

DIVISION OF GERM CELL BIOLOGY



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Our research

Many animals, including mammals, produce a large number of sperm over a long period for reliable reproduction. On the other hand, each sperm correctly replicates and transmits the genetic information to the next generation. The ultimate goal of the Division of Germ Cell Biology is to reveal how seemingly contradictory, albeit essentially important, productivity and fidelity are both achieved. To this end, we pay special attention to the sperm stem cells. We are also interested in other aspects of spermatogenesis.

What are the sperm stem cells?

The continual sperm production is supported by the “sperm stem cells,” termed formally “SSCs” after spermatogonial or spermatogenic stem cells. SSCs maintain a delicate balance between self-renewal and differentiation to sustain life-long spermatogenesis. It has been a central question which fraction of *spermatogonia*, the “early stage of cells before meiosis, are SSCs and where and how they behave in the testis.

The backbone of mammalian SSC research was established from the 1950s to the 1970s, relying on morphological analyses of fixed testis specimens. This led to a proposal of the prevailing stem cell model called the “A_s model.” This model proposed that singly isolated spermatogonia (A-single or A_s cells) act as SSCs. On the other hand, all syncytia are all committed to differentiation.

Our lab has introduced the *scale of time* using updated technologies, *i.e.*, live-imaging and pulse-labeling studies, which revealed many different aspects of dynamical behaviors of SSCs (Figure. 1).

A_s cells and syncytia together comprise a single SSC pool

Our live-imaging studies combined with mathematical and statistical analysis evidenced that SSCs reversibly change their morphology between singly-isolated and syncytia. This is mediated by incomplete cell division and breakdown of syncytia (Nakagawa *et al.*, Science 2010; Hara *et al.*, Cell Stem Cell 2014).

Cells inclined for differentiation can revert to SSCs

It is generally thought that once stem cells have been destined for differentiation, they never replicate themselves again. We found that such cells maintain the potential to self-renew up to a particular stage of differentiation and revert to SSCs with high frequency, particularly during regeneration after tissue damage (Nakagawa *et al.*, Developmental Cell 2007).

SSCs follow variable fate behaviors

Stem cells are often thought to invariably undergo asymmetric division, which produces one stem cell and one differentiating cell to maintain homeostasis. However, we found that individual SSCs follow highly variable fates. Still, collectively, they achieve a perfect balance between self-renewal and differentiation at a population level (Klein *et al.*, Cell Stem Cell 2010; Hara *et al.*, Cell Stem Cell 2014).

SSCs show active motion in an “open niche”

In many tissues, stem cells receive self-renewing signals in defined niche regions. We found that the mouse testis has no such areas, with SSCs scattered among differentiated cells and moving around actively. Further, their distribution is biased near blood vessels. Such microenvironment is called an “open (or facultative) niche” in contrast to the aforementioned “closed (or defined) niche” (Yoshida *et al.*, Science 2007; Hara *et al.*, Cell Stem Cell 2014).

SSC heterogeneity underpins the asymmetric fates of SSCs

In an open niche, some SSCs remain undifferentiated while others differentiate in response to broadly distributed signaling molecules. We found that some SSC fractions resist differentiation-promoting Wnt signal by expressing a Wnt-inhibitor, Shias 6. Another subset responds to retinoic acid (RA) by upregulating an RA receptor to differentiate (Ikami *et al.*, Development 2015, Tokue *et al.*, Stem Cell Reports 2017).

SSCs compete for self-renewal factors in an open niche

We also question the mechanism that maintains a constant SSC density in the open niche of the testis. Evidence suggests that SSC density homeostasis is achieved through competing with each other for limited amounts of self-renewal factors (*e.g.*, FGFs). This mechanism was proposed as the “mitogen competition model” (Kitadate *et al.*, Cell Stem Cell 2019).

Periodic differentiation of SSCs

Strikingly, differentiation of SSCs and subsequent differentiation to mature into sperm occur synchronously every 8.6 days. This is called the seminiferous epithelial cycle. We propose that the periodic differentiation is triggered by periodic synthesis and degradation of RA (Sugimoto *et al.*, Mechanisms of Development 2012).

