover, in line with the qualitative evaluation, KIT-expressing SPG were divided into two clusters: MAGEA4^{low} /KIT^{high} SPG with a smaller nuclear size (9.1±0.8 µm) (cluster 3) and MAGEA4^{medium}/KIT$^{med/low}$ SPG characterized by a larger nuclear size (12.8±1.8 µm) (cluster 2) (Fig. 4B, C).

We, therefore, investigated the stages of the seminiferous epithelial cycle in which these two SPG populations would be detected. The MAGEA4^{low}/KIT^{high} SPG were present in all the stages (Fig. 4D). During the cycle, their number progressively increased, paralleled by a constant reduction in their nuclear size (Fig. 4D, E). Interestingly, the MAGEA4^{medium}/KIT$^{med/low}$ SPG with large nuclei were found only from stage II to stage VII intermingled with the other generations of B spermatogonia (Fig. 4D). Their nuclear size remained constant from stage II-VII (Fig. 4E).

Considering our previous findings of a small SPG population expressing intermediate levels of MAGEA4, UCHL1 and KIT (Fig. 1), we hypothesized that the large MAGEA4^{medium}/KIT$^{med/low}$ SPG population represented a population of spermatogonia in transition between the undifferentiated and differentiating compartment. To directly test this hypothesis, we performed triple immunofluorescence for UCHL1, KIT and acrosin to analyze the stage distribution of positive cells (Fig. 4F, S5B). As expected, we found UCHL1^{+}/KIT^{-} SPG (undiff-SPG), UCHL1^{-}/KIT^{+} SPG (diff-SPG) and a small population of UCHL1^{+}/KIT^{+} SPG (Fig. 4G) Notably, the latter showed a large nucleus as well as low/medium UCHL1 and KIT expression levels (Fig. 4F), and these cells were found from stage III to VII, before spermiation and prior to the appearance of diff-SPG (Fig. 4G, S5C). Lastly, we investigated whether the large MAGEA4^{medium}/KIT$^{med/low}$ SPG were engaged in the cell cycle (Fig. 4H, S5D). Almost all KIT^{+} SPG were MKI67^{+}, with only 3% being MKI67^{-} (Fig. S5E). Strikingly, quiescent SPG were KIT$^{med/low}$ with a larger nuclear size (around 12 µm) compared to KIT^{high}/MKI67^{+} SPG (Fig. 4H), and they showed a stage-specific distribution, being detected from stages III to stage VI-VII (Fig. 4I). Interestingly, this population disappeared at stage VIII concomitantly with the appearance of the KIT^{high} SPG population with a similar nuclear diameter.

These results strongly suggest the presence of an early differentiating SPG population detectable from stage III to stage VI-VII in a quiescent state, characterized by a medium level of MAGEA4, UCHL1 and KIT expression and a large nuclear size. We conclude that this population is committed to differentiation and gives rise to the first generation of diff-SPG without cell division.

The cell cycle kinetic of the first generation of B spermatogonia in cynomolgus monkey

In mice, the first generation of diff-SPG (A1) gradually derive from quiescent undiff-SPG (Aal). During this transition Aal grow in size, acquire KIT expression then differentiate into A1 and enter S-phase (Kluin and de Rooij, 1981; Schrans-Stassen et al., 1999).

To pinpoint the stage of the cycle at which the first generation of differentiating SPG undergo S phase in the cynomolgus monkey, intact seminiferous tubules were pulsed with EdU for two hours in vitro and then co-stained to detect EdU, KIT and ACROSIN (Fig. 5A). As expected, EdU^{+}/KIT^{+} SPG were present in almost all the stages of the cycle (Fig. 5B). Interestingly, the first cohort of EdU^{+}/KIT^{+} SPG were detected at stage VI-VII, along with primary spermatocytes in S-phase (Fig. 5C), with a labelling index of around 60% (Fig. S6A). Although preleptotene spermatocytes stained positive for KIT, they could be discerned from KIT^{+} SPG due to their nuclear morphology (smaller and highly condensate nuclei) and their lower level of KIT expression (S6A-E). The first cohort of EdU^{+}/KIT^{+} SPG showed a large nuclear size (12.5±0.2 µm) (Fig. S6F) and were arranged mostly as 2- or 4-cell clones (Fig. 5C, D). Finally, we determined the stage of the cycle at which the first generation of B spermatogonia divided to originate B2 SPG. Since differentiating SPG divide synchronously, they generate several mitotic peaks at specific stages of the cycle. Therefore, we stained intact tubules for KIT to detect B SPG, with PHH3 to detect mitosis, and with ACROSIN to

distinguish the epithelial stage (Fig. 5E). The first peak of mitosis was detected at stage VIII, followed by peaks at stages XI, II, IV, V. (Fig. 5F).

These data show that the first generation of B SPG undergo S phase along the preleptotene spermatocytes at stage VI-VII and divide at stage VIII to generate B2. Therefore, the number of B SPG generations should be now considered five, not four as previously described. The mitotic peaks of B2, B3, B4 and B5 SPG occur at stage XI, II, IV and V/VI respectively.

Comparative analysis in human spermatogonial compartment

The spermatogonial compartments in human and nonhuman primates share important similarities (Boitani et al., 2016). We therefore investigated whether some of our novel relevant findings obtained in nonhuman primates could be extended to humans (Fig. 1A).

