© 2017. Published by The Company of Biologists Ltd | Development (2017) 144, 3022-3030 doi:10.1242/dev.146571

REVIEW

The nature and dynamics of spermatogonial stem cells

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ABSTRACT

Spermatogonial stem cells (SSCs) are crucial for maintaining spermatogenesis throughout life, and understanding how these cells function has important implications for understanding male infertility. Recently, various populations of cells harbouring stem cell-like properties have been identified in rodent seminiferous tubules, but deciphering how these cells might fuel spermatogenesis has been difficult, and various models to explain SSC dynamics have been put forward. This Review provides an overview of the organization and timing of spermatogenesis and then discusses these models in light of recent studies of SSC markers, heterogeneity and cell division dynamics, highlighting the evidence for and against each model.

KEY WORDS: Mouse, Spermatogenesis, Spermatogonia, Spermatogonial stem cells, Stem cells

Introduction

Spermatogenesis, the process that generates spermatozoa (mature male gametes), takes place in the wall of the seminiferous tubules and involves a multitude of sequential cell types (Fig. 1). First, there are undifferentiated spermatogonia, among which lies a population of spermatogonial stem cells (SSCs) that can both self-renew and give rise to large numbers of spermatogonia, thereby supporting the daily production of sperm. The undifferentiated spermatogonia, which are diploid, then undergo differentiation and further rounds of division to give rise to spermatocytes, which enter the meiotic process. During the lengthy meiotic prophase, recombination takes place and, through two sequential divisions, parental and maternal chromosomes are distributed randomly into haploid daughter cells, which are termed spermatids. Finally, the spermatids develop from round cells into highly specialized spermatozoa that are ultimately released into the seminiferous tubule lumen (Russell et al., 1990).

Seminiferous tubules also contain a number of other cell types (Russell et al., 1990). Within the seminiferous tubules, in between the germ cells on the basal lamina, are somatic Sertoli cells that play a supportive role in spermatogenesis. In the wall of the seminiferous tubules are peritubular myoid cells and macrophages. Finally, in the interstitial tissue are Leydig cells that produce testosterone, which plays an important role in Sertoli cell function. As will be discussed below, these various cell types may all play a role in the regulation of SSC behaviour and may be involved in the establishment of SSC niches.

SSCs are crucial for the maintenance of spermatogenesis throughout life; thus, understanding how they function is important for finding the cause of particular cases of infertility and will open up possibilities to stimulate or inhibit the spermatogenic process. In recent years, a number of studies have examined various factors that are expressed in SSCs as well as those that regulate them. Exciting new data also now show the presence of heterogeneity with respect to self-renewal capacity among SSCs and give insight into the characteristics of these SSCs. This Review focuses on these studies, discussing the proliferation of SSCs and the formation of differentiating types of spermatogonia. I begin by providing an overview of the organization of the spermatogonial compartment and the timing of spermatogenesis. I then describe the various schemes and models that have been proposed for spermatogonial multiplication and stem cell renewal, before discussing more recent studies that challenge these models. Our knowledge of SSCs in rodents is far more detailed and advanced than that of primate SSCs; the nature of SSCs in primates is still unclear and their behaviour seems quite different from that in rodents (de Rooij and Russell, 2000). Therefore, this Review will focus on rodents. However, it is inconceivable that such a fundamental process as spermatogenesis will be principally different in primate and non-primate mammals, so it is likely that much of what we learn about spermatogenesis will apply to primates too.

The organization and timing of spermatogenesis

Spermatogenesis is a precisely organized process, and in every area of the seminiferous epithelium spermatogonial proliferation produces a new batch of spermatocytes at regular intervals (for example, every 8.6 days in the mouse). In addition, the steps in the development of spermatogonia, spermatocytes and spermatids are precisely timed. As a result, every area in the epithelium always goes through a similar sequence of events; in the mouse, such an area thus looks the same every 8.6 days. This series of events is called the cycle of the seminiferous epithelium (Fig. 2). The cycle can be subdivided into stages, each of which represents a distinct complement of the various cell types that arise during spermatogenesis. These stages, which are indicated by Roman numerals, are based on the first 12 clearly recognizable steps in the development of the acrosome of spermatids (Fig. 3). For researchers, the advantage of this level of organization is that when one recognizes one of the cell types in a particular stage, one knows which other cell types will be present as well. Indeed, many of the studies of SSCs that have been performed to date, including those discussed below, refer to these stages of the cycle.

Spermatogonial cell types and localization

Spermatogenesis begins with SSCs that, besides maintaining their own numbers by self-renewing divisions, give rise to differentiating cells that enter a series of sequential divisions. In 1971, Huckins and Oakberg proposed a scheme of spermatogonial multiplication and stem cell renewal (Fig. 4) that was endorsed by most researchers in the field and that held for over 40 years (de Rooij, 1973; Huckins, 1971; Lok et al., 1982; Oakberg, 1971). This proliferation scheme was developed by studying whole-mounts of seminiferous tubules instead of sections, which enables one to observe the topographical arrangement of the spermatogonia on the tubule basal lamina (Clermont and Bustos-Obregon, 1968). Huckins described a

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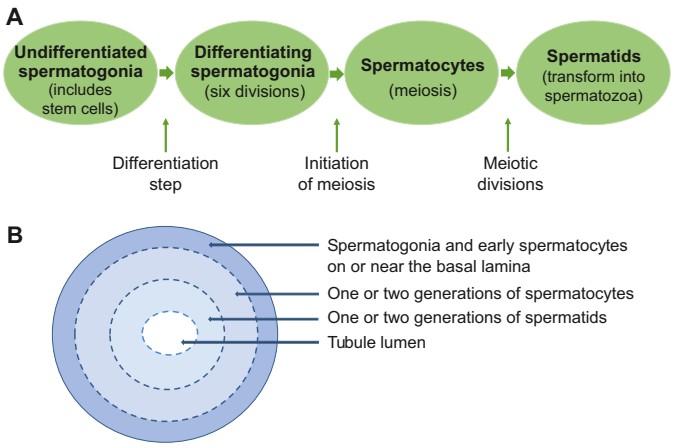

