Elucidating the identity and behavior of spermatogenic stem cells in the mouse testis

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Abstract

Spermatogenesis in mice and other mammals is supported by a robust stem cell system. Stem cells maintain themselves and continue to produce progeny that will differentiate into sperm over a long period. The pioneering studies conducted from the 1950s to the 1970s, which were based largely on extensive morphological analyses, have established the fundamentals of mammalian spermatogenesis and its stem cells. The prevailing so-called A_{single} (A_s) model, which was originally established in 1971, proposes that singly isolated A_s spermatogonia are in fact the stem cells. In 1994, the first functional stem cell assay was established based on the formation of repopulating colonies after transplantation in germ cell-depleted host testes, which substantially accelerated the understanding of spermatogenic stem cells. However, because testicular tissues are dissociated into single-cell suspension before transplantation, it was impossible to evaluate the A_s and other classical models solely by this technique. From 2007 onwards, functional assessment of stem cells without destroying the tissue architecture has become feasible by means of pulse-labeling and live-imaging strategies. Results obtained from these experiments have been challenging the classical thought of stem cells, in which stem cells are a limited number of specialized cells undergoing asymmetric division to produce one self-renewing and one differentiating daughter cells. In contrast, the emerging data suggest that an extended and heterogeneous population of cells exhibiting different degrees of self-renewing and differentiating probabilities forms a reversible, flexible, and stochastic stem cell system as a population. These features may lead to establishment of a more universal principle on stem cells that is shared by other systems.

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Morphological basics of mouse spermatogenesis and its stem cells

Mouse spermatogenesis occurs in seminiferous tubule of the testis. Fig. 1A and B illustrate a simplified architecture of the seminiferous tubules (Russell *et al.* 1990, Yoshida 2010). The anatomical framework of the tubules consists of the peripheral basement membrane and two types of somatic cells, i.e. Sertoli and peritubular cells. Sertoli cells comprise an epithelium inside the basement membrane, whose tight junctions provide an anatomical basis of blood–testis barrier, while peritubular cells cover the outside of the basement membrane. All the stages of germ cells from stem cells to spermatozoa are located between and nourished by Sertoli cells (Fig. 1B and C). Spermatogonia, which are defined as mitotic stages of spermatogenic cells including stem and differentiating cell types, occupy the basal compartment between basement membrane and the Sertoli cells' tight junction.

Characteristically, spermatogonia form syncytia between their mitotic sisters due to an incomplete cytokinesis, which causes the synchronization of the subsequent divisions. As a result, spermatogonia are typically found as single isolated cells (designated as A_{single} (A_s)) or syncytia of 2^n (2, 4, 8, 16, etc.) cells. The syncytia continue to extend along with subsequent course of differentiation. Spermatogonia of mouse and other rodent are often classified into two: the so-called 'undifferentiated' and 'differentiating' spermatogonia. 'Undifferentiated' spermatogonia (sometimes designated as A_{undiff}) consist of A_s , A_{paired} (A_{pr} , syncytia of two cells), and A_{aligned} (A_{al} , syncytia of 4, 8, 16, and occasionally 32 cells). 'Differentiating' spermatogonia are derived from 'undifferentiated' spermatogonia and proceed highly organized differentiation process and include A_1 , A_2 , A_3 , A_4 , In, and B spermatogonia, showing synchronous division accompanying the extension of the syncytia. The term 'undifferentiated' may be misleading because formation and extension of spermatogonial syncytia are

Figure 1 Anatomy of the seminiferous tubules and the process of spermatogenesis. (A) Architecture of seminiferous tubules shown by actual tissue image and (B) schematic representation. (C) Scheme of the spermatogenic cell types observed in the mouse testis. All the spermatogonia, including stem cells, occupy the basal compartment of the tubule, while subsequent differentiating cells are stratified to the lumen. See text for the details. Modified from Yoshida S 2010 Stem cells in mammalian spermatogenesis. *Development, Growth & Differentiation* 52 311–317. Reproduced with permission from John Wiley and Sons. Copyright 2010 Japanese Society of Developmental Biologists.

considered as an important signature of differentiation (de Rooij & Russell 2000). However, this term will be used in this review not only because no other terms correctly indicate this collective entity but also because this population seems to retain potential of self-renewing and formation of syncytia does not indicate irreversible commitment for differentiation (discussed later). When spermatogonia enter meiosis, large syncytia of germ cells – now designated as spermatocytes – translocate across the tight junction into adluminal compartment. After meiosis, haploid round and then elongating spermatids form and mature into spermatozoa as the cells move towards the lumen. Clearly, stem cells comprise a subpopulation of spermatogonia in the basal compartment, but elucidation of their identity and behaviors has long been a big challenge.

Spermatogenic stem cell models

Several models have been proposed with respect to the identity and behavior of stem cells, essentially on the basis of intensive morphological investigations of fixed specimens (for review Russell *et al.* (1990), Meistrich & van Beek (1993) and de Rooij & Russell (2000)). Among them the so-called ' A_s model' became prevailing, which proposed that singly isolated A_s spermatogonium is the only cell type that act as stem cells (Fig. 2A). Whereas, interconnected syncytia of A_{pr} and A_{al} , as well as more matured differentiating spermatogonia, are considered no longer capable of self-renewal and irreversibly committed for differentiation (Huckins 1971, Oakberg 1971). A second model hypothesized that some spermatogonial syncytia are fragmented and replenish the stem cell pool, based on the presence of syncytia

with the cell numbers of other than 2^n at a higher frequency after irradiation (Erickson 1978).

