DIVISION OF GERM CELL BIOLOGY

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Mammalian spermatogenesis represents a highly potent and robust stem cell system. Decades of research, including detailed morphological examinations, post-transplantation repopulation, and *in vitro* culture, have made it one of the most intensively studied mammalian tissue stem cell systems. However, the nature of the stem cells and their control, as well as their niche, remains largely unknown. The Division of Germ Cell Biology aims to fully understand the mammalian spermatogenic stem cell system, mainly using mice. In 2009, our second year at NIBB, we have tackled a number of important issues in mouse spermatogenic stem cells.

I. Background: The mammalian spermatogenesis

Mammalian testes continually produce numerous sperm during the reproduction period. Lines of investigations that emerged in the 1950s and involve detailed morphological analyses established the backbone of mammalian spermatogenesis research. The morphologically most primitive spermatogonia in the adult mouse testis are A_s or A_{single} spermatogonia (single, isolated spermatogonia). Their progeny remain interconnected due to incomplete cytokinesis, forming syncytial cysts of 2^n cells (2, 4, 8, 16 etc.). It has been experimentally established that "undifferentiated spermatogonia" (or "A_{undiff}" hereafter), which contribute <1% of the entire testicular cells and consist of A_s, A_{pr} (A_{paired}; interconnected two-cell cysts), and A_{al} ($A_{aligned}$; cysts of 4, 8, 16 or occasionally 32 cells) contain stem cells.

Then, which subfraction of A_{undiff} consists of the actually self-renewing stem cell compartment in homeostasis and how do they behave (proliferate, self-renew or die) in the testis? The prevailing, so-called "A_s model", which was originally proposed in 1971, proposes that A_s is the only cell type that can act as stem cells, while the interconnected population of A_{undiff} (A_{pr} and A_{al}) is devoid of stem cell capacity.

II. Toward the nature of the mouse spermatogenic stem cell system that includes reversibility

Despite that the "A_s model" is comprehensive and persuasive, this model is not based on the direct analyses of the cells' behavior. Thus, the A_s model warrants functional evaluations.

Similarly, corollaries of this model are that all the A_s cells are functionally equivalent and uniformly act as the stem cells, and that this defined population of 'stem cells' plays an active role in every aspect of the stem cell functions, i.e., maintenance of homeostasis, post-transplantation colony formation, and post-injury tissue regeneration.

We have previously shown that no single stem cell population acts in every aspect of stem cell function: Cells that support the steady-state spermatogenesis are different from those that support colony-formation and/or regeneration (Nakagawa et al., Dev. Cell, 2007). Based on this finding, we propose the functional hierarchy in the spermatogenic stem cell compartments as shown in Figure. 1. We propose that in addition to the actually self-renewing population (actual stem cells), an extended population of cells that retain the self-renewing potential but are destined for differentiation (potential stem cells) exist, which plays an important role for the continuity and robustness of the stem cell system. In case of actual stem cell loss, potential stem cells might revert to the self-renewing mode and replenish the actual stem cell pool (Figure 1).

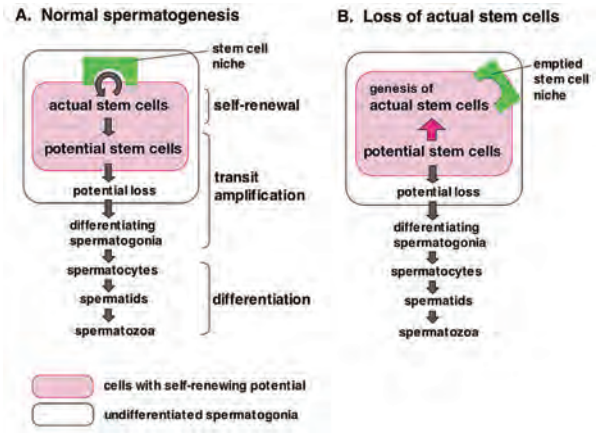

Figure 1. Proposed model of the functional compartments in mouse spermatogenesis (Nakagawa et al., 2007).

In 2009, by revisiting the A_s model, we investigated the cell population of A_{undiff}. For this attempt, we introduced a gene expression profile for describing the heterogeneity of the cells, in addition to the number of chained cells or the length of the cysts, on which the "A_s model" entirely depended on. Several studies, including ours, have been establishing that the populations of cysts that compose the same number of spermatogonia, which are classically considered to be homogeneous, are indeed heterogeneous in terms of patterns of gene expression. Therefore, a new scope has been raised that the entire A_{undiff} population is more heterogeneous than has previously been considered.