We had previously shown that among the different undiff-SPG subsets, only those expressing GFRA1 were engaged in the cell cycle, suggesting that this fraction likely includes the spermatogonial stem cells (Di Persio et al., 2017). More recently, scRNA-seq suggested that GFRA1 is not expressed in the most primitive undiff-SPG, which are instead identified by UTF1 and PIWL4 expression (Guo et al., 2018; Sohni et al., 2019). At present, however, the proliferative index of PIWIL4-expressing cells in human is unknown. As in cynomolgus monkeys, in humans, too, an overlap in the immunoreactivity for GFRA1 and PIWIL4 was found to be limited to around 16% of SPG, and most PIWIL4$^+$ cells did not stain for GFRA1 (Fig. 6A, B). The absence of co-staining for MKI67 and PIWIL4 indicates that all the PIWIL4$^+$ SPG in humans are quiescent, as they are in cynomolgus monkey (Fig. 6C).

Our results indicate that both in human and nonhuman primates, PIWIL4$^+$ SPG represent a subset of quiescent SPG, and the proliferative activity in the undifferentiated spermatogonial compartment is driven by GFRA1$^+$ SPG.

As another interesting finding of this study was the novel identification of an early differentiating SPG in cynomolgus monkeys, we therefore wondered whether a similar SPG population would also be detectable in humans. Interestingly, we had already described the presence of a small fraction of UCHL1$^+$/KIT$^+$ SPG, suggesting the presence of an intermediate population between undifferentiated and differentiated SPG also in humans (Di Persio et al., 2017). To directly quantify this SPG fraction, we performed a triple staining for UCHL1, KIT and MAGEA4 (Fig. 6D). We found that UCHL1$^+$/KIT$^+$ SPG were very few (around 5% of all SPG), with low expression levels of KIT and UCHL1 but a medium expression level of MAGEA4 (Fig. 6D, E). To further characterize this intermediate SPG population, we evaluated the expression of MAGEA4, KIT and the nuclear diameter (Fig. 6F). As we found for cynomolgus monkeys, we found that MAGEA4 and KIT expression levels in humans correlated inversely (Fig. S7). The PCA analysis confirmed the presence of the three different SPG clusters: MAGEA4^{high}/KIT$^-$/large nuclei (cluster 1); MAGEA4^{medium}/KIT$^{med/low}$/large nuclei (cluster 2); MAGEA4^{low} /KIT^{high}/small nuclei (cluster 3) (Fig. 6G, H; S2).

These data suggest that, as we found for cynomolgus monkeys, in human seminiferous tubules, there is an intermediate population in transit from undifferentiated and differentiating spermatogonia characterized by large nuclei and intermediate levels of MAGEA4, UCHL1 and KIT.

Figure 6I shows a schematic comparison of the SPG subsets in cynomolgus monkeys and humans, using data from the current study and previously published studies (Di Persio et al., 2017).

Discussion

In this study, we performed the first evaluation of the amplification and differentiation of the spermatogonial compartment in mature cynomolgus monkeys based on marker protein expression instead of classical histological evaluation. Using qualitative and quantitative immunofluorescence, we generated a dataset that advances our understanding of the expansion of the spermatogonial compartment in primates. Our results reveal that, as previously demonstrated in human testis, in cynomolgus monkeys the undiff-SPG are largely quiescent, and immunoreactivity toward the GFRA1 antibody defines the only undiff-SPG engaged in the cell cycle (Di Persio et al., 2017). Moreover, we show that PIWIL4$^+$ SPG, considered the most primitive undiff-SPG in scRNA-seq studies, are quiescent (Di Persio et al., 2021; Lau et al., 2020; Sohni et al., 2019). We also provide evidence that in cynomolgus monkeys, the first generation of differentiating spermatogonia does not arise by mitotic division of undifferentiated progenitors (A_{pale}) but by differentiation, therefore challenging the current model of spermatogonial expansion in primates (Ehmcke et al., 2005a). Our findings also indicate that in cynomolgus monkeys there are five generations of differentiating spermatogonia, not four as previously described (Fouquet and Dadoune, 1986).

To gain insight into the arrangement of the undiff-SPG compartment, we employed UCHL1 as a general marker along with selected markers such as GFRA1, UTF1 and PIWIL4. In line with our previous results obtained in humans, we found that in cynomolgus monkeys the undiff-SPG show a high phenotypic heterogeneity in terms of protein expression (Di Persio et al., 2017). Our data are also in line with a high-resolution scRNA-seq analysis of cells from adult cynomolgus monkey testis (Lau et al., 2020). However, since there is no evidence that the mRNA expression pattern matches with the protein expression pattern, it is difficult to establish a direct correlation between the clusters identified by scRNA-seq and the subsets identified by marker analysis. A notable difference between humans and cynomolgus monkeys is that in humans GFRA1 is expressed in a larger proportion of undiff-SPG than in cynomolgus monkeys (80% vs. 40%, respectively). The protein GFRA1 is the co-receptor for GDNF, one of the best-characterized niche components regulating SSCs (Makela and Hobbs, 2019; Meng et al., 2000). Thus, the species' differing proportion of the GFRA1$^+$ subset within the undiff-SPG can be attributed to species-specific transcriptional profile differences or to different rates of mRNA/protein stability (Lau et al., 2020; Shami et al., 2020). Interestingly, we found that the distribution of SPG subsets does not fluctuate significantly between stages, suggesting that the phenotypic heterogeneity in primitive spermatogonia is not directly correlated to the epithelial stages.