However, it was theoretically impossible to obtain a final conclusion solely based on the morphology of spermatogonia. First, precise judgment of spermatogonial interconnection in whole-mounted seminiferous tubule specimen (Clermont & Bustos-Obregon 1968) is difficult because intercellular bridges are not always visible under microscope, and identification of syncytia was practically based on the distance between nuclei with similar appearance that reflect their cell cycle phase (de Rooij & Russell 2000). More fundamentally, what one needs to know more is the behavior of spermatogonia over time (i.e. proliferation, differentiation, death, etc.) in order to fully understand the stem cell system.

In the field of mammalian spermatogenesis, the syncytia of spermatogonia have often been called a 'clone', which by definition indicates a group of cells derived from a single ancestor cell. This term is appropriate if we rely on the A_s model. However, this may be confusing if one considers fragmentation of a syncytium, which gives rise to multiple syncytia that belong to a single 'clone'. Indeed, such fragmentations have recently been observed by live imaging (Nakagawa *et al.* 2010). Our recent literature used 'cysts', which are widely used for germline of *Drosophila* and sometimes for other animals including mammals (Nakagawa *et al.* 2010). However, in *Drosophila*, syncytia of spermatogonia are encapsulated by somatic cells, while 'cysts' are used for this entire structure of spermatogonia and the surrounding somatic cells (called cyst cells). Given that mammalian spermatogonia are not encapsulated, I realize that 'cyst' may also be confusing. Therefore, 'syncytia' or 'spermatogonial syncytia' will

Figure 2 Schematic representations of undifferentiated spermatogonia subsets and their functional link. (A) The A_s model. This is entirely based on the anatomical classification or number of interconnected cells within individual syncytia, proposing that A_s is the only cell type that can act as stem cells while interconnected cells are irreversibly committed for differentiation. (B) A simplified diagram for subpopulations based on gene expression analyses. Syncytia of the same morphological entity are classified by their gene expression as shown. (C) Flows of a bulk of cells between indicated subpopulations. Processes shown by solid arrows are directly observed by live imaging, while dotted arrows indicated imaginary processes. (D) Cell flow between A_{undiff} subpopulations under steady state, which include reversion shown by yellow arrows. Modified from Nakagawa T, Sharma M, Nabeshima Y, Braun RE & Yoshida S 2010 Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. *Science* 328 62–67. Reproduced with permission from The American Association for the Advancement of Science. Copyright 2010 The American Association for the Advancement of Science.

be used in this review. Of note, the spermatogonial syncytia comprised mitotic sisters and cousins as a result of incomplete divisions, which is contrary to other examples of multinucleated syncytia formed by cell fusion (skeletal muscles, syncytial trophoblasts, etc.).

Heterogeneous composition of ‘undifferentiated’ spermatogonia in their gene expression

‘Undifferentiated’ spermatogonia, which include A_s, A_{pr} and A_{al}, are anatomically heterogeneous, with regards to the number of cells within individual syncytia (Fig. 2A). However, they share a very similar characteristic nuclear morphology that is distinct from that of differentiating spermatogonia, i.e. minimal heterochromatin condensation (Chiarini-Garcia & Russell 2001, 2002), suggesting that this entity of cells (A_s, A_{pr} and A_{al}) shares some important features. Indeed, undifferentiated spermatogonia are characterized by unique gene expression, i.e. *Plzf*^{pos}/*E-cadherin*^{pos}/*Kit*^{neg}. This is distinct from ‘differentiating’ spermatogonia, which are *Plzf*^{neg}/*E-cadherin*^{neg}/*Kit*^{pos} (Schrans-Stassen *et al.* 1999, Buaas *et al.* 2004, Costoya *et al.* 2004, Tokuda *et al.* 2007). However, expression of some genes such as *Gfra1* (also known as *GFRα1*), *Nanos2*, and *Neurog3* (also known as *Ngn3* or *Neurogenin3*) exhibits internal heterogeneity in undifferentiated spermatogonia population (Yoshida *et al.* 2004, Hofmann *et al.* 2005b, Tokuda *et al.* 2007, Suzuki *et al.* 2009, Zheng *et al.* 2009, Nakagawa *et al.* 2010, Yoshida 2010), as shown in a simplified manner in Fig. 2B. The *Gfra1*^{pos}/*Nanos2*^{pos} (*Neurog3*^{neg}/*Nanos3*^{low}/*E-cadherin*^{pos}/*Plzf*^{pos}/*Kit*^{neg}) population contains a majority of the A_s and A_{pr} spermatogonia, while the *Neurog3*^{pos} population (*Gfra1*^{neg}/*Nanos2*^{neg}/*Nanos3*^{high}/*E-cadherin*^{pos}/*Plzf*^{pos}/*Kit*^{neg}) largely corresponds to the A_{al} spermatogonia. Interestingly, this heterogeneity is different from morphological heterogeneity, namely the number of cells within a syncytium. Importantly, A_s spermatogonia population, which has been generally considered to be uniformly the stem cells, was shown to be a heterogeneous entity: while many of them are *Gfra1*^{pos}/*Nanos2*^{pos}/*Neurog3*^{neg}, some are *Gfra1*^{neg}/*Nanos2*^{neg}/*Neurog3*^{pos}.

It is also likely, however, that the above-mentioned heterogeneity of gene expression is not the only heterogeneity in the A_{undiff} population. For example, interestingly, *Id4* is reported to be expressed only in A_s spermatogonia (Oatley *et al.* 2011). Accumulation of gene expression pattern would reveal more detailed heterogeneity in this population.

Transplantation: functional assessment of spermatogenic stem cells

Experiments such as artificial cryptorchid testes and testes of vitamin A-deficient animals (Nishimune *et al.* 1978, van Pelt & de Rooij 1990, Hogarth & Griswold 2010) had functionally indicated that ‘undifferentiated’ spermatogonia population is responsible for the stem cell activity: in these situations, spermatogenesis arrests and only undifferentiated spermatogonia were observed, which is enough to reestablish complete

spermatogenesis after testes are relocated back into scrotum or dietary vitamin A is restored.