Fig. 1. An overview of the spermatogenic process. (A) The principle cell types involved in spermatogenesis are depicted together with the most important developmental steps and events. (B) Spermatogenesis takes place in the seminiferous tubules. The schematic shows a cross-section through a tubule highlighting the general positions of the various spermatogenic cell types.

population of early spermatogonia, which together are termed undifferentiated spermatogonia, consisting of single spermatogonia (A_{single}, A_s), pairs of spermatogonia (A_{paired}, A_{pr}) and chains of four, eight and 16 cells ($A_{aligned}$, A_{al}). She proposed, in a model that is now referred to as the 'A_s model' (Fig. 4), that the A_s spermatogonia are the SSCs. Then, after the SSCs divide, their daughter cells either migrate away from each other and become two new SSCs or they stay together, connected by an intercellular bridge through incomplete cytokinesis, constituting the first step along the differentiation pathway. Subsequently, the pairs can proliferate further to form clones of 4, 8 or 16 interconnected A_{al} spermatogonia (Fig. 4). The A_{al} spermatogonia then differentiate into so-called differentiating spermatogonia that are irreversibly committed towards meiosis and carry out six sequential divisions, becoming spermatocytes and then spermatids.

These various spermatogonial cells types also exhibit distinct localisations. Notably, the A_s, A_{pr} and A_{al} ($A_{s,pr,al}$) spermatogonia are not randomly distributed over the tubule basal lamina within the seminiferous tubule. Indeed, using special fixation techniques, it has been shown that increased numbers of $A_{s,pr,al}$ spermatogonia are found on those parts of the tubule basal lamina opposing interstitial tissue (Chiarini-Garcia et al., 2001, 2003). More recently, using a live-imaging set-up after GFP labelling of $A_{s,pr,al}$ spermatogonia, this niche was further narrowed down to those areas of the tubule basal lamina opposing interstitial arterioles and venules (Yoshida et al., 2007). Many of the GFP-labelled $A_{s,pr,al}$ spermatogonia can be seen close to the interstitial blood vessels. Apparently, the SSCs in these locations are able to self-renew and produce daughter cells that become pairs and chains that follow the differentiation path.

Models for spermatogonial stem cell renewal

As described above, the A_s model for SSC renewal has been the most popular model for many years. However, during the last decade, two alternative models have been proposed. In one of these models, all A_s, A_{pr} and A_{al} spermatogonia have stem cell potential and stem cell renewal is achieved by fragmentation of pairs and chains (Hara et al., 2014; Klein et al., 2010; Nakagawa et al., 2007, 2010). This model can be called the 'fragmentation model' (Fig. 5). More recently, another scheme has been proposed in which only some of the A_s spermatogonia have the potential for long-term self-renewal whereas other A_s spermatogonia have a restricted capacity to do so, indicating the presence of a SSC hierarchy (Aloisio et al., 2014; Chan et al., 2014; Helsel and Oatley, 2017; Helsel et al., 2017; Komai et al., 2014; Oatley et al., 2011; Sun et al., 2015). This model can thus be called the 'hierarchical A_s model' (Fig. 6).

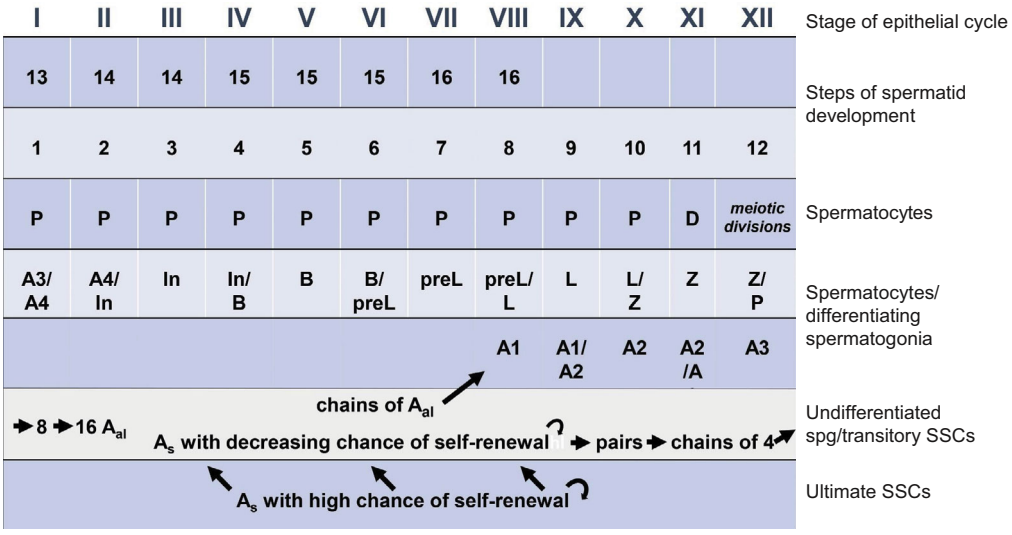

Fig. 2. Spermatogenesis and the cycle of the seminiferous epithelium. All developmental steps during spermatogenesis have a fixed duration, and every area of the epithelium always goes through a similar cycle of events, called the cycle of the seminiferous epithelium, and looks the same every 8.6 days. The epithelial cycle can be subdivided into 12 specific stages (I-XII), each of which represents a distinct repertoire of cell types, and was initially classified according the first 12 developmental steps of spermatid development. The process starts with spermatogonial stem cells (SSCs) that have a strong tendency to self-renew but which also produce SSCs with a decreasing self-renewal capacity that, besides exhibiting some self-renewal, divide to form pairs (A_{pr}) and chains (A_{al}) of spermatogonia. The SSCs and A_{pr} and A_{al} spermatogonia together are called undifferentiated spermatogonia (spg). The chains of A_{al} spermatogonia can then differentiate into A1 spermatogonia, a class of differentiating spermatogonia that divides six times to ultimately form spermatocytes via A1-A4 to intermediate (In) to B spermatogonia. The spermatocytes go through G1 and S phase (becoming pre-leptotene spermatocytes, preL) and then enter meiotic prophase, during which time they progress through leptonema (L), zygonema (Z), pachynema (P) and diplonema (D) followed by the two meiotic divisions that will render spermatids.