In the present study, we provide evidence that in both cynomolgus monkeys and humans, spermatogonia immunoreactive to PIWIL4 antibodies are quiescent. Moreover, in line with previous data, we show that among the undiff-SPG, only GFRA1$^+$ SPG are engaged in the cell cycle, suggesting that in adult primates, GFRA1 is required to trigger spermatogonial proliferation and expansion (Di Persio et al., 2017). It has been recently proposed that in the adult mouse, quiescent and activated SSCs interconvert upon modulation of the MAPK/AKT signaling pathway (Suzuki et al., 2021). Whether a similar mechanism relates the PIWIL4$^+$ and GFRA1$^+$ SPG subsets in primates remains unknown. Yet, the observation that PIWIL4 mRNA but not GFRA1 mRNA is expressed in embryonic and fetal-infant male germ cells suggests that an alternative mechanism must be in place for prespermatogenic germ cell expansion (Guo et al., 2021).

We found that only 15% of undiff-SPG express MKI67, showing that, as in humans, most of the undifferentiated spermatogonia in cynomolgus monkeys are quiescent (Di Persio et al., 2017). Taking advantage of cynomolgus monkeys linear distribution of stages along the seminiferous tubules, we analyzed the proliferation rate and clonal arrangement of proliferating undiff-SPG during the epithelial cycle. Our results indicate that undiff-SPG slowly divide at stages XII-III and increase their MKI67 proliferation rate at stages IV-XI. In the first part of the cycle, they are arranged only as single-cell clones and thereafter as single-cell clones, clone pairs and rarely as 4-cell clones. This suggests that upon division, GFRA1$^+$ SPG can generate both single and chained clones. Clones of 4 cells in the S phase were detected only in the second half of the epithelial cycle, in line with the situation in mice, where the longer chains of GFRA1$^+$ SPG (Aal8) are only found in stages IX–XI (Grasso et al., 2012).

The large body of data available about the kinetics of spermatogonial self-renewal and differentiation in primates has been obtained by the classical nuclear recognition of A_{pale} (Ehmcke and Schlatt, 2006; Hermann et al., 2009; Plant, 2010). However, the lack of specific spermatogonial markers could have hindered the interpretation of data in early studies (Clermont and Antar, 1973; Ehmcke et al., 2005a; Ehmcke et al., 2005b; Fouquet and Dadoune, 1986; Simorangkir et al., 2009). Here, we show that in the second half of the epithelial cycle, the S phases of undiff-SPG and diff-SPG largely overlap. Importantly, at stage VI-VII, EdU$^+$/KIT$^+$ and EdU$^+$/GFRA1$^+$ SPG show similar nuclear dimensions and comparable clonal sizes being arranged mostly as 2-cell or 4-cell clones. Therefore, EdU-labeled undiff-SPG and diff-SPG cannot be discriminated, even by the most experienced observers, unless specific markers are employed for their recognition in co-staining experiments.

Based on marker analysis, we provide evidence that the first generation of diff-SPG is derived from a small SPG population that emerge during the first half of the cycle. This novel SPG population i) expresses low levels of UCHL1 and medium/high levels of MAGEA4, ii) is quiescent and iii) is characterized by low levels of KIT and large nuclear diameters. We propose that these cells represent early diff-SPG that are in transition to become B1 SPG. During this transition, they re-enter cell cycle, undergo S phase along with the S phase of preleptotene spermatocytes (stages VI-VII) and divide at stage VIII to generate B2. This transition is similar in rodents, where the first generation of diff-SPG (A1 SPG) are generated by transformation of undiff-SPG ($A_{aligned}$ SPG) without cell division (de Rooij and Russell, 2000; Nakagawa et al., 2010; Schrans-Stassen et al., 1999). Also in humans, we found a population of spermatogonia characterized by intermediate levels of MAGEA4, UCHL1 and KIT expression and large nuclear sizes. Unfortunately, since in humans the epithelial stages occupy only a small area of the tubule basal lamina and adjacent areas are in randomly different epithelial stages, we could not ascertain the stage distribution of this specific SPG population. However, it is tempting to speculate that this population may represent early diff-SPG in transition to become B1. The transition between undiff-SPG to diff-SPG is a key step during spermatogenesis that drives the synchronous initiation of spermatogenesis in both immature and adult testis (de Rooij and Russell, 2000). In mice, this transition is regulated by retinoic acid (RA) (Endo et al., 2015; Zhou et al., 2008) but at present it is not known whether RA is involved in this transition in primates. Because scRNA-seq analyses indicate that STRA8, a major RA target gene involved in the transition between undiff-SPG to diff-SPG in mice (Endo et al., 2015), is not expressed in prepuberal human testis nor in more

primitive SPG subsets of adult humans and monkeys, this key passage during spermatogenesis in primates may be controlled by alternative pathways (Guo et al., 2020; Shami et al., 2020). For example, *in vitro* culture experiments suggest that AKT signaling is involved in human SPG differentiation (Tan et al., 2020).

Based on the proliferative status of SPG and the expression of PIWIL4, GFRA1 and UTF1, we propose a tentative model for the relationship among subsets during spermatogenesis in adult primates, also taking into consideration the complex transition highlighted in scRNA-seq studies (Di Persio et al., 2021; Lau et al., 2020; Sohni et al., 2019) (Fig. 7). We propose that GFRA1^{+} SPG are the only cells able to self-renew and to generate the other SPG subsets. Following mitotic amplification, daughter cells may return to quiescence and start to increase UTF1 and/or PIWIL4 expression, or they may move further toward differentiation (differentiation-primed SPG). We also speculate that quiescent SPG may acquire GFRA1 expression and re-enter the cell cycle or move toward differentiation with no further clonal amplification, both in physiological conditions and after an inflicted germ cell loss.