We have also investigated the behaviors of these A_{undiff} subpopulations in homeostasis (steady-state spermatogenesis) and during regeneration after tissue insult. We revealed that a particular subset of A_{undiff} changes their behavior dynamically between homeostasis and regeneration: In steady-state, they differentiate without self-renewal, but in

regeneration, they are willing to get back into a self-renewing stem cell state. This finding provides a novel view for stem cell biology and explains why the stem cells can change the behavior between homeostasis and regeneration so smoothly. In homeostasis, the number of stem cells should be constant, while in regeneration, they increases to recover the stem cell pool quickly. This feature is crucial for the continuity of tissue functions.

III. Investigating the temporal control of differentiation and establishment of the testicular tissue

In seminiferous tubules, spermatogenic differentiation occurs in a periodical manner, which results in the formation of beautiful stratification of different steps of differentiating cells within the seminiferous tubules (Figure 2A). Essentially, this is caused by the periodical differentiation of A_{undiff} with a regular interval of 8.6 days (white arrows in Figure 2B).

We have investigated how this periodical event is controlled. This question can be generalized to how the timing of A_{undiff} differentiation is regulated. We paid special attention to the involvement of Sertoli cells and the role of retinoic acid (RA) signaling. The importance of RA has been recognized for decades based on the finding that deficiency in vitamin A (VA), the dietary precursor of RA, causes a severe spermatogenesis defect in which differentiation of A_{undiff} is affected, and differentiating cell types are all lost.

We have revisited this VA-deficient (VAD) model and revealed the central role of RA not only in the control of the timing of A_{undiff} differentiation, but also in the regulation of the functional change of the supporting somatic Sertoli cells, which are huge cells that nourish the stratified germ cells. The local retinoid metabolism in the seminiferous tubules involves multiple cell types of germ and somatic cells. Therefore, co-operation between different cell types that are located adjacently may control the differentiation of A_{undiff}.

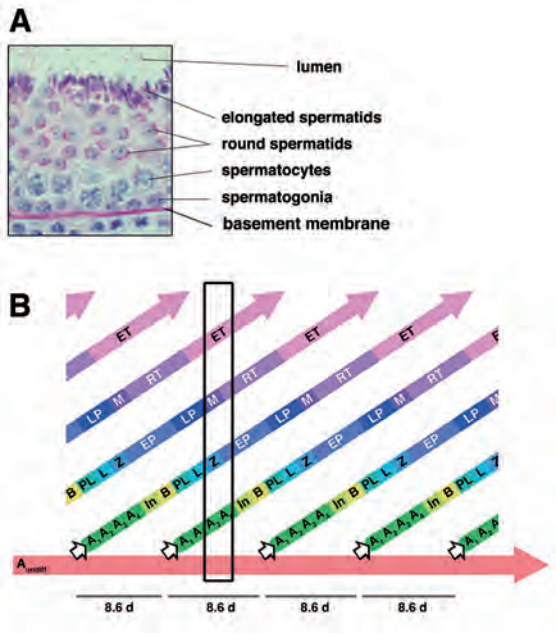

Figure 2. Stratified tissue architecture in mouse seminiferous tubules.

(A) Appearance of a representative part of the seminiferous tubules. Note the beautifully stratified germ cells at different steps of differentiation.

(B) A scheme that explains the formation of the stratified architecture shown in (A). The population of A_{undiff} (red) persists for a long period and gives rise to A1 differentiating spermatogonia periodically with a regular interval of 8.6 days. As a result, a regular stratification of the differentiating germ cells is formed, as indicated by the rectangle.

IV. Investigating the nature of the stem cell niche

Evidence suggests an intimate relationship between stem cells and the niche microenvironment in seminiferous tubules. It is difficult to identify the nature and function of the niche, however, because seminiferous tubules do not exhibit suspicious sub-structures. Moreover, actual stem cells can be identified only functionally, and their histological detection has not yet been achieved. Therefore, our current aim has been to clarify the niche of A_{undiff}.