Then, post-transplantation repopulation of spermatogenesis established by Brinster and colleagues opened the door for the functional and quantitative assay of spermatogenic stem cells (Brinster & Avarbock 1994, Brinster & Zimmermann 1994). Like hematopoiesis, self-renewing, and differentiating, abilities of a single stem cell became assayable by direct measurement of the post-transplantation colony formation. Using this experimental system, it was rediscovered that a vast majority of the stem cell activity resides in *Kit*^{neg} 'undifferentiated' spermatogonia (Shinohara *et al.* 2000, Ohbo *et al.* 2003). Sorted fractions of *Kit*^{pos} 'differentiating' spermatogonia (A₁ to A₄, In and B) also showed some low activity of post-transplantation colony formation (Shinohara *et al.* 2000, Ohbo *et al.* 2003, Barroca *et al.* 2009). The origin of this activity is interesting, while the purity of the fractions warrants careful evaluation.

In undifferentiated spermatogonia, transplantable stem cell activity was detected both in *Gfra1*^{pos} and in *Gfra1*^{neg} (likely to be *Neurog3*^{pos}) fractions (Hofmann *et al.* 2005a, Nakagawa *et al.* 2007, Grisanti *et al.* 2009). It is noteworthy that *in vitro* culture that retains the stem cell activity, another big breakthrough in this field, has been developed taking advantage of this transplantation-based stem cell assay (Kanatsu-Shinohara *et al.* 2003, Kubota *et al.* 2004).

On transplantation, the donor testes are dissociated into single cell suspensions, making it impossible to link the morphological classification of spermatogonia (i.e. A_s, A_{pr}, or A_{al}) to their stem cell activity. In addition, the host germ cells need to be depleted before transplantation. Therefore, while transplantation reveals the ability of self-renewal, it may not reflect the stem cell functionality in the steady-state spermatogenesis that undergoes in undisturbed testes. Indeed, the same donor cells give rise to much larger number of colonies when transplanted into young hosts, indicating that detection of stem cell potential is context-dependent (Shinohara *et al.* 2001). Therefore, while it is no doubt that transplantation and *in vitro* cultures have provided important implications for the stem cell behavior *in vivo*, methods that enable to analyze the stem cell function under normal tissue architecture have also been warranted.

Pulse chase and live imaging: stem cell analysis in undisturbed testes

In principle, two types of experiments are capable of analyzing particular cells' behavior in undisturbed tissues or organs. First is the 'pulse-chase' experiment, in which irreversible and inheritable label is introduced to the cells of interest followed by detection of labeled cells after a certain period of time (Fig. 3). If stem cells

are pulse labeled, the label is expected to persist in both the stem and the differentiating descendants for prolonged period (Fig. 3B). On the other hand, if transit amplifying cells are induced, the label will be observed in differentiating progeny only transiently (Fig. 3C). Several kinds of genetic labeling technology have been developed mainly in mice and fruitflies (Fox *et al.* 2008, Buckingham & Meilhac 2011). In mice, tamoxifen-inducible Cre recombinase–LoxP system is the most widely used system, which recombines the reporter transgene integrated in the genome for permanent labeling on administration of tamoxifen. Potentially, unexpected effects of tamoxifen (or its active metabolite 4-OH tamoxifen) could disturb the steady state: we have evaluated that the dose used in our experiments does not affect the steady state spermatogenesis (Nakagawa *et al.* 2007 and data not shown).

Secondly, a more direct method of cell fate analysis is live-imaging. GFP and other fluorescent proteins have enabled visualization of the cells of interest in living organisms when expressed under an appropriate promoter. Thus, filming of living cells' action in living animals became a realistic challenge. In 2007, our group established an *in vivo* live-imaging system for GFP-labeled spermatogonia in a testis of anesthetized mice (Yoshida *et al.* 2007). Live imaging is quite informative: entire pedigree of a cell lineage, morphological change and movements, and cell death are only available from this technique. However, prolonged cell fate analyses (for months and sometimes for years) are necessary for the stem cell assessment, which are available only from pulse-chase experiments. So, combination of these complementary strategies is very useful. By these experiments, a number of properties of mouse undifferentiated spermatogonia have been uncovered in recent years (Nakagawa *et al.* 2007, 2010, Yoshida *et al.* 2007, Sada *et al.* 2009).

Stem cells supporting the steady state

To dissect the mouse spermatogenic stem cell system, pulse-labeling experiments have been applied for *Nanos2*^{pos} and *Neurog3*^{pos} subpopulations of undifferentiated spermatogonia, using transgenic mice expressing tamoxifen-inducible Cre under the control of *Nanos2* and *Neurog3* regulatory sequences (Nakagawa *et al.* 2007, 2010, Sada *et al.* 2009). Many of the labeled *Nanos2*^{pos} spermatogonia persisted in the tissue and continued to produce differentiating progeny for several months, indicating the central role of *Nanos2*^{pos} (also *Gfra1*^{pos}) spermatogonia in maintenance of steady state (Sada *et al.* 2009). On the other hand, a vast majority of the labeled *Neurog3*^{pos} spermatogonia proliferated and differentiated but did not stay in the tissue (Nakagawa *et al.* 2007, 2010). It is also supported that *Nanos2*^{pos}/*Gfra1*^{pos}/*Neurog3*^{neg} spermatogonia give rise to *Neurog3*^{pos} (also *Nanos2*^{neg}/*Gfra1*^{neg}/*Nanos3*^{high})