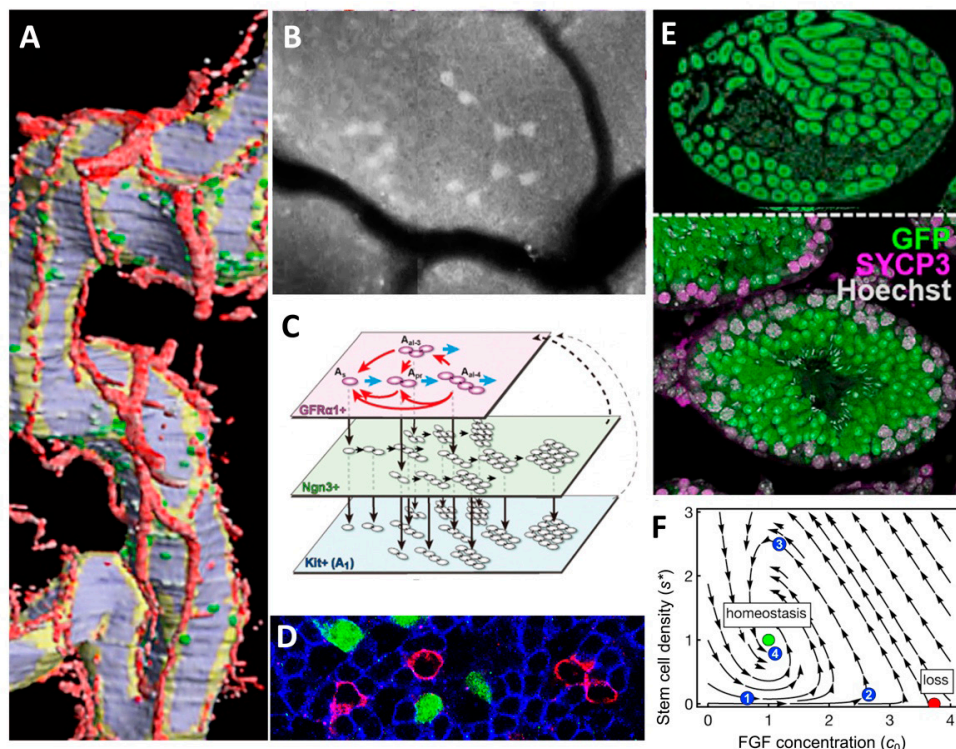


Figure 1. Different looks of mouse sperm stem cells (SSCs)

- A. 3D-reconstructed seminiferous tubules, showing SSCs in green, blood vessels in red.
B. An intravital live-imaging filming of GFP⁺ SSC syncytia undergoing differentiation.
C. Scheme of SSC dynamics in multi-compartment, interconverting singly-isolated and syncytial states.
D. Whole-mount immunofluorescence, showing SSCs (magenta) intermingled with primed (green) and committed (blue) progenies.
E. Sectioned host testis showing repopulated by transplanted donor SSCs (green), augmented by transient RA inhibition.
F. A phase portrait showing the dynamics of homeostatic SSC density regulation in the mitogen competition model.

Clonal analysis and tuning of post-transplantation SSC fate

When SSCs are transplanted into host animals' seminiferous tubules whose germ cells have been depleted, SSCs engraft and regenerate spermatogenesis (Brinster *et al.*, 1994). What happens during repopulation was largely elusive. We found that donor SSCs follow stochastic fate with significant similarity with homeostasis. We further succeeded in tuning the donor SSC fate to increase the repopulation efficiency, which would promise the practical use of this technology (Nakamura *et al.*, Cell Stem Cell 2021).

Identification of a tiny, most primitive fraction of SSCs

An important advance gained in FY2021 is that we have detailed gene expression heterogeneity of SSCs (precisely, a population called “undifferentiated spermatogonia”) and their

dynamical state transitioning in supporting the homeostatic and continual sperm production (Nakagawa *et al.*, Cell Reports 2021). This is a collaborative study with Nagasawa (Osaka) and Simons (Cambridge) labs. We identified that a gene *Plvap* (plasma-membrane vesicle associated protein) is expressed in a tiny, most primitive fraction of mouse SSCs (Figure 2A).