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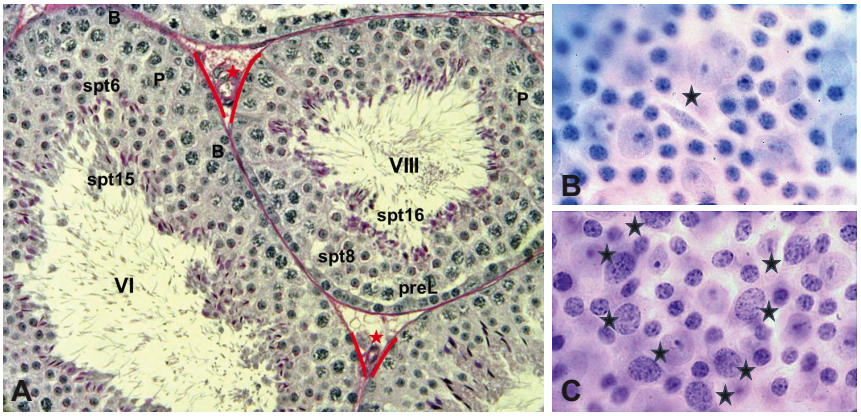

Fig. 3. Sections through seminiferous tubules and views on the tubule basal lamina. (A) The image shows a section through mouse seminiferous tubules, one of which is in epithelial stage VI whereas the other is in stage VIII. Using the diagram in Fig. 2, one can look for the cell types present in these tubules. In the cross-section of the stage VI tubule, we can find B spermatogonia on the basal membrane (labelled B). More towards the lumen, one can see pachytene spermatocytes (P), round spermatids in step 6 of their development (spt6) and already-elongated step 15 spermatids (spt15) that are already close to the lumen. In the stage VIII tubule, we can find pre-leptotene spermatocytes (preL) still on the basal lamina, pachytene spermatocytes (P), round spermatids in step 8 (spt8) and step 16 elongated spermatids (spt16) about to be released into the tubule lumen. In between the tubules, one can see triangles with interstitial tissue and some blood vessels (marked by stars). As the niche for A_s, A_{pr} and A_{al} spermatogonia is close to the interstitial blood vessels, one can expect these niches to be localised in the areas indicated by red lines. (B) View of the basal lamina of a whole-mount seminiferous tubule of a Chinese hamster. In this field, one can see Sertoli cells, pre-leptotene spermatocytes and an A_s spermatogonium (star). (C) Another field of the basal lamina of a seminiferous tubule. This one shows B spermatogonia and one clone of eight A_{al} spermatogonia (stars) synchronously in early prophase of mitosis.

The fragmentation model

The fragmentation model is based on a series of experiments performed by the Yoshida group, who developed a live-imaging set-up to follow the behaviour of spermatogonia on the basal lamina of mouse seminiferous tubules for a period of about 3 days (Hara et al., 2014; Nakagawa et al., 2007, 2010). Spermatogonia were *lacZ* labelled for the expression of the differentiation marker neurogenin 3 (*Ngn3*; *Neurog3*), which marks $A_{s,pr,al}$ spermatogonia that may have taken a first step towards differentiation, or GFP labelled for the expression of GDNF family receptor alpha 1 (*Gfra1*), which marks early $A_{s,pr,al}$ spermatogonia that do not yet express a differentiation marker. In their most detailed study, the authors examined proliferative activity and fragmentation of individual clones of $A_{s,pr,al}$ spermatogonia (Hara et al., 2014). It was found that A_s spermatogonia almost always divide into A_{pr} spermatogonia and that the pairs and chains of spermatogonia can fragment into singles and pairs. These findings led the authors to propose that stem cell renewal primarily takes place by fragmentation of clones of two to eight A_{pr} and A_{al} spermatogonia into singles and pairs (Fig. 5). Furthermore, lineage-tracing experiments suggested that some of the cells expressing *Ngn3* were able to form patches of cells consisting of all generations of spermatogenic cell types. Some of these patches were still present in the epithelium after 14 months (Nakagawa et al., 2007). Although NGN3 is a differentiation marker, apparently some Ngn^+ spermatogonia had stem cell capacity as they were still capable of self-renewal. This suggests that, despite showing signs of being in the differentiation pathway, as evidenced by the expression of NGN3, some cells can revert back and acquire stem cell properties again and can subsequently form long-term patches containing the full array of cells types found during spermatogenesis for a very long time.

However, this model does not take into account the cell proliferation needed for the normal production of differentiating spermatogonia. In the live-imaging experiments, it was found that A_s, A_{pr} and A_{al} spermatogonia proliferate slowly as on average they divided only once per 10, 12 and 13 days, respectively (Hara et al., 2014). This would indicate that clones of A_s, A_{pr} and A_{al} divide considerably less often than once per cycle of the seminiferous epithelium. Such a low proliferative activity is incompatible with normal steady-state kinetics of the seminiferous epithelium. In five strains of mice, the percentage of $A_{s,pr,al}$ spermatogonia differentiating into differentiating type A1 spermatogonia varied between 70 and 88% (Tegelenbosch and de Rooij, 1993), whereas in the Chinese hamster it was 80% and in the ram 71% (Lok and de Rooij, 1983; Lok et al., 1982). These data, which are based on cell counts as well as on cell kinetic studies using ^{3}H-thymidine labelling, indicate that there have to be two or three divisions of $A_{s,pr,al}$ spermatogonia per epithelial cycle, i.e. division must occur once per 3-4 days, in order to ensure both self-renewal and the normal production of A1 spermatogonia. Finally, $A_{s,pr,al}$ spermatogonia have been observed to migrate (Hara et al., 2014) but to compensate for the loss of differentiating cells via this mechanism, one would have to assume that there are areas that harbour $A_{s,pr,al}$ spermatogonia exhibiting a much higher proliferative activity than that observed in the live-imaging experiments. This also suggests considerable differences between different areas of the basal lamina in $A_{s,pr,al}$ spermatogonial behaviour and the presence of a constant stream of $A_{s,pr,al}$ spermatogonia (from areas with a very high proliferative activity to areas with a low proliferative activity) but there is currently no evidence to suggest that this does indeed occur.