Figure 3 Pulse-chase experiment to dissect the stem cell system. (A) A conceptual stem cell system, which includes self-renewing stem cells, TA (transit-amplifying) cells, and differentiated cells. (B) When a stem cell is pulsed labeled, the labeled descendants will include both self-renewing and differentiating progeny, so that all the cells in a particular part of the tissue will be continuously labeled. (C) When a TA cell is pulsed, the labeled descendants will proliferate and differentiate but do not persist within the tissue. (B' and C') Examples of patches derived from pulse-labeled stem (B') and TA (C') cells in mouse seminiferous tubules. In these cases, cells labeled with *Lacz* expression were visualized in blue. Insets, cross sections. (D) If stem cells replace with each other, some stem cell cohorts (such that shown in red) will be extinct, while others (such that shown in blue) replenish them and expand their territories. Such a stem cell replacement is actually observed in mouse spermatogenesis (Fig. 4). (B') and (C'). Modified from Nakagawa T, Nabeshima Y & Yoshida S 2007 Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. *Developmental Cell* 12 195–206. Reproduced with permission from Elsevier. Copyright 2007 Elsevier.

spermatogonia, and that a bulk of *Neurog3*^{pos} spermatogonia differentiates into *Kit*^{pos} differentiating spermatogonia (Sada *et al.* 2009, Nakagawa *et al.* 2010). Altogether, it appears that *Gfra1*^{pos}/*Nanos2*^{pos} spermatogonia mainly self-renew their population and give rise to *Neurog3*^{pos} spermatogonia, which in turn mostly act as transient amplifying cells and differentiate into being *Kit*^{pos} (Fig. 2C).

Given that the *Gfra1*^{pos}/*Nanos2*^{pos} and the *Neurog3*^{pos} populations largely correspond to *A*_s/*A*_{pr} and *A*_{al} spermatogonia, respectively, this behavioral hierarchy in steady state seems to parallel the morphological hierarchy that appeared in the classic 'A_s model' (compare Fig. 2A and C). It is noteworthy that live imaging of *Neurog3*^{pos} spermatogonia directly observed the extension of spermatogonial syncytia for the first time (e.g. *A*_{pr} → *A*_{al-4} → *A*_{al-8}), which had long been believed to occur based on the snapshot analyses (Nakagawa *et al.* 2007, Yoshida *et al.* 2007).

However, some observations in undisturbed testis were not supposed by the A_s model (Fig. 2D). First, contrary to the idea that all the A_s are equivalent and act as stem cells, *Neurog3*^{pos} subset of A_s spermatogonia preferentially differentiated. Secondly, it is shown that a portion of *Neurog3*^{pos} cells sometimes 'revert' into being *Gfra1*^{pos} and act as self-renewing stem cells

(Nakagawa *et al.* 2007, 2010). Moreover, syncytia of *Neurog3*^{pos} spermatogonia occasionally fragmented into single cells and shorter syncytia (Nakagawa *et al.* 2010). However, it is an important question but still to be determined whether the resultant A_s cells after fragmentation of syncytial spermatogonia do replenish the stem cell pool or not.

Regeneration is supported by different population from that acting in steady state

It is generally considered that a single population of stem cells both supports the cell turnover in steady state and contributes to regeneration after tissue injury or transplantation. However, the lack of adequate experimental systems has prevented researchers from determining whether these cells, both can be designated as 'stem cells', are truly the same.

Nakagawa *et al.* (2007, 2010) analyzed the behavior of pulse-labeled *Neurog3*^{pos} spermatogonia population under three different conditions, i.e. steady state, regeneration after insult, and colony formation after transplantation (an extreme case of regeneration). The results suggest that *Neurog3*^{pos} spermatogonia (mostly found in syncytia), which differentiate without self-renewal in steady state with a high probability, shift

their mode to self-renewal in response to tissue insult or transplantation. This accompanied increased 'reversion' of *Neurog3*^{pos} spermatogonia into being *Gfra1*^{pos} (reversion in terms of gene expression): increased fragmentation of syncytia (reversion in terms of morphology) was also expected to occur at a high frequency, but this is yet to be examined experimentally.

On the other hand, behavior of *Gfra1*^{pos} spermatogonia under regeneration or after transplantation is still to be elucidated. Grisanti *et al.* (2009) showed that transplantable stem cell activity of *Gfra1*^{pos} fraction is not as high as *Gfra1*^{neg} (probably *Neurog3*^{pos}) fraction, suggesting that, unlike steady state, contribution of *Gfra1*^{pos} spermatogonia in post-transplantation colony formation would not be so crucial. Systematic analyses of the *Gfra1*^{pos} population after transplantation and on regeneration will be warranted to address this important issue.

These data demonstrate that a unique population of 'stem cells' does not play a role in every aspect of stem cell function; rather, different cell populations are recruited into stem cell pools in different contexts. In regeneration, behaviors of the stem cell compartment become largely different from what was proposed by the 'A_s model' and reminiscent of the 'clone fragmentation model', which were based on the stem cell behavior during regeneration (Erickson (1976, 1981) and Meistrich & van Beek (1993) for review). Therefore, the classical 'A_s model' and 'clone-fragmentation model' appear to represent different aspects of a reversible and flexible stem cell system. Similar reversion, namely fragmentation of syncytial spermatogonia into germline stem cells, has been observed in *Drosophila* spermatogenesis and oogenesis (Brawley & Matunis 2004, Kai & Spradling 2004).

Hierarchy and reversibility of the stem cell compartments

The above-mentioned evidence clearly indicates that the mouse spermatogenic stem cell compartment includes heterogeneous cell types with different degrees of stem cell potential (i.e. probability of self-renewal vs differentiation), rather than a small population of a single kind of stem cells. Interestingly, these different cell populations are recruited to the stem cell pool in different situations, which may be beneficial for tissue homeostasis in that the stem cell pool can be recovered quickly when necessary.

The spermatogenic stem cell system involves at least two types of heterogeneity: morphology (number of connected cells within a syncytium) and gene expression (*Gfra1*^{pos}/*Nanos2*^{pos} and *Neurog3*^{pos} populations). These two features are related to each other – the longer the syncytia are, the higher the proportion of *Neurog3*^{pos} cells is – but they are not perfectly matched (Fig. 2A).