Because of the lack of genetic approaches to perform lineage tracking or loss-/gain-of-function studies in primates, experimental validation of this model is currently out of reach. Nevertheless, this model may represent a starting point for future studies, including those addressing the causes of male infertility.

Limitations of the study

Based on marker expression and morphometric analysis, we have identified a novel subset of spermatogonia, detectable from stage III to stage VII of the seminiferous epithelial cycle, that we proposed to be early differentiating spermatogonia. The potential of this novel subset will remain untested until it is possible to perform in vivo or in vitro studies where the fate of this spermatogonial subset can be tested. Thus, our data do not preclude the presence of alternative fate models.

Materials and Methods

Testicular biopsies

Monkey testicular tissue samples from cynomolgus monkey (*Macaca fascicularis*) were obtained from the institutional breading facility of WWW Münster, Germany. Material from six mature animals was used for the study (Table 1). Ethical approval for the use of cynomolgus monkey (license # 39.32.7.1) was obtained according to German federal law on the care and use of laboratory animals. Each testicular biopsy was divided in two portions, one for histology analysis and the other for short in vitro pulse with EdU (5-ethynyl-2´-deoxyuridine) and whole mount staining (see below). For histological analysis, samples were fixed overnight at 4°C in Bouin fixative, washed, dehydrated, and routinely embedded in paraffin. To perform histological analysis, 5μm sections were cut and stained with Mayers Hematoxylin and Eosin (Sigma-Aldrich). All the samples included in this study showed well-preserved testicular tissue and a normal spermatogenesis.
Human testicular biopsies were used from heart-beating organ donors (n=3) at the hospital Policlinico Umberto I (Rome, Italy). For each donor, the free and informed consent of the family concerned, was obtained. The Ethical Committee of the hospital approved the use of human material according to national guidelines for organ donation as issued by the Italian Ministry of Public Health. Biopsies were collected as previously described (Muciaccia et al., 2013). For histological analysis, samples were fixed overnight at 4°C in Bouin fixative, washed, dehydrated, and routinely embedded in paraffin. To perform histological analysis, 5μm sections were cut and stained with Mayers Hematoxylin and Eosin (Sigma-Aldrich). All the samples included in this study showed well-preserved testicular tissue and a normal spermatogenesis.

EdU incorporation and detection

Monkey testicular samples obtained from biopsies were cultured in αMEM medium containing 4 mM glutamine, 1% non-essential amino acids, 2% penicillin/streptomycin, 0.08% gentamicin, 15 mM HEPES (pH 7.7) and 10 μM EdU (5-ethynyl-2′-deoxyuridine) at 34°C in 5% CO2 for 2 h (all reagents were from Thermo Fisher Scientific). After incubation, fragments were gently disentangled to obtain isolated seminiferous tubules and fixed in 4% paraformaldehyde at 4°C for 4h. EdU detection was performed the Click-iT EdU Imaging Kits following manufacturer recommendation (Thermo Fisher Scientific). Following EdU detection, tubules were employed to detect various antigens using whole-mount immunofluorescence (Di Persio et al., 2017)**.**

Whole-mount immunofluorescence

Monkey and human seminiferous tubules were gently disentangled from testicular biopsies and immediately fixed in 4% Paraformaldehyde (PFA) at 4°C for 4 h. Fixed tubules were permeabilized with 0.5% Triton X100, treated with 1 M glycine for 1 h, and with 0.1% TritonX-100, 1% Bovine Serum Albumin (BSA) and 5% normal donkey serum in Phosphate buffered saline (PBS) overnight at 4°C. Next day, tubules were washed in wash buffer (1% BSA, 0.1% Triton X-100 in PBS) three times for 30 min and incubated overnight at 4°C with appropriate primary antibodies (Table 2). In negative controls (NC) samples, the primary antibody and EdU detection were omitted. The following day, tubules were washed as above and incubated with species-specific secondary antibodies conjugated to Alexa 488-, Cy3- or Cy5-conjugated fluorochromes overnight at 4°C. Primary and secondary antibodies were diluted in 1% BSA and 0.1% Triton X-100 in PBS. After the secondary antibody, tubules

were washed in wash buffer as above, and nuclei were stained with TO-PRO-3. Tubules were mounted onto slides using Vectashield mounting medium and observed using a Leica TCS SP2 or Zeiss Airyscan 2 confocal microscope.