Taken together, these observations suggest that the 'fragmentation model' cannot fully explain the general behaviour of $A_{s,pr,al}$ spermatogonia in the mouse seminiferous epithelium. It is possible that the observations reported do not faithfully reflect the true *in vivo* situation. For example, the conditions during the live-imaging experiments might be damaging to spermatogonia, possibly because of phototoxic damage to the cells. This may inhibit the proliferative activity of $A_{s,pr,al}$ spermatogonia and/or disrupt intercellular bridges causing a subsequent fragmentation of spermatogonial clones. Second, and perhaps more likely, the areas with $A_{s,pr,al}$ spermatogonia selected for live imaging might not be representative of the entire population of $A_{s,pr,al}$ spermatogonia. In the live-imaging set-up used, only those seminiferous tubules that run directly under the testicular tunica albuginea can be observed

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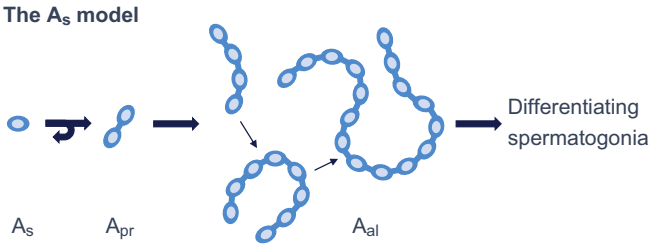

Fig. 4. The A_s model for spermatogonial multiplication and stem cell renewal. In the A_s model, the single A spermatogonia (A_s) are the spermatogonial stem cells. Upon division, the A_s can self-renew and produce two new singles or the daughter cells can form an interconnected pair, which represents a first differentiation step. Subsequently, chains of four, eight and 16 spermatogonia (A_{al}) are formed, all of which can differentiate into differentiating spermatogonia (de Rooij, 1973; Huckins, 1971; Lok et al., 1982; Oakberg, 1971).

(Hara et al., 2014; Klein et al., 2010). Alongside these tubules are stretches of interstitial tissue with its blood vessels, and it is known that the $A_{s,pr,al}$ spermatogonial niche is localised directly under these areas. Thus, the $A_{s,pr,al}$ spermatogonia in this niche cannot be seen in the current live-imaging system, as it only allows one to see cells on the basal lamina but not germ cells covered by interstitial cells or arterioles and venules. Also, most of the niche area is in a plane perpendicular to that followed in the live-imaging set-up. Indeed, the Yoshida group studied those areas in which clones of $A_{s,pr,al}$ spermatogonia seem to spill out from under the interstitial cells, and these areas probably correlate with those stages in which these cells are most numerous, i.e. stages II-VII. In these stages, the $A_{s,pr,al}$ spermatogonia are largely quiescent (Lok and de Rooij, 1983), which would explain the low proliferative activity observed in the live-imaging set-up. Furthermore, the observed fragmentation of pairs and chains could be related to their differentiation into A1 spermatogonia, as this is known to be accompanied by increasing internuclear distances between cells composing a clone (Lok et al., 1982). Finally, it should be noted that the A_{al} spermatogonia in stages II-VII are already poised to differentiate into A1 spermatogonia and normally do so in stage VIII in response to increasing retinoic acid (RA) levels (Hogarth and Griswold, 2010). It was also recently shown that one injection of RA is sufficient to induce these cells to become A1 spermatogonia within 24 h (Endo et al., 2015). It thus seems implausible that such cells poised for differentiation are just as likely to become stem cells again.

In conclusion, the data obtained so far to support the fragmentation model do not account for the cell production that is necessary to fuel spermatogenesis.

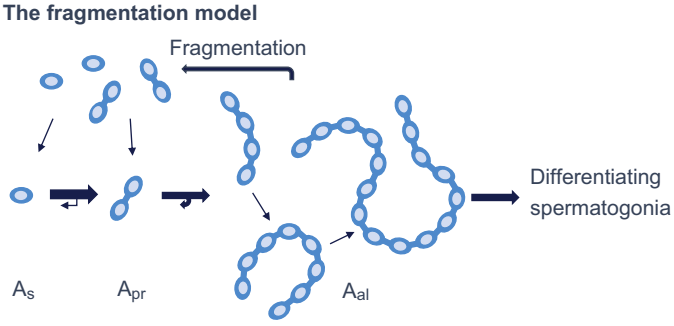

Fig. 5. The fragmentation model for spermatogonial multiplication and stem cell renewal. In the fragmentation model, most A_s form pairs. The pairs subsequently produce chains of four A_{al} spermatogonia that can divide into larger chains. Both A_{pr} and chains of A_{al} spermatogonia can fragment into smaller clones again, composed of one A_s or two A_{pr} cells. Note that in this model, A_s spermatogonia rarely self-renew directly by forming two new A_s (Hara et al., 2014; Klein et al., 2010; Nakagawa et al., 2007, 2010).

