Division of Germ Cell Biology

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Overview of our research

Production of numerous sperm for a long period in testis is fundamental for continuity of life across generations. This process of spermatogenesis is supported by the robust function of "stem cells", which both maintain the undifferentiated cells, while generating differentiated cells in a nicely balanced manner. The Division of Germ Cell Biology aims to fully understand the mouse sperm stem cell system under the context of *in vivo* testicular tissue. Our particular interests have been laid on the "undifferentiated spermatogonia", which are responsible for the stem cell functions. Our study has revealed several key properties of this interesting population.

First, we found that this stem cell system includes a functional hierarchy. It is comprised of an "actual" stem cell compartment that is prone to self-renew, and a differentiation-primed, "potential" stem cell compartment. Regarding the "actual" stem cells, we have been investigating their cellular identity, their *in vivo* behavior at a single-cell resolution, and the underlying mathematical principles. This lead to the discovery of neutral competition between the stem cells. We are currently investigating the mechanisms underlying the constant size of this population, and its connection to its tissue environment. "Potential stem cells" are also of our enthusiastic interest: In undisturbed, steady-state spermatogenesis, they largely contribute as transient amplifying cells and differentiate rather than self-renew. However, when tissues are insulted or testicular cells are transplanted to a host testes, their probability of self-renewal jumps up and they effectively replenish the lost "actual" stem cells. Such a flexible feature of stem cell dynamics has been found paradigmatic for many other stem cell-supported tissues.

Key references for these studies that are currently public include Nakagawa et al., Dev. Cell 2007; Yoshida et al., Science 2007; Nakagawa et al., Science 2010; Klein et al., Cell Stem Cell 2010; and Hara et al., Cell Stem Cell 2014.

I. The stem cell dynamics

Morphologically, the population of A_{undiff} includes singly isolated cells (A_s), or syncytia consisting mainly of 2 (A_{pr}), 4 (A_{al-4}), 8 (A_{al-8}), or 16 (A_{al-16}) cells. The formation of syncytia is due to incomplete cell division, a germline-specific cell division process where cytokinesis does not complete and the cytoplasmic connection between daughter cells persists via intercellular bridges. The prevailing stem cell theory proposed in 1971 states that stem cell activity is restricted to the population of A_s cells, while interconnected A_{pr} and A_{al} syncytia are irreversibly committed to differentiation and no longer contribute to the stem cell pool (Huckins, 1971; Oakberg, 1971), known as the "A_s model".

Figure 1 represents our latest model for the functional structure of the spermatogenic stem cell system, which indeed proposes an alternative for the "A_s model". This is the simplest interpretation of the results of our functional analyses of GFRα1$^+$ spermatogonia, which act as the "actual" stem cells. These include intravital live-imaging experiments, clonal fate analysis of pulse-labeled cells, and biophysical modeling analysis of the results.

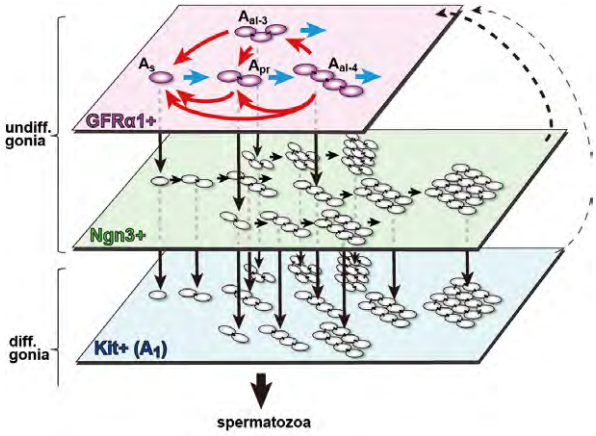

Figure 1. A proposed stem cell dynamics. On the top of the differentiation hierarchy, GFRα1$^+$ spermatogonia comprise a single stem cell pool, in which cells continually and reversibly interconvert between states of A_s, A_{pr} and A_{al} spermatogonia through incomplete cell division (blue arrows) and syncytial fragmentation (red arrows), while giving rise to Ngn3$^+$ cells. After leaving the GFRα1$^+$ compartment, differentiation-destined cells follow a series of transitions (GFRα1$^+$→Ngn3$^+$→Kit$^+$; downward black arrows) that accompany the extension of syncytial length (rightward black arrows). Ngn3$^+$ and, to a lesser extent, Kit+ cells retain the capacity to revert back into the GFRα1+ compartment in a context-dependent fashion (broken arrows). (Reprinted from Hara et al., Cell Stem Cell 2014.)

As crystalized in this model, our results suggest that the GFRα1+ sub-population of A_{undff} spermatogonia, which include both A_s cells and syncytia (A_{pr} and A_{al}) comprises a single stem cell pool, in which cells continually interconvert between these morphologically heterogeneous states through stochastic incomplete division and fragmentation of syncytia. The incomplete division and syncytial fragmentation causes the expansion of this population, while the excess cells over a particular "quota" would overflow to become the

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2015. The former title is indicated by an asterisk (*).