Imaging and quantification

To quantify the relative proportion of spermatogonial subtypes, their clonal size and proliferation index intact seminiferous tubules from at least three different testicular samples were co-stained for relevant antibodies. Due to the convoluted nature of monkey and human seminiferous tubules, in order to image the entire spermatogonial layer, z-stacks were acquired using Leica TCS SP2 confocal microscope or Zeiss Airyscan 2 with a 40×oil immersion objective. In each analysis, 25-30 fields (250.0 x 250.0 µm) were randomly selected from at least six different seminiferous tubules for each animal. Confocal focus on the basal layer was obtained looking at nuclei staining. Damaged or distorted tubules were not included in the analysis. For each field, confocal z-stacks were acquired (at 1 µm increments between z-slices). All the representative immunofluorescence staining showed in this study were processed as Maximum Intensity Projection. Regarding the clonal analysis of EdU^+ SPG, the number of spermatogonia per clone was determined using the method reported by Huckins (Huckins, 1971), considering positive cells as part of a clone when their internuclear distance was not more than 25 µm. In mouse, SPG belonging to a clone are connected by intercellular bridges that can be visualized by immunostaining of TEX14, an essential component of intercellular bridges (Greenbaum et al., 2006). However, our attempts to stain intercellular bridges with two different anti-TEX14 antibodies were unsuccessful (Fig. S8A). Therefore, in our clonal analysis we used the criterion of the internuclear distance and not the direct visualization of intercellular bridges. As additional criteria, the clones had to show the same or a very similar intensity and pattern of EdU labelling indicating that the timing of S-phase in these cells is synchronized, as expected for germ cell belonging to the same clone (Ehmcke et al., 2005b). Spermatogonial counts from cynomolgus monkey were normalized to Sertoli cell nuclei, as they are the only post-mitotic cell of the seminiferous epithelium (Sharpe et al., 2003). For each monkey, the number of Sertoli cell nuclei/field was quantified using SOX9 as marker (Fig.S8B and Table1). Next, SPG counts were performed using the same parameters as for Sertoli cell quantification (objective, frame size, number of fields). Finally, to homogenize the results, data from each monkey were normalized to 100 Sertoli cells. Spermatogonial counts from human seminiferous tubules were normalized for frame (250.0 x 250.0 µm). All quantifications were performed on stored images using the LAS AF Software and ZEN BLUE edition. The mean fluorescence intensity (MFI) of individual cells were quantified using LAS AF Software or ZEN 3.2. To evaluate the MFI, we first selected and measured three different non-fluorescent regions of interest (ROI) from the same image to obtain the mean value of the background's MFI. This background value was then subtracted from the MFI of ROIs manually draw around single immunoreactive cells. For each analysis, at least 100 cells were selected and analyzed from at least three different experiments. The MFI and the nuclear diameter of single cells were employed to perform Principal Component Analysis (PCA) as detailed below. An overview of all quantitative data for each analysis is provided in Supplementary Raw Data Tables.

Determination of the stages of the epithelial cycle in cynomolgus monkey whole mounted seminiferous tubules.

To determine the stage of the epithelial cycle in intact seminiferous tubules, longitudinal optical sections were acquired from the outer layer of peritubular cells towards the lumen,

using Leica TCS SP2 confocal microscope or Zeiss Airyscan 2 with a 40×oil immersion objective. Fig. S9A shows a representative Z-stack imaging series of stage II in a seminiferous tubules stained for ACROSIN and UTF1. Stage identification was based on the analysis of acrosomal development using immunostaining for ACROSIN and TOPRO3 DNA staining of elongating and elongated spermatids (Di Persio et al., 2017; Muciaccia et al., 2013). In whole mounted tubules, the immunostaining for ACROSIN allows the visualization of acrosomal vesicle form step I to VI of haploid spermatids and the Golgi granules in pachytene spermatocytes (Fig.S9B). The nuclear germ cell morphology and their relative arrangement within the seminiferous epithelium was also used as parameter for the recognition of stages for seminiferous epithelial cycle (Dreef et al., 2007). *Stage I:* this stage is characterized by two generation of spermatids: step 1 round spermatids and step 13 elongated spermatids. Step 1 spermatids do not show ACROSIN staining. *Stage II:* this stage is characterized by two generation of spermatids. Step 2 round spermatids show small proacrosomic granules positively stained for ACROSIN. *Stage III:* this stage contains two generation of spermatids. Step 3 round spermatids was identified when a spherical acrosomic vesicle containing a single acrosomic granule was observed near the nucleus. *Stage IV:* this stage is characterized by two generation of spermatids. Step 4 spermatids show a doughnut-shaped acrosomal vesicle near the nucleus. *Stage V:* recognition of this stage was dependent upon the presence of the acrosomic system in step 5 spermatids, which is characterized by the early formation of the head cap. Two different steps of spermatids are present. *Stage VI:* this stage is characterized by two generation of spermatids: step 6 round spermatids and step 14 elongated spermatids. In step 6 spermatids, the acrosome covered more than one third of the nucleus. During this stage the spermatids were released from the seminiferous epithelium into the lumen of the tubule. Bright stained nuclei spermatocyte in preleptotene step of meiotic prophase are present. Spermatocytes at pachytene step were also present. *Stage VII:* in this stage one generation of spermatids can be found. Elongated spermatids are not present. Step VII spermatids are characterized by an acrosomic system that covers almost half of the nucleus that cannot be detected by ACROSIN staining. Leptotene step of the spermatocytes show a larger nucleus. *Stage VIII:* this stage is characterized by only one generation of spermatids. Round spermatids of step VIII show no ACROSIN staining. Though, the pachytene step of the spermatocytes show a preacrosomal granule positively labelled for ACROSIN. *Stage IX:* this stage contains only one generation of spermatids. Step IX spermatids nuclei changed their form from a spherical to an oval shape. The primary spermatocytes of the early generation usually entered the zygotene step of meiotic prophase. Pachytene spermatocytes show a preacrosomal granule positive labelled for ACROSIN. *Stage X:* this stage is characterized by one generation of spermatids. Nucleus of step 10 spermatids assume a drop-shaped form. Pachytene spermatocytes show a preacrosomal granule positive labelled for ACROSIN. *Stage XI:* only one generation of spermatids is present in this stage. Step XI spermatids show an elongated nucleus. Preacrosomal granule of pachytene spermatocyte remains positive stained for ACROSIN. *Stage XII:* This stage is usually identified by the presence of meiotic divisions of primary and secondary spermatocytes. In step XII spermatids completed their elongation process