The hierarchical model

Recent reports indicate that A_s spermatogonia are heterogeneous with respect to their capacity for self-renewal, revealing the presence of a hierarchy amongst SSCs, and this led to the concept of a 'hierarchical model' for SSC renewal (Fig. 6). The development of this notion started with the identification of inhibitor of differentiation 4 (ID4) as a marker for SSCs. It was found that ID4 is almost exclusively expressed in A_s spermatogonia and in a few A_{pr}, that the expression of this protein is regulated by GDNF, and that ID4 is required for proper self-renewal of cultured SSCs (Oatley et al., 2011). However, only about 6000 of the ∼35,000 A_s spermatogonia in a mouse testis express ID4 and, intriguingly, these cells are not localized in the $A_{s,pr,al}$ spermatogonial niche (Chan et al., 2014; Tegelenbosch and de Rooij, 1993). In addition, upon transplantation into recipient mouse testes devoid of endogenous spermatogenesis, almost only $ID4^+$ cells can form repopulating spermatogenic colonies. In accordance, lineage-tracing experiments revealed the formation of long-term patches exhibiting full spermatogenesis by cells initially expressing ID4, demonstrating the stem cell capacity of $ID4^+$ cells (Sun et al., 2015). Importantly, it was shown that ID4 levels are heterogeneous amongst SSCs and that this heterogeneity correlates with heterogeneous levels of self-renewal capacity. For instance, the Oatley group used an *Id4-eGfp* transgenic mouse line and reported that about 20% of A_s spermatogonia are $ID4^{Bright}$, 40% are $ID4^{Dim}$ and 40% have an intermediate level of staining (Helsel et al., 2017). The authors considered it likely that all A_s spermatogonia show some level of ID4-eGFP expression. Furthermore, a few A_{pr} might be $ID4^+$ but it is not clear whether or not these were true A_{pr} spermatogonia, connected by an intercellular bridge, as they may also be A_s spermatogonia lying close together. No clear ID4-eGFP$^+$ A_{al} spermatogonia were seen. Purification of these cells by fluorescence-activated cell sorting revealed a 5.5-fold difference in the number of colonies formed after transplantation between $ID4^{Bright}$ and $ID4^{Dim}$ cells. The authors thus concluded that the $ID4^{Bright}$ population has the greatest capacity of self-renewal, that there is a gradient in ID4 expression among A_s spermatogonia from bright via intermediate to dim, and that concomitantly the capacity for self-renewal decreases (Helsel et al., 2017). Interestingly, a comparison of the transcriptomes of $ID4^{Bright}$ and $ID4^{Dim}$ spermatogonia revealed that a number of key genes, including *Bcl6b*, *Gfra1* and *Etv5*, are highly expressed in $ID4^{Bright}$ cells and low in $ID4^{Low}$ cells. In contrast, the expression of *Kit* and *Ngn3* is significantly lower in $ID4^{Low}$ cells than in $ID4^{Bright}$ cells (Helsel et al., 2017).

Another factor that seems to indicate self-renewal capacity is paired box 7 (PAX7) (Aloisio et al., 2014). *Pax7* is expressed in A_s spermatogonia, and in lineage-tracing experiments $Pax7^+$ cells were found to be able to form long-term spermatogenic patches and repopulating colonies after transplantation. These cells also co-express *Id4* and are actively proliferating, although they are even rarer than $Id4^+$ cells as there are only about 400 of these cells per mouse testis.

B cell-specific Moloney murine leukaemia virus integration site1 (*Bmi1*) is also expressed, almost exclusively, in A_s spermatogonia (Komai et al., 2014). The number of $Bmi1^+$ cells was estimated to be

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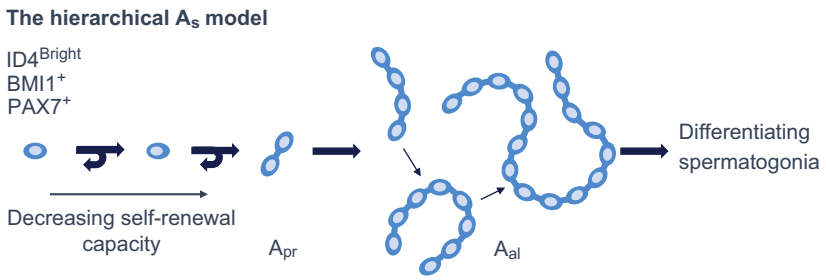

Fig. 6. The hierarchical A_s model. In this model, the population of A_s spermatogonia is heterogeneous. About 20% of the As spermatogonia express high levels of ID4 (i.e. they are ID4^{Bright}) and also BMI1, and some of these cells (around 10%) express PAX7. This type of SSC is capable of repopulating a recipient mouse testis after transplantation (Aloisio et al., 2014; Chan et al., 2014; Komai et al., 2014). In A_s spermatogonia that are produced subsequently, ID4 levels drop and these cells exhibit a decreasing chance of self-renewal and an increasing chance of forming A_{pr} spermatogonia that subsequently form clones of A_{al} spermatogonia. The cells with the highest chance of self-renewal have been called 'ultimate' SSCs and when their chance of self-renewal drops they are termed 'transitory' SSCs (Helsel and Oatley, 2017).

4800 per mouse testis. Lineage-tracing experiments have shown that *Bmi1*$^+$ cells, which also express *Id4*, can form long-term spermatogenic patches. Finally, the gene Skp1-Cullin-F-box (SCF)-type ubiquitin ligase (*Fbxw7*) also appears to play an important role in the regulation of self-renewal and colony formation (Kanatsu-Shinohara et al., 2014). Decreased expression of FBXW7 enhances and overexpression inhibits the colony-forming capacity of testicular germ cells, suggesting that FBXW7 plays an important role in A_s spermatogonia and possibly has to be downregulated in ID4$^+$/BMI1$^+$/(PAX7$^+$) cells to keep these cells from differentiation.

In summary, we can conclude that the strong expression of ID4 marks cells able to form colonies after transplantation and that ID4$^+$ cells can form long-term spermatogenic patches. In addition, PAX7$^+$ and BMI1$^+$ cells can also form long-term patches in lineage-tracing experiments. PAX7$^+$ cells are very rare but BMI1$^+$ cells occur in numbers comparable to ID4$^+$ cells. Both *Pax7*$^+$ and *Bmi1*$^+$ cells also express *Id4*$^+$, although whether a population that expresses all three markers – PAX7, BMI1 and ID4 – exists, remains to be tested. Taken together, these results suggest the presence of A_s spermatogonia that are uniquely efficient in forming colonies after transplantation and that form long-term patches in lineage-tracing experiments. These cells, which are marked by high levels of *Id4* and *Bmi1*, were named 'ultimate' SSCs by Helsel et al. (Helsel et al., 2017) and, for the sake of simplicity, I will use this term hereafter. By contrast, the A_s spermatogonia that showed a lower ID4-eGFP signal, and that exhibited lower self-renewal capacity, were termed 'transitory' SSCs.

The question then is, are the ID4$^+$/BMI1$^+$/(PAX7$^+$) A_s spermatogonia the true stem cells in this context and do the other A_s spermatogonia only form A_{pr} spermatogonia at their next division? Clearly, that cannot be the case as, at most, only about one in five A_s spermatogonia are ID4^{Bright}, and it is also known that, on average, the A_s spermatogonia have to divide two to three times per epithelial cycle in order to produce the necessary amount of A_{pr} spermatogonia and to replenish themselves (Lok and de Rooij, 1983; Lok et al., 1983; Tegelenbosch and de Rooij, 1993). Thus, if only ID4$^+$/BMI1$^+$ cells are able to self-renew, one-fifth of the A_s population would have to carry out all self-renewing divisions, which would be an unrealistic number of divisions for these cells. Therefore, it is more likely that the ID4$^-$ A_s spermatogonia that do not stain brightly for GFP in the experiments of Helsel et al. still have a diminished or diminishing chance of self-renewal and an enhanced chance of differentiation (Helsel and Oatley, 2017).