Of note, both differentiation-related traits can be reversible. Then, which feature is more closely related to the regulation of cellular function, morphology, or gene expression? Data suggest that the gene expression shows a more close relationship with differentiation: in steady state, *Neurog3*^{pos} spermatogonia are clearly directed for differentiation regardless of their morphology (even A_s spermatogonia that are *Neurog3*^{pos} are destined for differentiation), and *Gfra1*^{pos} cells barely become differentiating spermatogonia directly (Nakagawa *et al.* 2010). *Neurog3*^{pos} spermatogonia represent a unique property: while they are destined for differentiation, they clearly retain their potential of self-renewal. This is consistent with the idea of 'potential stem cells' that was conceptually proposed by Potten & Loeffler (1990).

On the other hand, it is no doubt that syncytium formation, which is a characteristic for germ cells widely in animal kingdom (Pepling *et al.* 1999), is an important property associated with differentiation. The relationship between morphology and gene expression in the stem cell self-renewal and differentiation is one of the important questions for the complete understanding of the stem cell system. With this regard, elucidation of the live behaviors of *Gfra1*^{pos} population will be very much warranted.

Stem cell replacement in steady state

From a classical viewpoint, every single stem cell is supposed to strictly repeat asymmetric division to form one self-renewing and one differentiating daughters, and it is carefully protected in tissue as schematically shown in Fig. 4A. The *Drosophila* gonads have been representing such a typical stem cell system and have provided a theoretical basis widely for stem cell research (Fuller & Spradling 2007, Losick *et al.* 2011, Spradling *et al.* 2011). On the other hand, investigation has been warranted for other systems including mammalian spermatogenesis.

A study of pulse chase of individual mouse spermatogenic stem cell-derived cohorts over 1 year gave unexpected results (Nakagawa *et al.* 2007). If you assume the above-mentioned scenario of repeated asymmetric division, all the individual stem cell lineages are expected to persist over time and their size should be constant (Fig. 4A). In reality, however, a majority of stem cell lineages that had persisted over, for example, 3 months became extinct in the following months (Fig. 4B), while the surviving lineages expanded (Fig. 4C). This indicates that stem cells regularly lose their function (perhaps due to differentiation of all the descendants) and are replenished by stem cells that were born from neighboring stem cell cohorts (Figs 3D and 4D). Therefore, although spermatogenesis is a highly stable process, its stem cells are continuously turning over with each other. Mathematical analyses suggested

Figure 4 Stem cell replacement in steady-state spermatogenesis. (A) Conceptual illustration of stem cell fate based on the assumption that stem cells always undergo strict asymmetric division and never replace with each other. Lineage of initial stem cells should be stable over time (five stem cell lineages are shown in different colors). (B and C) Chase of labeled single stem cell-derived patches in seminiferous tubules: many of the stem cell lineages become extinct (B) while others expand (C) over months. Reproduced from Klein AM, Nakagawa T, Ichikawa R, Yoshida S & Simons BD 2010 Mouse germ line stem cells undergo rapid and stochastic turnover. *Cell Stem Cell* 7 214–224. Reproduced with permission from Elsevier. Copyright 2010 Elsevier. (D) Scheme for stem cell replacement that explains the actual pulse-chase data shown in (B) and (C).

that stem cells have an average longevity of <2 weeks and that such a frequent replacement occurs stochastically (Klein *et al.* 2010). The stochastic features of stem cell replacement obtained from this macroscopic lineage tracing may suggest that the behavior of individual stem cells is equipotent regardless of whether they are ‘bona fide’, ‘copied’, or ‘reverted’ stem cells. However, this does not mean that individual stem cells are completely the same and behave independently from their microscopic history and local situations. It is one of the most interesting and the biggest challenges to link such macroscopic scale of population dynamics of stem cells with microscopic scale of behaviors of individual spermatogonia.

Flexibility and stochasticity may be common features of stem cells

The above-mentioned findings may shift our view for the mouse spermatogenic stem cells. ‘Stem cells’ may not be a rare entity of specialized cells that always undergoes asymmetric division, but may be a population of cells showing flexible and stochastic features. Similar characteristics are recently emerging from other stem cell systems, too. The behavior of pulse-labeled stem cells in mammalian skin and intestinal epithelia revealed essentially the same class of mathematical signatures, suggesting that stochasticity commonly underlies the stem cell systems (Clayton *et al.* 2007, Doupe *et al.* 2010,

Lopez-Garcia *et al.* 2010, Snippert *et al.* 2010, Klein & Simons 2011, Simons & Clevers 2011).

Interestingly, similar flexible stem cell behavior is also suggested in *Drosophila* germline stem cells, which was classically considered as a ‘stereotypic’ system. *Drosophila* spermatogenic stem cells turn over with age, which was shown by pulse-chase experiment (Wallenfang *et al.* 2006). In addition, recent success of long-term live imaging of testis organ culture visualized interesting behaviors of stem cell daughters (Sheng & Matunis 2011): although a stem cell always divided in a geographically asymmetric manner, namely perpendicular to the niche (hub), as had been observed (Yamashita & Fuller 2005, Losick *et al.* 2011, Spradling *et al.* 2011), the subsequent fates of daughter cells were not always asymmetric. According to Sheng and Matunis (2011), ~7 and ~13% of stem cell divisions produced daughter pairs that both self-renew (symmetric renewal) and daughter pairs that both differentiate (symmetric differentiation) respectively. The probabilities of symmetric renewal and symmetric differentiation were increased and decreased, respectively, and the reversion from syncytia also takes place when regeneration was induced. These altogether result in the recovery of stem cell pool after tissue insult. It may be an issue of semantics whether this stem cell replacement is an exceptional event or whether it represents regular turnover. The observed frequency looks high enough to support the latter, although the possibility of

an artifact caused by *ex vivo* cultivation needs to be assessed carefully. I think that mathematical analysis of pulse-labeled stem cell cohorts *in vivo* is warranted in this system as well.