Principal component analysis (PCA) and hierarchical clustering

The principal component analysis (PCA) and clustering were performed using the hierarchical clustering on principal components (HCPC) method (Husson et al., 2010). R 4.1.3 and the R packages FactoMineR (Version 2.4) and factoextra (Version 1.0.7) were used. For the PCA shown in Figure 1C the MAGEA4, UCHL1 and KIT mean fluorescence intensities measured in 97 macaque testicular cells were used as variables. For the PCAs shown in Figure 4B and in Figure 6G the variables included in the analysis were the

MAGEA4 and KIT fluorescence intensities and the diameter of the nuclei of 126 macaque and 151 human testicular cells, respectively. An overview of all included parameters for each analysis is provided in Figure Data Table. The PCA was performed to reduce the dimensionality of the data into fewer continuous variables using the function PCA() from the FactoMineR (Version 2.4) package. Prior to the analysis, the data were scaled to avoid dominance by variables with large measurement units. The results of the PCA were visualized using the factoextra (Version 1.0.7) package. The hierarchical clustering was performed using the Ward's criterion on the selected principal components using the function HCPC() from the FactoMineR (Version 2.4) package. The partition in different clusters was initially performed by cutting the hierarchical tree, the K-means clustering was then used to improve the initial partition. The function fviz_cluster() in factoextra (Version 1.0.7) package was used to visualize individual clusters.

Statistical analysis

All quantitative data are shown as the mean± standard error of the mean (SEM). Normality and Equal Variance tests were performed for all variables. To define the significance of the differences between two groups, data were analysed using a *t*-test. To compare many groups, data were analysed using one-way analysis of variance (ANOVA) followed by a post hoc Tuckey or Kruskal-Wallis Method. Pearson analysis was performed to analyze correlation. The significance level was fixed at P=0.05. Statistical analyses were executed using SigmaPlot 14.0 and graphs were obtained by GraphPad Prism 9. Details regarding the statistical analysis are provided in Supplementary Raw Data Tables.

Acknowledgment

This study was supported by Ateneo Sapienza Funds and Ministry of Research MIUR (2017TK7Z8L) to EV. Also, this work was supported by the German Research Foundation (CRU326, grants to N.N. (NE 2190/3-2).

Author contributions

Conceptualization: C.C, S.D.P., D.G.d.R, E.V.; Validation: M.P, S.F., J.W., S.S., N.N., D.G.d.R.; Formal Analysis: C.C., M.P., S.D.P., E.V.; Investigation: C.C., M.P., S.F.; Resources: G.S., G.F., J.W., S.S., N.N.; Data Curation: C.C.; Writing- Original Draft Preparation: C.C, E.V.; Writing - Review & Editing: C.C., M.P., S.D.P., S.F., G.S., G.F., J.W., S.S., N.N., D.G.r.R., E.V.; Visualization: C.C., S.D.P., E.V. Supervision: E.V.; Project Administration: E.V.; Funding Acquisition: N.N., E.V.

Declaration of interests

The authors declare no competing interests.

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Legends

Figure 1. Classification of the spermatogonial compartments in cynomolgus monkey based on marker combinations

(A) Experimental outline. Monkey and human testis biopsies were processed as detailed in the material and methods section. Isolated seminiferous tubules were immunostained for various SPG markers and analyzed by confocal microscopy. Immunostainings were quantified followed by morphometric and PCA analyses. Roman numerals represent different stages of seminiferous epithelial cycle.
(B) Representative staining of MAGEA4 (cyan) /UCHL1 (yellow) /KIT (magenta) /nuclear staining (grey) on whole mounted intact seminiferous tubules of cynomolgus monkeys. Insets show examples of the different SPG: the arrowhead indicates MAGEA4$^+$/UCHL1$^+$/KIT$^-$ SPG, the asterisk MAGEA4$^+$/UCHL1$^+$/KIT$^+$ SPG and the arrow MAGEA4$^+$/UCHL1$^-$/KIT$^+$ SPG. Negative controls (NC) show no staining. Scale bars, 50 µm.
(C) Box plot shows the percentage of MAGEA4$^+$/UCHL1$^+$/KIT$^-$, MAGEA4$^+$/UCHL1$^+$/KIT$^+$ and MAGEA4$^+$/UCHL1$^-$/KIT$^+$ SPG. More than 4900 cells were counted from 3 animals.
(D) Cluster analysis performed on a total of 97 cells from 3 animals. Each dot represents a cell, and the three colors represent the resulting clusters: cluster 1 (blue), cluster 2 (purple), cluster 3 (magenta).
(E) Box plots representing MAGEA4, UCHL1 and KIT fluorescence intensity (FI) in the 3 clusters. a*** p<0,001 cluster 1 vs cluster 2 and 3; b*** p<0,001 cluster 2 vs cluster 3.

Figure 2. Undifferentiated spermatogonial subsets are detected along all the stages of the epithelial cycle

(A, D, G) Representative images of PIWIL4 (blue)/ UTF1 (green) /nuclei (grey) (A), PIWIL4 (blue)/GFRA1 (orange) /nuclei (grey) (D), and UTF1 (green) /GFRA1 (orange)/ nuclei (grey) (G) stainings. Insets show examples of positive and negative SPG. NC show no staining. Scale bars, 20 µm (main) and 10 µm (insets).
(B,C) Quantification of the percentage of UTF1$^+$/PIWIL4$^+$ and UTF1$^+$/PIWIL4$^-$ SPG and their distribution in each stage of seminiferous epithelial cycle. Total cells counted: 2700 from 3 animals. In (C) data are shown as mean ±SEM.
(E, F) Percentage of PIWIL4$^+$/GFRA1$^-$ PIWIL4$^+$/GFRA1$^+$ and PIWIL4$^-$/GFRA1$^+$ SPG and their distribution in each stage of seminiferous epithelial cycle. Total cells counted: 2000 from 3 animals. In (F) data are shown as mean ±SEM.
(H, I) Percentage of UTF1$^+$/GFRA1$^-$ and UTF1$^+$/GFRA1$^+$ SPG and their distribution in each stage of seminiferous epithelial cycle. Total cells counted: 2410 from 3 animals. In (I) Data are shown as mean ±SEM.