Insights from long-term lineage-tracing experiments

Lineage tracing involves marking a few cells in a tissue. Subsequently, the descendants of the marked cell will form a patch of similarly marked cells and by studying the development of such a patch one can follow self-renewal and the formation of differentiating cells by the marked cell. Such lineage-tracing experiments have now been carried out for spermatogonia expressing *Ngn3*, *Gfra1*, *Id4*, *Pax7* and *Bmi1* (Aloisio et al., 2014; Hara et al., 2014; Komai et al., 2014; Nakagawa et al., 2007; Sun et al., 2015). In all these situations, A_s spermatogonia were marked, patches of marked spermatogenesis were formed, and the number and size of the patches were followed with time. Interestingly, however, the results from these approaches seem to fall into two groups with rather different outcomes.

Patches formed by *Ngn3*- or *Gfra1*-expressing cells

In experiments in which *Ngn3* and *Gfra1* expression was marked, the numbers of patches rather quickly decreased with time and kept decreasing for at least a year (Hara et al., 2014; Nakagawa et al., 2007). This ongoing decrease in the number of patches indicates that the SSCs that were labelled in this context (i.e. those expressing *Ngn3* and *Gfra1*) are not fully able to maintain themselves. Instead, these cells and their descendants have a relatively high chance of differentiation and therefore keep disappearing from the epithelium. Taken together, this suggests that these cells do have some capacity of self-renewal, as patches were formed by *Ngn3*$^+$ and *Gfra1*$^+$ cells, but that they seem to function mainly as 'transitory' SSCs, i.e. cells that cannot maintain themselves long term.

Patches formed by *Id4*-, *Bmi1*- or *Pax7*-expressing cells

By contrast, a rather different picture is seen when cells expressing *Id4*, *Bmi1* or *Pax7* are marked. In these cases, the number of patches remains about constant (Aloisio et al., 2014; Komai et al., 2014; Sun et al., 2015), in stark difference to the results obtained by detection of *Ngn3*$^+$ or *Gfra1*$^+$ expression. Apparently, the self-renewal capacity of *Id4*-, *Bmi1*- or *Pax7*-expressing cells is so high that little or no loss of patches occurs. Furthermore, after tagging for expression of *Bmi1*, the patches were seen to grow to an average length of almost 2 mm after 48 weeks (Aloisio et al., 2014), and when *Id4*-expressing cells were marked, long patches also formed and grew, for example, to about 4 mm after 13 months (Sun et al., 2015). The presence of such large clones is puzzling. If only the ultimate SSCs are tagged, one can imagine that these cells keep producing tagged transitory stem cells that will replace transitory stem cells that fail to self-renew and are lost to differentiation.

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In such a way, the patch that forms should reach a maximum size that is determined by the distance between individual, tagged and non-tagged, ultimate SSCs in the neighbourhood, i.e. the non-tagged ultimate SSCs should keep forming non-tagged transitory SSCs that will prevent the descendants from neighbouring ultimate SSCs from forming an expanding patch of tagged cells. Thus, if the density of ultimate SSCs is indeed about a fifth of the A_s spermatozoa present in the area, the maximum size of the patches cannot be more than a couple of hundreds of microns or even less. However, this is clearly not the case, and the patches do grow to a length of about several millimetres (Aloisio et al., 2014; Sun et al., 2015). Two explanations for this seem possible. First, it may be possible that some ultimate SSCs (tagged and untagged) are much more active than others and their patches overgrow in comparison with the patches of their neighbours. Alternatively, it is possible that the number of ultimate stem cells is actually lower than a fifth of the number of A_s spermatogonia. Indeed, it may be possible that the real number of ultimate SSCs is closer to the number of $PAX7^+$ cells than that of the ID4- and BMI1-positive cells, although further studies are clearly needed to ascertain if this is true.

The heterogeneity of spermatogonial stem cells

The data described above indicate that A_s spermatogonia are heterogeneous with respect to their capacity to self-renew or differentiate. This begs the question of which cells should be called SSCs and which ones should be termed progenitor cells? Should they only be called SSCs when they can produce repopulating colonies after transplantation? Although such cells can be thought of as being the most primitive SSCs, 50% of their daughter cells must still enter the differentiation pathway as otherwise SSC tumours would be formed. Notably, Helsel et al. suggest that self-renewal capacity slowly diminishes as ID4 expression decreases (Helsel et al., 2017). So, should SSCs no longer be called SSCs, but instead be referred to as progenitor cells, when their chance of self-renewal is down from 50 to 40%, or 30%? Indeed, Helsel et al. called the $ID4^{Bright}$ cells 'ultimate SSCs' whereas the A_s spermatogonia displaying less bright fluorescence for ID4-eGFP were termed 'transitory SSCs' and slowly lose their self-renewal capacity as the expression of ID4 diminishes. However, they leave open the possibility that ID4 levels might change in such a way that transitory SSCs may become ultimate SSCs again, perhaps after a cytotoxic insult. Thus, perhaps all A_s spermatogonia should be referred to as SSCs as long as they have some self-renewal capacity, no matter whether or not they are able to form repopulating colonies after transplantation or express early differentiation genes such as *Ngn3*. This would be in contrast to the popular view that only cells that are capable of maintaining themselves perpetually should be called stem cells, and that subsequent cells should be termed progenitor cells; perhaps things are just not so black and white in spermatogenesis.