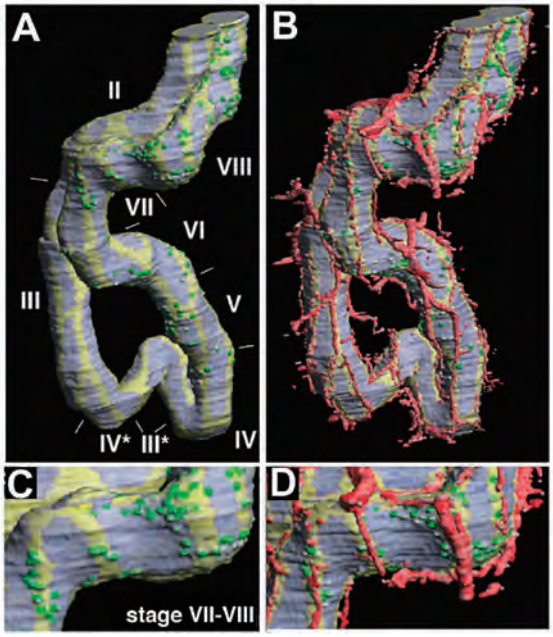

Figure 3. Localization of A_{undiff} revealed by three-dimensional reconstruction (Yoshida et al., Science, 2007)

Computationally reconstituted three-dimensional images of the seminiferous tubules based on 280 serial sections. A_{undiff} (green) show biased localization to the blood vessel network (red) and the area adjacent to the interstitium (yellow). (A, C) and (B, D), without or with blood vessels, respectively. Roman numerals indicate the stage of the seminiferous epithelium.

The seminiferous tubules exhibit a convoluted tubular structure with a diameter of ~200μm: Individual tubules connect to the common outlet of the mature sperm (rete testes) with both ends and form loops. Spermatogenesis occurs evenly throughout the inner surface of the tubules. Therefore, in mouse testis, an overall 'polarity' that covers the entire organ cannot be recognized, making a good contrast to the *Drosophila melanogaster* germline stem cell system.

By taking advantages of live-imaging and three-dimensional reconstruction, we previously revealed that A_{undiff} preferentially localizes to the limited regions of seminiferous tubules that are adjacent to blood vessels and interstitial cells that surround the tubules (Figure. 3). In addition, the dynamic migration of spermatogonia from the vasculature proximity to spread throughout the tubules was also observed upon differentiation of A_{undiff} (Yoshida et al., Science, 2007). Based on these findings we proposed that this area may act as the niche for A_{undiff} (Figure 4). We are tackling the cellular and molecular identification of this vasculature-associated niche.

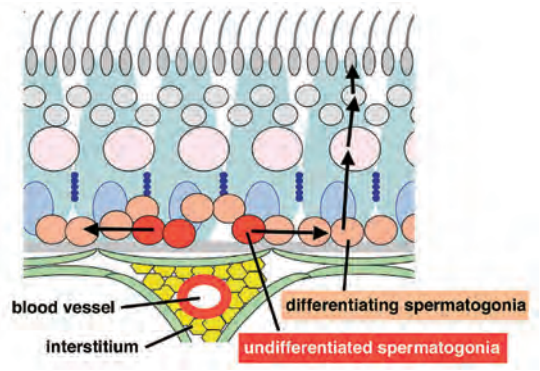

Figure 4. A proposed niche microenvironment for A_{undiff}.

Although the seminiferous tubule is a repeat of the same structure, the proximity to the surrounding blood vessels and interstitial cells specializes the particular region of the basal compartment of the tubules, so that it can act as the niche for undifferentiated A_{undiff} population.

Publication List

〔Original papers〕

- Hara, K., Kanai-Azuma, M., Uemura, M., Shitara, H., Taya, C., Yonekawa, H., Kawakami, H., Tsunekawa, N., Kurohmaru, M., and Kanai, Y. (2009). Evidence for crucial role of hindgut expansion in directing proper migration of primordial germ cells in mouse early embryogenesis. Dev. Biol. *330*, 427-439.
- Suzuki, H., Sada, A., Yoshida, S., and 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. Dev. Biol. *336*, 222-231.

〔Original paper (E-publication ahead of print)〕

- Uemura, M., Hara, K., Shitara, H., Ishii, R., Tsunekawa, N., Miura, Y., Kurohmaru, M., Taya, C., Yonekawa, H., Kanai-Azuma, M., and Kanai, Y. (2009). Expression and function of mouse Sox17 gene in the specification of gallbladder/bile-duct progenitors during early foregut morphogenesis. Biochem. Biophys. Res. Commun. 2009 Nov. 12

〔Review Articles〕

- Yoshida, S. (2009). Spermatogenic stem cell system in the mouse testis. Cold Spring Harb. Symp. Quant. Biol. *73*, 25-32.
- Yoshida, S. (2009). Casting back to stem cells. Nat. Cell Biol. *11*, 118-120.