Ngn3$^+$ state of A_{undff}, which then further differentiate into Kit$^+$ "differentiating" spermatogonia that are largely devoid of self-renewing potential. Currently, we are investigating the mechanism that determines the quota or tissue capacity of GFRα1$^+$ cells, as well as the detailed nature of the heterogeneity of this population.

II. Mechanisms of the different behavior of "actual" and "potential" stem cells

In 2015, we challenged one key question: Why a particular "actual" stem cell population of A_{undiff} (i.e., GFRα1$^+$ cells) remain undifferentiated, while the other, "potential" stem cells (Ngn3$^+$) differentiate, despite that both cells are located in the same tissue "open niche" environment and exposed to the same differentiation-inducing signal? (Ikami et al., Development 2015)

2-1. Architecture of the mouse testis – the "open niche"

It was generally considered that the behavior of stem cells is regulated by a tissue microenvironment, or the stem cell niche. In some tissues, like *Drosophila* gonads or mammalian small intestines, stem cells cluster in an anatomically specialized ("closed" or "definitive") niche that determines the stem cell fates. Because niche-derived signals appear to be spatially restricted, cells that are located within the niche can be maintained in an undifferentiated state, and their displacement from the niche leads to differentiation. In mouse testis, in contrast, spermatogenic stem cells appear to be distributed over the basal compartment and intermingled with differentiating

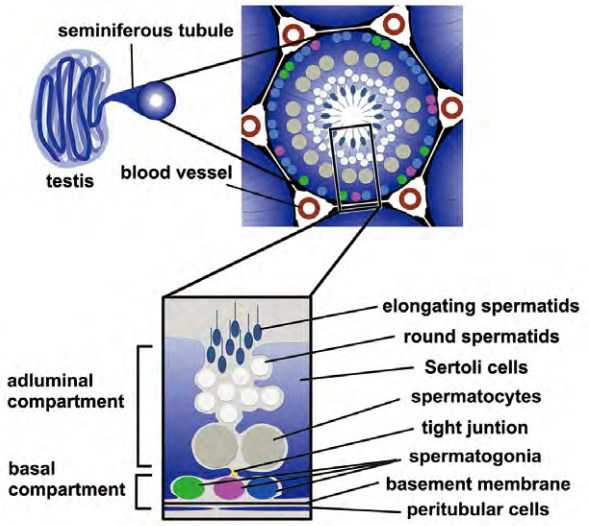

Figure 2. Anatomy of seminiferous tubules and seminiferous epithelium. GFRα1$^+$ and Ngn3$^+$ cells and KIT$^+$ differentiating spermatogonia, reside in the basal compartment (between the basement membrane and the tight junction of Sertoli cells). Modified from Ikami *et al*., Development (2015).

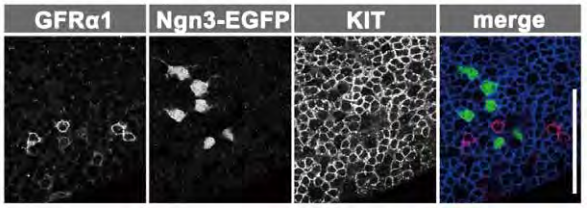

Figure 3. Intermingling of GFRα1$^+$, Ngn3$^+$, and KIT$^+$ spermatogonia. Representative images of triple immuno-stained whole-mount seminiferous tubules of an *Ngn3-EGFP* mouse. Scale bars = 50 μm. Reproduced from Ikami *et al*., Development (2015)

progeny, designated as an "open" niche. Here, the details of the mechanisms that determine whether stem cells differentiate or remain undifferentiated are unknown.

Mouse spermatogenesis occurs in seminiferous tubules and represents a typical example of an open niche-supported stem cell system (Figure 2) (Russell *et al*., 1990; Stine and Matunis, 2013). Here, GFRα1$^+$ (roughly corresponding to "actual" stem cells), Ngn3$^+$ (nearly "potential" stem cells), and Kit$^+$ ("differentiating" spermatogonia that have lost most stem cell potential) populations of spermatogonia are intermingled with each other in the basal compartment of the seminiferous tubules (Figure 3). These cells are uniformly exposed to retinoic acid (RA), an essential differentiation-inducing signaling molecule that is synthesized in a spatially ubiquitous but temporally periodic manner. Intriguingly, however, all of A_{undiff} cell do not differentiate, rather some A_{undiff} always remain undifferentiated. Although this process is extremely important for the long-term maintenance of the stem cells and the integrity of spermatogenesis, the underlying mechanism has been largely unknown.