The role of the SSC niche

Generally, tissue stem cells are localized in a specific area – a niche – in which they preferentially self-renew whereas daughter cells that spill out of the niche differentiate (Schofield, 1978; Snippert et al., 2010; Stange and Clevers, 2013). As already mentioned above, $A_{s,pr,al}$ spermatogonia in the seminiferous tubules are preferentially located in those areas of the basal lamina opposing the interstitial tissue, especially where interstitial venules and arterioles are present (Chiarini-Garcia et al., 2001, 2003; Yoshida et al., 2007). This suggests that this is where SSCs reside and where A_{pr} and A_{al} spermatogonia are formed. However, ultimate SSCs have been found to only be localized outside of these areas (Chan et al., 2014). This implies that there may be separate niches for ultimate SSCs in areas of the basal lamina of seminiferous tubules opposing other tubules. The idea that emerges then is that ultimate SSCs self-renew and form transitory SSCs that move to the $A_{s,pr,al}$ spermatogonial niche; there, the transitory SSCs both self-renew and form A_{pr} spermatogonia that enter the differentiation pathway forming chains of A_{al} spermatogonia. However, further studies are clearly needed to determine if this is indeed the case.

It is also not yet clear which cell types might constitute the potential $A_{s,pr,al}$ spermatogonial and ultimate SSC niches. It seems logical that Sertoli cells play an important role as they produce a number of growth factors, such as GDNF, FGF2, CXCL12 and WNT5A, that influence spermatogonial behaviour (reviewed by de Rooij, 2009, 2015). However, as yet, no morphological differences have been observed between Sertoli cells in- and outside of the $A_{s,pr,al}$ spermatogonial niche. Besides Sertoli cells, a number of other cell types contribute to the establishment of the niche. Peritubular myoid cells, for instance, produce colony-stimulating factor 1 (CSF1) and GDNF, both of which have a role in the regulation of SSC behaviour. Peritubular macrophages also are important in the $A_{s,pr,al}$ spermatogonial niche, producing CSF1 and expressing enzymes involved in the biosynthesis of RA, which regulates spermatogonial differentiation (Chen et al., 2014, 2016; DeFalco et al., 2015; Kokkinaki et al., 2009; Meistrich and Shetty, 2015; Oatley et al., 2009; Potter and DeFalco, 2017). However, although Sertoli cells, peritubular myoid cells and peritubular macrophages together can regulate spermatogenesis, they have not yet been reported to form recognizable associations/structures that could correspond to two distinct niches – one for the ultimate SSCs and one for the transitory SSCs/$A_{s,pr,al}$ spermatogonia.

SSC markers: from expression to function

As highlighted above, factors such as ID4, PAX7 and BMI1 can be considered as marker proteins for ultimate SSCs. However, it is not yet clear whether these factors are important for ultimate SSC function and/or behaviour. This is because the effects of deficiency for these proteins are surprisingly small. In mice deficient for ID4, for example, spermatogenic problems do arise but fertility is only significantly decreased after more than half a year; at 8 months of age, the epididymal sperm concentration in these mice is still at 20% of the normal concentration (Oatley et al., 2011). In addition, no effects on spermatogenesis can be seen in mice in which PAX7 is specifically knocked out in germ cells (Aloisio et al., 2014). Finally, in mice deficient for BMI1, epithelial stages are disturbed in only 1% of tubule cross-sections.

The reason for the lack of acute effects and the relatively small effects at later ages/times of deficiency for *Id4*, *Pax7* and *Bmi1* may lie in a redundancy of these genes i.e. other genes may take over. However, it could also be caused by the system itself. As has now become clear, the seminiferous epithelium harbours few ultimate SSCs and a great many transitory stem cells. Ultimate SSCs produce transitory SSCs, which have a higher chance of differentiation, to the extent that they are not completely capable of maintaining themselves. It may thus be possible that, in the normal epithelium, the transitory SSC population slowly loses stem cells, which are replenished by daughter cells from the rare ultimate SSCs, and as long as the population of transitory SSCs does not get seriously depleted, little visible effect of a depletion of ultimate SSCs can be expected. Taken together, it may be possible that more or less stable or slowly deteriorating spermatogenesis is possible in the absence of ultimate SSCs, as long as no appreciable cell loss is inflicted.

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Implications for SSC culture and spermatogenesis *in vitro*

Much of our knowledge about stem cell regulation in many tissues has been gathered by culturing stem cells under various conditions and studying their differentiation. With respect to spermatogenesis, the latter has proven to be very difficult probably because of the complexity of the spermatogenic process. Nevertheless, important progress has been made. Mouse SSCs isolated from pre-pubertal mouse testes, which are enriched for SSCs, can be propagated during long-term cultures (Kanatsu-Shinohara et al., 2003; Kubota and Brinster, 2008), even in serum-free and feeder cell-free media (Kanatsu-Shinohara et al., 2011, 2005, 2014). The presence of SSCs in such cultures is generally determined by way of a SSC transplantation assay, in which cell suspensions containing SSCs are transplanted into recipient mouse testes devoid of endogenous spermatogenesis; the donor SSCs then form repopulating spermatogenic colonies in the recipient testes. Given that ultimate SSCs are virtually the only cell type that can form a repopulating colony after transplantation, findings on SSC characteristics assayed by colony counts after culture are likely to apply to, and reflect the behaviour of, ultimate SSCs. Indeed, only about 1-2% of germ cells in culture can give rise to colonies after transplantation, suggesting that many of the cells in these cultures are transitory SSCs and even pairs and chains, although the nature of the rest of the spermatogonia is not known (Kanatsu-Shinohara et al., 2005; Kanatsu-Shinohara and Shinohara, 2013).

Currently, many of these *in vitro* studies have aimed to assess numbers of colonies formed after transplantation of a fixed number of cells from SSC cultures, cultured under different circumstances. Therefore, these studies are often investigating the effect of gene and growth factor deficiencies on the self-renewal of cultured ultimate SSCs. For example, it has been shown that ultimate SSCs can self-renew without the presence of GDNF, and that FGF2 can promote self-renewal of these cells in the absence of GDNF (Takashima et al., 2015). Furthermore, reactive oxygen species (ROS) levels have been shown to play a role in the self-renewal of ultimate SSCs, with increased levels of ROS promoting ultimate SSC self-renewal (Morimoto et al., 2013, 2015). By contrast, inhibiting the expression of the transcriptional repressor *Bcl6b* in cultured SSCs greatly diminishes the colony-forming ability of cells after transplantation (Oatley et al., 2006). Further work will be needed to learn whether these effects on self-renewal are unique for ultimate SSCs or also apply to transitory SSCs.