Remaining questions: spatio-temporal regulation of the stem cells

Stem cells' behavior is regulated properly in the seminiferous tubules. Stem cell niche, which is generally defined as the microenvironment that regulates stem cell function, is considered as an indispensable element of the stem cell system (Morrison & Spradling 2008). Mouse seminiferous tubules do not represent clearly specialized niche structure cells unlike *Drosophila* testes (hub cells) and ovaries (cap cells), or mouse intestine (base of the crypts). This is why the study of the mouse spermatogenic stem cell niche has been based on the localization of A_{undiff} spermatogonia. Thanks to precise and insightful observations of testis sections, Chiarini-Garcia *et al.* discovered that these A_{undiff} spermatogonia preferentially localize to the area adjacent to the interstitium between seminiferous tubules (Chiarini-Garcia *et al.* 2001, 2003). Subsequently, three-dimensional reconstruction of serial sections of the tubules confirmed this biased localization of A_{undiff} spermatogonia to the blood vessels and accompanying interstitial cells (Yoshida *et al.* 2007). In addition, live imaging visualized that when Neurog3^{pos} spermatogonia transit into A₁ spermatogonia they depart this presumptive vasculature-associated niche region and spread to all over the basal compartment (Yoshida *et al.* 2007). These region-related distributions and movements of spermatogonia clearly indicate that seminiferous tubules, although seemingly uniform, comprised multiple functional compartments in a manner related to the vasculature pattern. In addition, alteration of vasculature pattern accompanies the reorganization of spermatogonia, suggesting that the blood vessels (and/or their associated cells) determine the niche region. Given that blood vessels and interstitial cells do not have direct contact with spermatogonia, some molecular mechanisms should link the vasculature/interstitium with the niche microenvironment that is proximal to the stem cells (Yoshida 2011). One of such molecules may be *Csf1*, which are expressed in interstitial cells and a subset of peritubular cells and increase the colony-formation activity in cultured spermatogonia (Oatley *et al.* 2009). However, the molecular and cellular nature of this specialized microenvironment remains to be mostly elucidated.

Another unsolved question is related to the seminiferous epithelial cycle: differentiation process from A₁ spermatogonia to mature sperm, which takes place 35 days, progresses in a periodical manner with the interval of 8.6 days. Sertoli cells also change their functions cyclically so that they can support the different

stages of germ cells properly (Oakberg 1956, Russell *et al.* 1990). The stem cell behaviors seem to be also related to this cyclic phenomenon (de Rooij 1998), suggesting that some cycle-related environmental cues regulate the undifferentiated spermatogonia. Of note, expression of GDNF, the ligand for *Gfra1*, shows an oscillating pattern along with the seminiferous epithelial cycle (Johnston *et al.* 2011, Sato *et al.* 2011, Grasso *et al.* 2012). It is also suggested that local retinoic acid concentration oscillates along with the cycle and induce transition from 'undifferentiated' to A₁ differentiating spermatogonia at stages VII and VIII (Sugimoto *et al.* 2012). In addition, retinoic acid also acts on Sertoli cells so that germ cell differentiation and Sertoli cells are well coordinated. Elucidation of the role of these and other cycle-related environmental factors on the stem cell regulation remains as an open question.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Barroca V, Lassalle B, Coureuil M, Louis JP, Le Page F, Testart J, Allemand I, Riou L & Fouchet P 2009 Mouse differentiating spermatogonia can generate germinal stem cells *in vivo*. *Nature Cell Biology* **11** 190–196. (doi:10.1038/ncb1826)
- Brawley C & Matunis E 2004 Regeneration of male germline stem cells by spermatogonial dedifferentiation *in vivo*. *Science* **304** 1331–1334. (doi:10.1126/science.1097676)
- Brinster RL & Avarbock MR 1994 Germline transmission of donor haplotype following spermatogonial transplantation. *PNAS* **91** 11303–11307. (doi:10.1073/pnas.91.24.11303)
- Brinster RL & Zimmermann JW 1994 Spermatogenesis following male germ-cell transplantation. *PNAS* **91** 11298–11302. (doi:10.1073/pnas.91.24.11298)
- Buass FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG & Braun RE 2004 Plzf is required in adult male germ cells for stem cell self-renewal. *Nature Genetics* **36** 647–652.