2-2. Heterogeneous differentiation competence in response to retinoic acid

To address this issue, we tested the response of Ngn3$^+$ and GFRα1$^+$ spermatogonia to RA by pulse-labeling these populations using the tamoxifen-inducible cre-loxP system. The results indicated that Ngn3+ cells rapidly and efficiently differentiated into KIT$^+$ cells in response to RA. In contrast, GFRα1$^+$ cells did not show effective differentiation induced by RA, but they generated Ngn3$^+$ cells in an RA-independent manner. Thus, GFRα1$^+$ and Ngn3$^+$ cells show distinctive differentiation competence in response to RA.

Next, through DNA microarray gene expression analysis of FACS-sorted GFRα1$^+$ and Ngn3$^+$ fractions, we found that the expression of Retinoic acid receptor gamma (RARγ) was highly specific in Ngn3$^+$ cells compared to GFRα1$^+$ cells (Figure 4). In contrast, the levels of other transcripts related to the RA signaling pathway were similar between these cells, Further, we found that enforced expression of RARγ provided the GFRα1$^+$ cells with differentiation competence to differentiate to KIT$^+$ cells in response to RA.

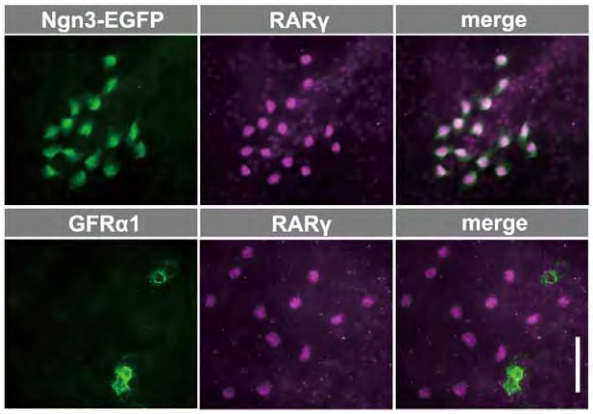

Figure 4. Predominant expression of RARγ in NGN3$^+$ spermatogonia compared with GFRα1$^+$ cells.
Representative images of whole-mount seminiferous tubules from *Ngn3-EGFP* mice stained for EGFP, GFRα1 and RARγ. Scale bars = 50 μm. Reproduced from Ikami *et al*., Development (2015)

2-3. Differential dynamics of GFRα1$^+$ and Ngn3$^+$ spermatogonia

To summarize, these results inferred that, when exposed to a high concentration of RA, RARγ drives the Ngn3$^+$ cells to differentiation, while the absence of RARγ allows the GFRα1$^+$ cells to remain undifferentiated. This heterogeneous differentiation competence, combined with the periodically but ubiquitously distributed RA, appears to be important for homeostatic spermatogenesis in the open niche environment of seminiferous tubules (Figure 5). This scenario would be paradigmatic for other open niche-supported tissue stem cells, since it would allow the stem cell population to maintain the undifferentiated pool while periodically producing differentiating progeny.

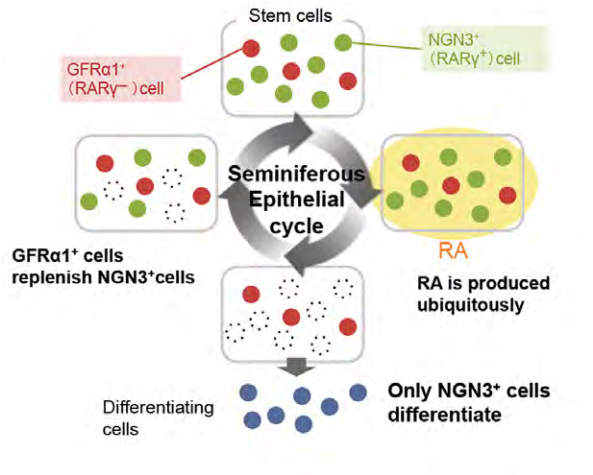

Figure 5. A model of stem cell dynamics during the seminiferous epithelial cycle.
Before the elevation of RA, the entire A_{undiff} population is composed of a mixture of GFRα1$^+$ (RARγ$^-$) and Ngn3+ (RARγ$^+$) cells (top). When the RA amount increases, to which both GFRα1$^+$ and Ngn3$^+$ spermatogonia appear to be equally exposed (right), only Ngn3+ cells that express RARγ respond and differentiate into Kit$^+$ cells, while GFRα1$^+$ cells remain undifferentiated (bottom). Then, the GFRα1$^+$ cells replenish Ngn3$^+$ cells through a RA-independent mechanism (left). Modified from Ikami *et al*., Development (2015)

Publication List:

〔Original paper〕

- Ikami, K., Tokue, M., Sugimoto, R., Noda, C., Kobayashi, S., Hara, K., and Yoshida, S. (2015). Hierarchical differentiation competence in response to retinoic acid ensures stem cell maintenance during mouse spermatogenesis. Development *142*, 1582-1592.

〔Review Article〕

- Yoshida, S. (2015). From cyst to tubule: innovations in vertebrate spermatogenesis. WIREs Developmental Biology, doi: 10.1002/wdev.204