Detailing the heterogeneity of mouse SSCs

Plvap shows overlapping expression with some other genes, including *Eomes*, *Pdx1*, *Shisa6*, and *T* (brachyury). By dimension reduction of single-cell (sc)RNA-seq data (La *et al.*, 2018), *Plvap*⁺ cells cluster at the undifferentiated end of SSCs, forming a discrete subfraction of cells expressing established undifferentiated markers, *GFRα1* and *Id4*. In contrast, differentiation-related genes (*e.g.*, *Sox3*, *Ngn3*, and *RARγ*) is expressed in cells near the opposite end (Figure 2B). Based on these, Figure 2C summarizes the heteroge-

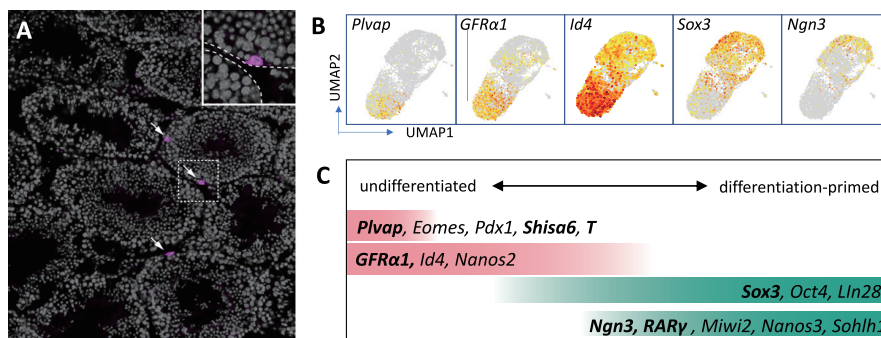


Figure 2. Heterogeneity of SSCs

- A. Immunostaining of a mouse testis section for *Plvap* protein (magenta), with DNA counterstained in gray. Only three cells located on the periphery of seminiferous tubules express *Plvap*.
B. UMAP analyses of scRNA-seq data showing the expression of indicated genes.
C. A summary of expression profile of a set of genes within SSCs. Genes shown in bold have been analyzed in our lab. Adopted from Cell Reports 37 (3), 109875.

neous expression of key SSC genes.

Contribution of multiple SSC fractions to self-renewing pool in homeostasis

By pulse-labeling using tamoxifen-dependent CreER, $Plvap^+$, $Sox3^+$, and $Ngn3^+$ cells were found to contribute to the self-renewing pool in homeostasis. In particular, after being sparsely labeled with GFP expression, these cells gave rise to cell patches comprised of GFP^+ germ cells in three months (Figure 3).

Interestingly, while the patch-forming efficiency varied between these fractions, the resultant individual patches showed indistinguishable size distribution irrespective of the original cell state. Further, strikingly, the same heterogeneous SSC population was established in every such patch, again, irrespective of the original cell state.

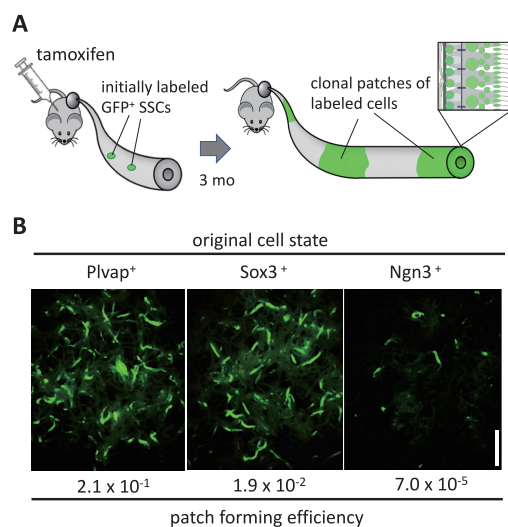


Figure 3. Pulse-labeling of multiple SSC fractions
A. Experimental design addressing the contribution of spermatogonia expressing particular genes to homeostasis, following a pulse-labeling by tamoxifen injection.
B. Results of the pulse-labeling of the $Plvap^+$, $Sox3^+$, and $Ngn3^+$ fractions. Note the differential patch forming efficiencies in contrast to the similarly-looking individual patches.