Although the cultures work fine and enable one to propagate SSCs from many animals, for example mouse, human, rat and cattle (Aponte et al., 2008; Hamra et al., 2017; Oatley et al., 2016; Sadri-Ardekani et al., 2009), as yet it has not been possible to achieve complete spermatogenesis – from SSCs up to spermatozoa – *in vitro*. However, this was possible using organ cultures of explanted immature mouse testes (Sato et al., 2011a,b). The efficiency of this method is not high but, hopefully, with the increasing knowledge about the spermatogonial compartment and the factors that drive SSC self-renewal and differentiation, the methodology can be further improved in the near future.

Conclusions

Based on the studies of murine spermatogenesis described above, the most likely scenario for spermatogonial proliferation in non-primate mammals seems to involve a class of relatively rare SSCs, which can be termed 'ultimate SSCs'. However, ultimate or not, in the steady-state situation only 50% of the stem cell divisions can be self-renewing to prevent these cells from becoming an ever-growing population of cells. The other 50% of ultimate stem cell divisions will produce transitory stem cells that also have a chance of producing A_{pr} spermatogonia. As most of the $A_{s,pr,al}$ spermatogonia are situated near the interstitial tissue and its blood vessels, whereas the ultimate stem cells are not, one has to assume that the transitory stem cells move to the $A_{s,pr,al}$ spermatogonial niche. It is possible that the ultimate stem cell niche is so small that only one daughter can remain in the niche whereas the other has to migrate out and become a transitory SSC, as occurs in the *Drosophila* germline (Sheng and Matunis, 2011). Indeed, this would constitute a very stable situation as there is no chance of the ultimate SSCs depleting themselves by stochastically going through an extra round of producing differentiating daughter cells. It would also explain the constant number of patches in the lineage-tracing experiments. However, further studies are clearly needed to gain a better understanding of this niche and of the dynamics of SSCs.

An important feature of 'ultimate' SSCs is that they are more resistant to irradiation and cytotoxic agents than are transitory SSCs (see Box 1) (Aloisio et al., 2014; Komai et al., 2014). It will thus be interesting to study the DNA damage repair mechanisms available to these cells in comparison with those in transitory SSCs. It is known that DNA damage repair proteins in germ cells vary considerably across cell types (Ahmed et al., 2007; Beumer et al., 1998; Hamer et al., 2003) but if or how these differ between ultimate and transitory SSCs need to be explored. The numbers of ultimate versus transitory SSCs per mouse testis is also not yet clear. There are 5000-6000 $ID4^+$ and $IBM1^+$ cells per testis but the increasing size of the live-imaging patches of BMI1- and PAX7-expressing cells predicts that the number of ultimate SSCs is much lower. It will be interesting to see whether $ID4^+/PAX7^-$ and $BMI1^+/PAX7^-$ SSCs are able to form colonies after transplantation into recipient mouse testes.

Box 1. Understanding SSC dynamics: implications for cancer treatments

Virtually all standard cancer therapies – both chemotherapies and radiotherapy – are highly toxic to the male germline and, as such, infertility is a common complication that is associated with cancer treatment. Understanding how cells in the germline respond to such therapies is thus of high importance. In recent years, it has become clear that the damage that irradiation and alkylating agents will do to a tissue is totally dependent on the resistance of its stem cells to these toxic agents. Recovery is only possible when sufficient stem cells survive. Interestingly, some findings suggest that ultimate SSCs are more resistant to the alkylating agent busulfan and radiation than are other types of spermatogonia, including transitory SSCs. It was found that the number of $PAX7^+$ cells increases after administration of busulfan or cyclophosphamide, and also after irradiation. In contrast, the number of cells that are positive for FOXO1, which marks all $A_{s,pr,al}$ spermatogonia, quickly decreases (Aloisio et al., 2014). Furthermore, both after irradiation and after administration of busulfan, it was found that $BMI1^+$ spermatogonia are resistant to the toxic effect of these treatments and are stimulated to proliferate (Komai et al., 2014). Although more detailed studies will be needed, these results suggest that ultimate stem cells are uniquely resistant to these toxic treatments.

These results might explain an experiment in mice in which it was shown that after a high dose of irradiation, the surviving SSCs almost only self-renew during at least their first six divisions (van Beek et al., 1990). It was puzzling how these cells could react so quickly after the irradiation. However, in the light of the resistance of ultimate SSCs, it is likely that only ultimate stem cells survived and subsequently showed their ability to produce new ultimate and transitory SSCs, which started to form repopulating colonies. This is a very promising development that could enable one to predict more reliably the effect of a cytotoxic treatment on the seminiferous epithelium.

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Molecularly, ultimate stem cells express ID4 and BMI1, and some express PAX7, but how these proteins function in SSCs remains unclear. Also, it remains to be established whether ID4 and BMI1 are always co-expressed and whether there are subtypes of ultimate SSCs, perhaps exhibiting different properties. Furthermore, although FBXW7 expression plays an important role in conferring the ability to form repopulating colonies in recipient mouse testes after transplantation, it is likely that many more genes play a role in ultimate SSC function. Among these, *Nanos2* and retinoblastoma (*Rb*; *Rb1*) should be mentioned, as deficiency for these genes causes a very rapid and complete depletion of the seminiferous epithelium (Hu et al., 2013; Sada et al., 2009). Lineage tracing for *Nanos2*-expressing cells renders spermatogenic patches that decrease in number with time but less quickly than do patches of *Gfra1*- or *Ngn3*-expressing cells. However, both NANOS2 and RB, besides being expressed in A_s and A_{pr} spermatogonia, are also expressed in later types of spermatogonia and these proteins may play a more general role in $A_{s,pr,al}$ spermatogonia.

These findings raise many new questions. What composes the niche of ultimate stem cells? What is the trigger for the daughter cells of ultimate stem cells to become transitory stem cells? What makes ultimate SSCs resistant to the cytotoxic drug busulfan and irradiation? Discovering the answers to these and other questions will strongly stimulate further research into spermatogonial multiplication and stem cell renewal.

Acknowledgements

The author is grateful to Drs G. Hamer (Amsterdam) and E. Vicini (Rome) for critical reading of the manuscript.

Competing interests

The author declares no competing or financial interests.

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