Figure 3. The proliferative activity among undifferentiated spermatogonia is entirely sustained by GFRA1-expressing SPG

(A) Representative image of MKI67 (green)/ PIWIL4 (blue)/ nuclei (grey) staining in cynomolgus monkey seminiferous tubules. Insets show examples of MKI67$^+$/PIWIL4$^-$ and MKI67$^-$/PIWIL4$^+$ SPG. NC show no staining. Scale bars, 20 µm (main) and 10 µm (insets).
(B) Representative image of UCHL1 (yellow)/ GFRA1 (orange)/ MKI67 (green) staining. Insets show examples of positive and negative SPG. NC show no staining. Scale bars, 20 µm (main) and 10 µm (insets).
(C) Box plot representing the percentage of UCHL1/GFRA1/MKI67 cell population. Data are from 600 cells scored in 3 animals.

(D) Distribution of the percentage of MKI67^{+}/GFRA1^{+} SPG along the stages of the seminiferous epithelial cycle. Data are from 1000 cells scored in 3 animals. Data shown as mean ±SEM.
(E) Representative image of EdU (green) /GFRA1 (orange) staining in a cynomolgus monkey. Inset shows ACROSIN (cyan) staging relative to the main figure. NC show no staining. Scale bars: 20 µm and 10 µm.
(F) Distribution of of EdU^{+}GFRA1^{+} SPG during the cycle of the seminiferous epithelium. A total of 150 EdU $^{+}$/GFRA1^{+} SPG were counted in 4 animals. Data shown as mean ±SEM.
(G, H) Representative images of EdU^{+} single, pairs and a 4-cell group (G). Clonal size evaluation of EdU^{+}GFRA1^{+} SPG during the cycle of the seminiferous epitheliuml. Scale bar: 20 µm. A total of 61 clones were detected in 4 animals. Data shown as mean ±SEM.

Figure 4. Early differentiating SPG are detected at stage III of the cycle
(A) Representative image of MAGEA4 (cyan)/ KIT (magenta)/ nuclei (grey) staining performed on whole mount intact tubules. Insets show examples of positive and negative SPG: i) example of MAGEA4^{+}/KIT^{-} SPG; ii) example of MAGEA4^{+}/KIT$^{med/low}$ SPG and iii) example of MAGEA4^{+}/KIT^{high} SPG. NC show no staining. Scale bars, 20 µm (main) and 10 µm (insets).
(B) Cluster analysis performed on 126 cells from 3 animals. Each dot represents a cell, and the three colors represent the resulting clusters: cluster 1 (blue), cluster 2 (purple), cluster 3 (magenta).
(C) Box plot representing MAGEA4/KIT fluorescence intensity (FI) and nuclear size in the 3 clusters. MAGEA4 FI: a*** p<0,001 cluster 1 vs cluster 2 and 3; b*** p<0,001 cluster 2 vs cluster 3. KIT FI: a*** p<0,001 cluster 1 vs cluster 2 and 3; b** p<0,01 cluster 2 vs cluster 3. NUCLEI µm: a*** p<0,001 cluster 1 vs cluster 2 and 3; b*** p<0,001 cluster 2 vs cluster 3.
(D) Distribution of the number of KIT^{+} SPG in each stage of the cycle of the seminiferous epithelium. Data are from 6000 cells scored from 3 different animals. Data are shown as mean±SEM.
(E) Nuclear diameters of KIT^{+} SPG in each stage of the cycle of the seminiferous epithelium. Data are expressed as mean±SEM. (n=3 animals).
(F) Representative picture of UCHL1^{+} (yellow)/KIT^{+} (magenta) SPG (white asterisk) Nuclear staining shown in grey. Insets show an example of UCHL1^{+}/KIT^{+} SPG. Dashed line indicates the nuclear diameter of selected SPG. NC show no staining. Scale bars, 20 µm (main) and 10 µm (insets).
(G) Line plot representing the distribution of the UCHL1^{+}/KIT^{-}, UCHL1^{+}/KIT^{+} and UCHL1^{-} /KIT^{+} cells during the stages of the cycle of the seminiferous epithelium. 7200 cells were counted from 3 animals. Data are shown as mean ± SEM.
(H) Representative image of the MKI67 (green)/ KIT (magenta) staining. Nuclear staining shown in grey. White asterisks indicate MKI67^{-} /KIT$^{med/low}$ SPG. Insets show example of MKI67^{-} /KIT$^{med/low}$ SPG. Dashed line indicates the nuclear diameter of selected SPG. NC show no staining. Scale bars, 20 µm (main) and 10 µm (insets).
(I) Distribution of KIT^{+} MKI67^{+}and MKI67^{-}SPG during the stages of the epithelial cycle. A total of 6000 cells were counted from 3 animals. Data are expressed as mean ± SEM.