We reasoned that SSCs transit continually and reversibly between multiple cell states, from the self-renewal-biased $Plvap^+$ state to the differentiation-primed $Ngn3^+$ state (Figure 3B). As a result, a heterogeneous SSC pool is always established, which both maintains itself and gives rise to irreversibly committed progenitor cells of Kit^+ spermatogonia as a result of cell division and state transition.

A multistate dynamics: Proposal of an inclusive model of mouse SSCs

Finally, we synthesized a mathematical model that captures the multistate SSC dynamics in homeostasis. This model assumes that the cells undergo cell division and state transition (either differentiation and reversion) in a stochastic (*i.e.*, probabilistic) manner, following particular rates specific to each state (Figure 4).

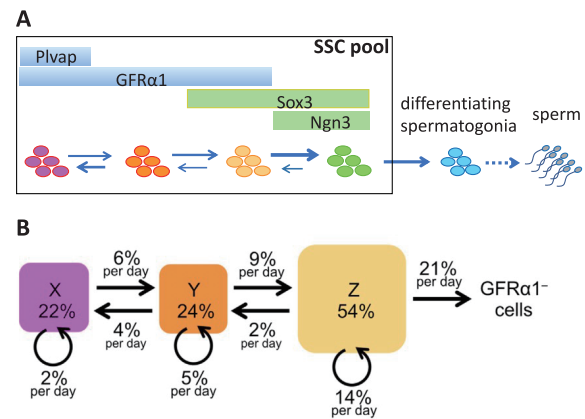


Figure 4. Multistate dynamics of mouse SSCs

A. A general perspective of the multistate dynamics, in which SSCs transit between multiple states reversibly to form a single SSC pool.
B. A numerically fit dynamics of mouse SSCs in homeostasis. The flows of cells between cell states and cell division at respective states are shown in values relative to the total $GFR\alpha1^+$ cells per day. Note that the $GFR\alpha1^-$ ($Ngn3^+$) to $GFR\alpha1^+/Sox3^+$ state is not defined due to its low frequency in this model. Adopted from Cell Reports 37 (3), 109875.

Interestingly, such a multistate dynamics is found capable of saving the cell divisions in the self-renewing pool in producing a certain number of differentiating cells. This is due to the differential cell division frequencies between the states; the more primitive the state is, the less frequently the SSC divide. See Nakagawa *et al.*, Cell Reports 2021 for detail.

Furthermore, this quantitative model can explain the seemingly contradictory models proposed for the SSC dynamics, and may provide inclusive insights into this long-held problem in the field of SSC research.

Publication List:

[Original Papers]

- Nakagawa, T., Jörg, D.J., Watanabe, H., Mizuno, S., Han, S., Ikeda, T., Omatsu, Y., Nishimura, K., Fujita, M., Takahashi, S., Kondoh, G., Simons, B.D., Yoshida, S., and Nagasawa, T. (2021). A multistate stem cell dynamics maintains homeostasis in mouse spermatogenesis. Cell Rep. 37, 109875. DOI: 10.1016/j.celrep.2021.109875
- Nakamura, Y., Jörg, D.J., Kon, Y., Simons, B.D., and Yoshida, S. (2021). Transient suppression of transplanted spermatogonial stem cell differentiation restores fertility in mice. Cell Stem Cell 28, 1443-1456. e7. DOI: 10.1016/j.stem.2021.03.016
- Osawa, Y., Murata, K., Usui, M., Kuba, Y., Le, H.T., Mikami, N., Nakagawa, T., Daitoku, Y., Kato, K., Shawki, H.H., Ikeda, Y., Kuno, A., Morimoto, K., Tanimoto, Y., Dinh, T.T.H., Yagami, K., Ema, M., Yoshida, S., Takahashi, S., Mizuno, S., and Sugiyama, F. (2021). EXOC1 plays an integral role in spermatogonia pseudopod elongation and spermatocyte stable syncytium formation in mice. eLife 10, e59759. DOI: 10.7554/eLife.59759

[Review article]

- Kitadate, Y., and Yoshida, S. (2022). Regulation of spermatogenic stem cell homeostasis by mitogen competition in an open niche microenvironment. Genes Genet. Syst. 97, 15-25. DOI: 10.1266/ggs.21-00062