- Buckingham ME & Meilhac SM 2011 Tracing cells for tracking cell lineage and clonal behavior. *Developmental Cell* **21** 394–409. (doi:10.1016/j.devcel.2011.07.019)
- Chiarini-Garcia H & Russell LD 2001 High-resolution light microscopic characterization of mouse spermatogonia. *Biology of Reproduction* **65** 1170–1178. (doi:10.1095/biolreprod65.4.1170)
- Chiarini-Garcia H & Russell LD 2002 Characterization of mouse spermatogonia by transmission electron microscopy. *Reproduction* **123** 567–577. (doi:10.1530/rep.0.1230567)
- Chiarini-Garcia H, Hornick JR, Griswold MD & Russell LD 2001 Distribution of type A spermatogonia in the mouse is not random. *Biology of Reproduction* **65** 1179–1185. (doi:10.1095/biolreprod65.4.1179)
- Chiarini-Garcia H, Raymer AM & Russell LD 2003 Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. *Reproduction* **126** 669–680. (doi:10.1530/rep.0.1260669)
- Clayton E, Doupe DP, Klein AM, Winton DJ, Simons BD & Jones PH 2007 A single type of progenitor cell maintains normal epidermis. *Nature* **446** 185–189. (doi:10.1038/nature05574)
- Clermont Y & Bustos-Obregon E 1968 Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted “in toto”. *American Journal of Anatomy* **122** 237–247. (doi:10.1002/aja.1001220205)
- Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ & Pandolfi PP 2004 Essential role of Plzf in maintenance of spermatogonial stem cells. *Nature Genetics* **36** 653–659.
- Doupe DP, Klein AM, Simons BD & Jones PH 2010 The ordered architecture of murine ear epidermis is maintained by progenitor cells with random fate. *Developmental Cell* **18** 317–323. (doi:10.1016/j.devcel.2009.12.016)
- Erickson BH 1976 Effect of 60Co gamma radiation on the stem and differentiating spermatogonia of the postpubertal rat. *Radiation Research* **68** 433–448. (doi:10.2307/3574325)
- Erickson BH 1978 Effect of continuous gamma-radiation on the stem and differentiating spermatogonia of the adult rat. *Mutation Research* **52** 117–128. (doi:10.1016/0027-5107(78)90100-8)
- Erickson BH 1981 Survival and renewal of murine stem spermatogonia following 60Co gamma radiation. *Radiation Research* **86** 34–51. (doi:10.2307/3575597)
- Fox DT, Morris LX, Nystul T & Spradling AC 2008 Lineage analysis of stem cells. In *StemBook*, edn 2010/07/09. Cambridge (MA): Harvard Stem Cell Institute.
- Fuller MT & Spradling AC 2007 Male and female *Drosophila* germline stem cells: two versions of immortality. *Science* **316** 402–404. (doi:10.1126/science.1140861)
- Grasso M, Fuso A, Dovere L, de Rooij DG, Stefanini M, Boitani C & Vicini E 2012 Distribution of GFRA1-expressing spermatogonia in adult mouse testis. *Reproduction* **143** 325–332. (doi:10.1530/REP-11-0385)
- Grisanti L, Falcatori I, Grasso M, Dovere L, Fera S, Muciaccia B, Fuso A, Bero V, Boitani C, Stefanini M *et al.* 2009 Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. *Stem Cells* **27** 3043–3052.
- Hofmann MC, Braydich-Stolle L, Dettin L, Johnson E & Dym M 2005a Immortalization of mouse germ line stem cells. *Stem Cells* **23** 200–210. (doi:10.1634/stemcells.2003-0036)
- Hofmann MC, Braydich-Stolle L & Dym M 2005b Isolation of male germline stem cells; influence of GDNF. *Developmental Biology* **279** 114–124. (doi:10.1016/j.ydbio.2004.12.006)
- Hogarth CA & Griswold MD 2010 The key role of vitamin A in spermatogenesis. *Journal of Clinical Investigation* **120** 956–962. (doi:10.1172/JCI41303)
- Huckins C 1971 The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. *Anatomical Record* **169** 533–557. (doi:10.1002/ar.1091690306)
- Johnston DS, Olivas E, DiCandeloro P & Wright WW 2011 Stage-specific changes in GDNF expression by rat Sertoli cells: a possible regulator of the replication and differentiation of stem spermatogonia. *Biology of Reproduction* **85** 763–769. (doi:10.1095/biolreprod.110.087676)
- Kai T & Spradling A 2004 Differentiating germ cells can revert into functional stem cells in *Drosophila melanogaster* ovaries. *Nature* **428** 564–569. (doi:10.1038/nature02436)
- Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S & Shinohara T 2003 Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biology of Reproduction* **69** 612–616. (doi:10.1095/biolreprod.103.017012)
- Klein AM & Simons BD 2011 Universal patterns of stem cell fate in cycling adult tissues. *Development* **138** 3103–3111. (doi:10.1242/dev.060103)
- Klein AM, Nakagawa T, Ichikawa R, Yoshida S & Simons BD 2010 Mouse germ line stem cells undergo rapid and stochastic turnover. *Cell Stem Cell* **7** 214–224. (doi:10.1016/j.stem.2010.05.017)
- Kubota H, Avarbock MR & Brinster RL 2004 Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *PNAS* **101** 16489–16494. (doi:10.1073/pnas.0407063101)
- Lopez-Garcia C, Klein AM, Simons BD & Winton DJ 2010 Intestinal stem cell replacement follows a pattern of neutral drift. *Science* **330** 822–825. (doi:10.1126/science.1196236)
- Losick VP, Morris LX, Fox DT & Spradling A 2011 *Drosophila* stem cell niches: a decade of discovery suggests a unified view of stem cell regulation. *Developmental Cell* **21** 159–171. (doi:10.1016/j.devcel.2011.06.018)
- Meistrich ML & van Beek ME 1993 Spermatogonial stem cells. In *Cell and Molecular Biology of the Testis*, pp 266–295. Eds C Desjardins & LL Ewing. New York, NY: Oxford University Press.
- Morrison SJ & Spradling AC 2008 Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell* **132** 598–611. (doi:10.1016/j.cell.2008.01.038)
- Nakagawa T, Nabeshima Y & Yoshida S 2007 Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. *Developmental Cell* **12** 195–206. (doi:10.1016/j.devcel.2007.01.002)
- Nakagawa T, Sharma M, Nabeshima Y, Braun RE & Yoshida S 2010 Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. *Science* **328** 62–67. (doi:10.1126/science.1182868)
- Nishimune Y, Aizawa S & Komatsu T 1978 Testicular germ cell differentiation *in vivo*. *Fertility and Sterility* **29** 95–102.
- Oakberg EF 1956 A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *American Journal of Anatomy* **99** 391–413. (doi:10.1002/aja.1000990303)
- Oakberg EF 1971 Spermatogonial stem-cell renewal in the mouse. *Anatomical Record* **169** 515–531. (doi:10.1002/ar.1091690305)
- Oatley JM, Oatley MJ, Avarbock MR, Tobias JW & Brinster RL 2009 Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development* **136** 1191–1199. (doi:10.1242/dev.032243)
- Oatley MJ, Kaucher AV, Racicot KE & Oatley JM 2011 Inhibitor of DNA binding 4 is expressed selectively by single spermatogonia in the male germline and regulates the self-renewal of spermatogonial stem cells in mice. *Biology of Reproduction* **85** 347–356. (doi:10.1095/biolreprod.111.091330)
- Ohbo K, Yoshida S, Ohmura M, Ohneda O, Ogawa T, Tsuchiya H, Kuwana T, Kehler J, Abe K, Scholer HR *et al.* 2003 Identification and characterization of stem cells in prepubertal spermatogenesis in mice. *Developmental Biology* **258** 209–225. (doi:10.1016/S0012-1606(03)00111-8)
- van Pelt AM & de Rooij DG 1990 Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. *Biology of Reproduction* **43** 363–367. (doi:10.1095/biolreprod43.3.363)
- Pepling ME, de Cuevas M & Spradling AC 1999 Germline cysts: a conserved phase of germ cell development? *Trends in Cell Biology* **9** 257–262. (doi:10.1016/S0962-8924(99)01594-9)
- Potten CS & Loeffler M 1990 Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **110** 1001–1020.
- de Rooij DG 1998 Stem cells in the testis. *International Journal of Experimental Pathology* **79** 67–80. (doi:10.1046/j.1365-2613.1998.00057.x)
- de Rooij DG & Russell LD 2000 All you wanted to know about spermatogonia but were afraid to ask. *Journal of Andrology* **21** 776–798.
- Russell L, Ettlin R, Sinha Hikim A & Clegg E 1990 Histological and histopathological evaluation of the testis. Clearwater, FL: Cache River Press.