Figure 5. Kinetics of the differentiating spermatogonia in cynomolgus monkey
(A) Representative images of EdU^{+} (green) KIT^{+} (magenta) SPG. Nuclear staining shown in grey. Inset shows ACROSIN (cyan) staging relative to the main figure. NC show no staining. Scale bars, 20 µm (main) and 10 µm (insets).
(B) Line plot showing the percentage of EdU^{+}/KIT^{+} SPG during the cycle of the seminiferous epithelium. A total of 1500 EdU^{+}/KIT^{+} SPG were scored in 3 animals. Data are shown as mean ±SEM. b*p<0,05 stage III vs stages I, VIII and XII; b** p<0,01 stage III vs stages II,

VI, VII,IX, X and XI; c*p<0,05 stage IV vs stages I, VI, VII, VIII, IX and X; c** p<0,01 stage IV vs stages II, XI and XII; a*** p<0,001 stage V vs stages I, II, VI, VII, VIII, IX, X, XI and XII.
(C) Representative images of EdU^+/KIT^+ SPG clones in stages VI-VII. EdU^+/KIT^+ SPG in S-phase are arranged as single cells, pairs and 4 cell clones. Scale bar 20 µm. (D) Box plot representing the clonal size of EdU^+/KIT^+ SPG at stage VI-VII of the seminiferous cycle. A total of 37 clones were scored from 3 animals.
(E) Representative image of PHH3 (green)/KIT (magenta) staining. Inset shows ACROSIN (cyan) staging relative to the main figure. NC show no staining. Scale bars, 20 µm (main) and 10 µm (insets).
(F) Distribution of $PHH3^+/KIT^+$ cells per stage. 1700 KIT^+ SPG were scored in 3 animals, Data are shown as mean ± SEM. c** p<0,01 stage II vs stage XI; c*** p<0,001 stage II vs stages III, VI, VII, VIII, IX, X and XII; b*** p<0,001 stage IV vs stages I, II, III, VI, VII, VIII, IX, X, XI and XII; a*** p<0,001 stage V vs stages I, II, III, IV, VI, VII, VIII, IX, X, XI and XII.

Figure 6. Comparative analysis shows conserved features of the spermatogonial compartment in human and nonhuman primates
(A) Representative image of PIWIL4 (blue)/GFRA1 (orange) /nuclei (grey) staining in human seminiferous tubules. Insets show examples of $PIWIL4^+/GFRA1^+$ and $PIWIL4^+/GFRA1^-$ SPG. NC show no staining. Scale bars, 20 µm (main) and 10 µm (insets).
(B) Box plot representing the percentage of $PIWIL4^+/GFRA1^-$, $PIWIL4^+/GFRA1^+$ and $PIWIL4^-/GFRA1^+$ SPG in human seminiferous tubules. 3104 cells were counted from 3 patients.
(C) Representative image of MKI67 (green/ PIWIL4 (blue)/ nuclei (grey) staining in human seminiferous tubules. Insets show examples of $MKI67^+/PIWIL4^-$ and $MKI67^-/PIWIL4^+$ SPG. NC show no staining. Scale bars, 20 µm (main) and 10 µm (insets).
(D) Representative image of MAGEA4 (cyan) /UCHL1 (yellow) /KIT (magenta) /nuclear staining (grey) on human seminiferous tubules. Insets show examples of $MAGEA4^+/UCHL1^+/KIT^+$ positive SPG (asterisks). NC show no staining. Scale bars, 20 µm (main) and 10 µm (insets).
(E) Box plot showing the percentage of $MAGEA4^+/UCHL1^+/KIT^-$, $MAGEA4^+/UCHL1^+/KIT^+$ and $MAGEA4^+/UCHL1^-/KIT^+$ SPG measured using human seminiferous tubules. More than 5117 cells were counted from 3 patients.
(F) Representative image of MAGEA4 (cyan)/ KIT (magenta)/ nuclei (grey) staining in human seminiferous tubules. Insets show examples of $MAGEA4^+/KIT^-$ and $MAGEA4^+/KIT^+$ cells. NC show no staining. Scale bars, 20 µm (main) and 10 µm (insets). (G) Cluster analysis performed on a total of 151 cells from 3 donors. Each dot represents a cell, and the three colors represent the resulting clusters: cluster 1(blue), cluster 2 (purple), cluster 3 (magenta). (H) Box plots representing MAGEA4/KIT fluorescence intensity (FI) and nuclear size in the 3 clusters. MAGEA4: a*** p<0,001 cluster 1 vs cluster 2 and 3; b*** p<0,001 cluster 2 vs cluster 3. KIT: a*** p<0,001 cluster 1 vs cluster 2 and 3; b*** p<0,001 cluster 2 vs cluster 3. NUCLEI: a ** p<0,01 cluster 1 vs cluster 2, *** p<0,0 cluster 1 vs cluster 3; b*** p<0,001 cluster 2 vs cluster 3. .
(I) Schematic representation comparing the proportions of SPG subsets between cynomolgus monkey and human. Each percentage is related to 100% of $MAGEA4^+$ SPG.

Figure 7. Model for the premeiotic spermatogonial expansion in primates
This model defines the relationship between subsets of adult primates SPG using markers co-staining analysis. Among the undifferentiated SPG, the $GFRA1^+$ SPG are the only cells able to self-renew and to give rise to the other subsets. After the mitotic amplification, daughter cells may become quiescent and start to express higher levels of UTF1 and/or PIWIL4 or they may move further towards differentiation (differentiation primed SPG). Early

differentiating SPG are quiescent, and characterized by a medium level of MAGEA4, UCHL1 and KIT expression and a large nuclear size. The first generation of differentiating SPG derive from early differentiating SPG by transition. SPG, spermatogonia; Bs, B spermatogonia; PL, preleptotene spermatocytes.