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, as well as their niche, remains largely unknown in the context of homeostatic spermatogenesis. The Division of Germ Cell Biology, which was launched in 2008, aims to fully understand the mammalian spermatogenic stem cell system, mainly using the mouse system. Being a brand-new research division of NIBB, our overall research view and current foci of interests will be described. These include 1) the cellular nature of the stem cell compartment and their behaviors in the homeostatic spermatogenesis, and 2) the anatomical basis and function of the mammalian spermatogenic stem cell niche.

Ⅰ. Identifying the mouse spermatogenic stem cell compartments

1-1. Background: The mammalian spermatogenesis

Mammalian testes continuously produce numerous sperm during the reproduction period. 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 chains 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 pairs), and A_{al} ($A_{aligned}$; chains of 4, 8, 16 or occasionally 32 cells) contain stem cells.

Which fraction 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 so-called "A_s model" is currently most widely considered to be true. This model 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. This comprehensive model, however, is based upon "snapshots" of fixed specimens and involves too many theoretical limits to be entirely conclusive regarding stem cell function because "stem cells" by definition maintain themselves while producing differentiating progeny for a long period.

Transplantation of a single cell suspension into the recipient testis enables the functional and quantitative analyses of the "stem cells" based on their repopulating activity in the host testis. Combined with the morphology-based A_s model, it is often considered to be the case that all A_s spermatogonia are equivalent and act as stem cells, which support both homeostatic spermatogenesis and post-transplantation repopulation, despite the inherent limitation due to the lack of direct functional evidence.

1-2. Heterogeneous composition of the stem cells

In order to understand the nature of the mouse spermatogenic stem cell system in testicular tissue, we have been investigating the behavior and function of A_{undiff}. Authentic identification of A_{undiff} is based on morphology under electron or high-resolution light microscopy. Using the genetic labeling of A_{undiff} by means of the *Ngn3* regulatory sequence (Yoshida et. al., *Dev. Biol.* 2004), we have established experimental systems to investigate their live behaviors without disturbing normal tissue architecture.

We first asked whether the "stem cells" detected by transplantation are identical to the "stem cells" that actually self-renew in homeostasis, or whether these two "stem cells" represent different groups of cells. In order to address this question, a tamoxifen-dependent Cre recombinase (CreER^{TM}) was expressed in the Ngn3+ A_{undiff} (Yoshida et al., *Development* 2006). Together with an appropriate reporter transgene, this has enabled the irreversible pulse-labeling of A_{undiff} spermatogonia upon transient administration of tamoxifen, and the first quantitative detection of "actual stem cells" (i.e., the cells that persist for a long time while producing differentiating progeny, thus supporting tissue homeostasis). Intriguingly, the contribution of the pulse-labeled subpopulation of A_{undiff} to "actual stem cells" and "post-transplantation colony-forming stem cells" represents a great difference (approximately 40 times higher in the latter than in the former). Therefore, these two "stem cells" represent different subpopulations of A_{undiff}. We concluded that, in addition to actual stem cells, an extended population exists that does not self-renew while retaining the potential of self-renewal, which was defined as a population of "potential stem cells" (Nakagawa et al., *Dev. Cell* 2007).

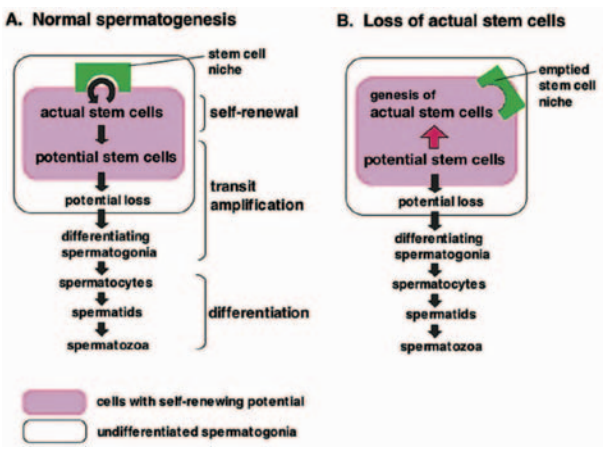

Figure 1. Proposed model of the functional compartments in the mouse spermatogenesis (Nakagawa et al., *Dev. Cell* 2007).

The "potential stem cells" were shown to rapidly turn over in homeostasis, suggesting that they consist of a transit-amplifying compartment.

Figure 1 shows our simplest interpretation of the hierarchical composition of the mouse spermatogenic stem cell system (Nakagawa et al., *Dev. Cell* 2007). In case of actual stem cell loss, potential stem cells might revert to the self-renewing mode and replenish actual stem cells. We are currently investigating the cell-biological characteristics of actual and potential stem cells.