- Sada A, Suzuki A, Suzuki H & Saga Y 2009 The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. *Science* **325** 1394–1398. (doi:10.1126/science.1172645)
- Sato T, Aiyama Y, Ishii-Inagaki M, Hara K, Tsunekawa N, Harikae K, Uemura-Kamata M, Shinomura M, Zhu XB, Maeda S *et al.* 2011 Cyclical and patch-like GDNF distribution along the basal surface of Sertoli cells in mouse and hamster testes. *PLoS ONE* **6** e28367. (doi:10.1371/journal.pone.0028367)
- Schrans-Stassen BH, van de Kant HJ, de Rooij DG & van Pelt AM 1999 Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. *Endocrinology* **140** 5894–5900. (doi:10.1210/en.140.12.5894)
- Sheng XR & Matunis E 2011 Live imaging of the *Drosophila* spermatogonial stem cell niche reveals novel mechanisms regulating germline stem cell output. *Development* **138** 3367–3376. (doi:10.1242/dev.065797)
- Shinohara T, Orwig KE, Avarbock MR & Brinster RL 2000 Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. *PNAS* **97** 8346–8351. (doi:10.1073/pnas.97.15.8346)
- Shinohara T, Orwig KE, Avarbock MR & Brinster RL 2001 Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. *PNAS* **98** 6186–6191. (doi:10.1073/pnas.111158198)
- Simons BD & Clevers H 2011 Strategies for homeostatic stem cell self-renewal in adult tissues. *Cell* **145** 851–862. (doi:10.1016/j.cell.2011.05.033)
- Snippert HJ, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, Barker N, Klein AM, van Rheenen J, Simons BD *et al.* 2010 Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* **143** 134–144. (doi:10.1016/j.cell.2010.09.016)
- Spradling A, Fuller MT, Braun RE & Yoshida S 2011 Germline stem cells. *Cold Spring Harbor Perspectives in Biology* **3** a002642. (doi:10.1101/cshperspect.a002642)
- Sugimoto R, Nabeshima Y & Yoshida S 2012 Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of Sertoli cells in mouse seminiferous epithelium. *Mechanisms of Development* **128** 610–624. (doi:10.1016/j.mod.2011.12.003)
- Suzuki H, Sada A, Yoshida S & Saga Y 2009 The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins Nanos2 and Nanos3. *Developmental Biology* **336** 222–231. (doi:10.1016/j.ydbio.2009.10.002)
- Tokuda M, Kadokawa Y, Kurahashi H & Marunouchi T 2007 CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. *Biology of Reproduction* **76** 130–141. (doi:10.1095/biolreprod.106.053181)
- Wallenfang MR, Nayak R & DiNardo S 2006 Dynamics of the male germline stem cell population during aging of *Drosophila melanogaster*. *Aging Cell* **5** 297–304. (doi:10.1111/j.1474-9726.2006.00221.x)
- Yamashita YM & Fuller MT 2005 Asymmetric stem cell division and function of the niche in the *Drosophila* male germ line. *International Journal of Hematology* **82** 377–380. (doi:10.1532/IJH97.05097)
- Yoshida S 2010 Stem cells in mammalian spermatogenesis. *Development, Growth & Differentiation* **52** 311–317. (doi:10.1111/j.1440-169X.2010.01174.x)
- Yoshida S 2011 Stem cell niche system in mouse spermatogenesis. In *Male Germline Stem Cells: Developmental and Regenerative Potential*, Stem cell biology and regenerative medicine series, pp 159–175. Humana Press.
- Yoshida S, Takakura A, Ohbo K, Abe K, Wakabayashi J, Yamamoto M, Suda T & Nabeshima Y 2004 Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. *Developmental Biology* **269** 447–458. (doi:10.1016/j.ydbio.2004.01.036)
- Yoshida S, Sueno M & Nabeshima Y 2007 A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. *Science* **317** 1722–1726. (doi:10.1126/science.1144885)
- Zheng K, Wu X, Kaestner KH & Wang PJ 2009 The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. *BMC Developmental Biology* **9** 38. (doi:10.1186/1471-213X-9-38)

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