Ⅱ. Testicular niche for A_{undiff}

2-1 Anatomy of the mouse testis and the research background

Spermatogenesis proceeds inside the seminiferous tubules, 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 seminiferous epithelium (the inner surface of the tubules). Therefore, in the 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.

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 is to clarify the niche of A_{undiff}.

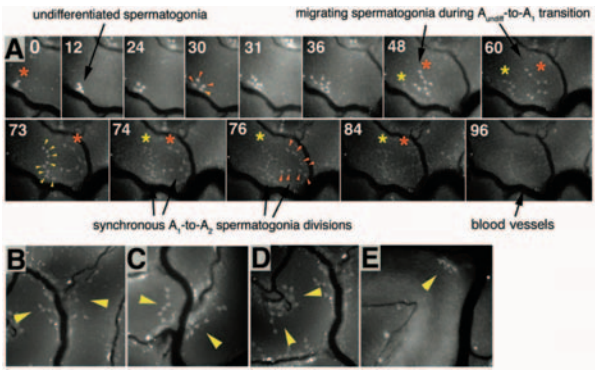

Figure 2. Localization of GFP-labeled Ngn3+ A_{undiff} and their relocation upon transition into differentiating spermatogonia (Yoshida et al., *Science* 2007).

(A) Live imaging of spermatogonia upon differentiation of A_{undiff}. Before differentiation (0 hour; the elapsed time indicated in each panel in hours), labeled A_{undiff} preferentially localized to the area adjacent to the blood vessels (seen as a black line) and surrounding interstitium. Upon differentiation, two chains of 8-cell cysts ($A_{al\text{-}8}$; indexed in yellow and orange) migrated from this position to spread all over the tubule (36-60h). Subsequently, the two cysts underwent synchronous mitotic division with 2-3 hours' interval, resulting in the formation of two 16-cell cysts of differentiating spermatogonia (73-74h).
(B-E) Examples of the vasculature-proximal localization of A_{undiff}. A_{undiff} (arrowhead) preferentially localized to areas adjacent to blood vessels, more characteristically to their branch points. In (B-C), A_{undiff} in neighboring seminiferous tubules show back-to-back localization over branching vessels.

2-2. Live imaging and the vasculature-associated niche for A_{undiff}

We have developed a live imaging system in which GFP-labeled Ngn3+ A_{undiff} and their progeny can be continuously filmed in undisturbed testes. It was revealed that A_{undiff} preferentially localize to the area adjacent to blood vessels and interstitial cells that surround the seminiferous tubules. In addition, the dynamic migration of spermatogonia from the vasculature proximity to spread throughout the tubules was also observed upon differentiation of A_{undiff} (Figure 2). The same relocation was also supported by three-dimensional reconstruction based on authentic morphological identification of A_{undiff} on serial sections (Figure 3). These extend the preceding observations from mouse and rat testis sections that A_{undiff} shows a significant biased localization to the interstitium, and we proposed such area as the niche for A_{undiff} (Yoshida et al., *Science* 2007).

We are currently investigating the cellular and molecular identification of this vasculature-associated niche.

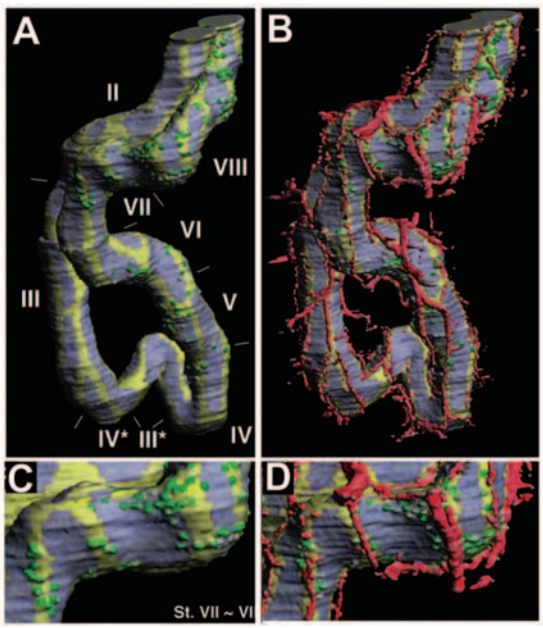

Figure 3. Localization of A_{undiff} revealed by three-dimensional reconstruction (Yoshida et al., *Science* 2007)

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.

Publication List

〔Original paper〕

- Sato, Y., Watanabe, T., Saito, D., Takahashi, T., Yoshida, S., Kohyama, J., Ohata, E., Okano, H., and Takahashi, Y. (2008). Notch mediates the segmental specification of angioblasts in somites and their directed migration toward the dorsal aorta in avian embryos. Dev. Cell. *14*, 890-901.