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 mouse sperm stem cell system.

In 2010, we revealed a couple of important features of mouse sperm stem cells. One is the discovery of a hierarchy between subpopulations of 'undifferentiated spermatogonia', in which the stem cell functions reside, and reversibility between these subpopulations. This was a proposal that warrants a re-evaluation of a long-held theory in this field. Second is the finding that stem cells are not in a state where they always experience asymmetric division and persist for the entire life span of an organism, but that stem cells replace each other frequently and support spermatogenesis as a population.

I. Hierarchy and reversibility within undifferentiated spermatogonia

Lines of morphological investigations that emerged in the 1950s 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" (A_{undif}), which contribute <1% of the entire testicular cell population 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. Undifferentiated spermatogonia then transform into A_1 stage of 'differentiating spermatogonia', as they lose their vast self-renewing potential (Figure 1).

The prevailing "A_s model" (Figure 2), which was originally proposed in 1971, supposes that A_s is the only cell type that can act as stem cells, while the interconnected

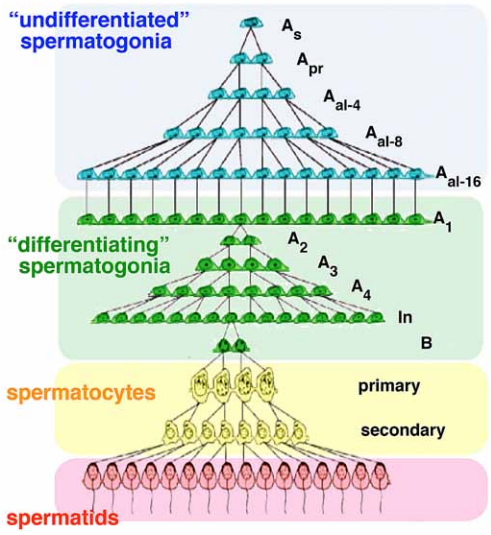

Figure 1. Spermatogenic cell types found in mature mouse testis. Spermatogonia, spermatocytes, and spermatids correspond to the mitotic, meiotic and haploid cells respectively. 'Undifferentiated' spermatogonia population harbors the stem cell functions. Reprinted from Develop. Growth Differ. *52*, 311-317 (2010), with permission.

population of A_{undiff} (A_{pr} and A_{al}) is devoid of stem cell capacity. 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 population plays active roles in every aspect of the stem cell functions, i.e., maintenance of steady state and regeneration after tissue insult and transplantation. However comprehensive and persuasive the "A_s model" is, this was not based on the direct analyses of the cells' behavior. Therefore, the A_s model warrants functional evaluations. It was previously shown by our group that no single stem cell population acts in every aspect of stem cell function: Cells supporting the steady state spermatogenesis are different from those that support regeneration (Nakagawa et al., Dev. Cell, 2007). However, the precise cellular identity that supports these stem cell functions remained to be elucidated.

In 2010, we revealed that a gene expression profile visualizes the heterogeneous nature of the undifferentiated spermatogonia population, in addition to the number of chained cells or the length of the syncytial cysts (Nakagawa

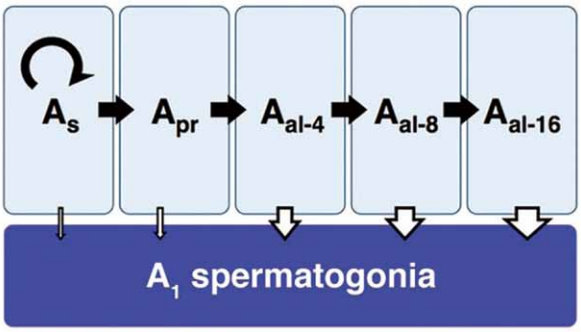

Figure 2. The 'A_s model'. Schematically shown on the basis of theories proposed by the groups of Huckins, Oakberg and de Rooij. Reprinted from Science *328*, 62-67 (2010), with permission

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2010. The former title is indicated by an asterisk (*).

et al., Science 2010). We established that the populations of cysts that compose the same number of spermatogonia are heterogeneous in their expression of GFRα1 (glial cell line-derived neurotropic factor receptor 1; shown in magenta in Figure 3) and Ngn3 (neurogenin3; green) genes.

We then investigated the steady-state behavior of these subpopulations of undifferentiated spermatogonia by means of pulse-labeling and live imaging. It was shown that the bulk of the Ngn3+ population differentiate into longer cysts (rightward black arrows in Figure 3) and Kit+ differentiating spermatogonia (shown in blue) as represented by downward white arrows, and that the Ngn3+ cells are supplied from GFRα1+ spermatogonia (downward black arrows). As a logical consequence, the GFRα1+ population is postulated to

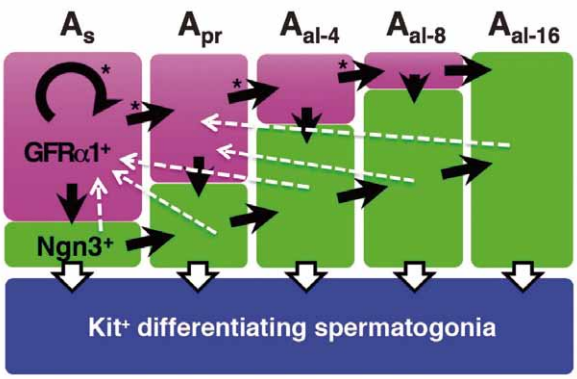

Figure 3. A modified version of 'As model'. Based on the proposal by Nakagawa et al. (2010). See text for the detail. Arrows with asterisks are not confirmed but sill hypothetical. Reprinted from Science *328*, 62-67 (2010), with permission.

function as the main body of the self-renewing compartment.

Interestingly, a very small portion of Ngn3+ cells did 'revert' back into being GFRα1+ and shorter chains (dotted arrows in Figure 3) in steady state. This accompanies fragmentation of syncytial cysts as shown in Figure 4: This phenomenon was not assumed in the mouse 'A_s model', while observed in fruit fly germlines. During regeneration,

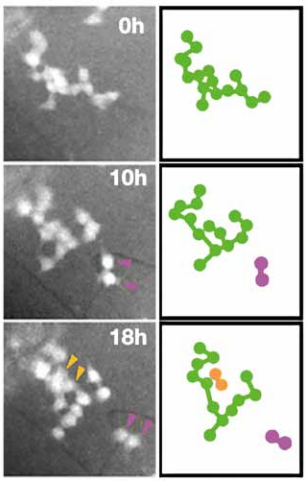

Figure 4. An example of fragmentation of A_{al-16} cyst spermatogonia observed by live imaging. Two pairs of spermatogonia (magenta and orange, morphologically defined as A_{pr}'s) were pinched off an A_{al-16} spermatogonia. Elapsed times are shown.

Ngn3+ cells return to a self-renewing stem cell state much more frequently than in steady state. These observations proposes modification of the A_s model and explain how the stem cell population change their behavior so that they remain constant in steady state, while increasing during regeneration to recover the stem cell pool quickly.

II. Rapid and stochastic turnover between the sperm stem cells

It has been believed that stem cells, including that of mammalian spermatogenesis, are preserved in steady-state cycling tissues as they repeat an asymmetric division that produces one self-renewing and one differentiating daughter cell (Figure 5a). Indeed, such a "stem cell-type" division has been observed only in a limited number of instances including fruit fly germline stem cells, but remains to be evaluated in most systems including mouse spermatogenesis.

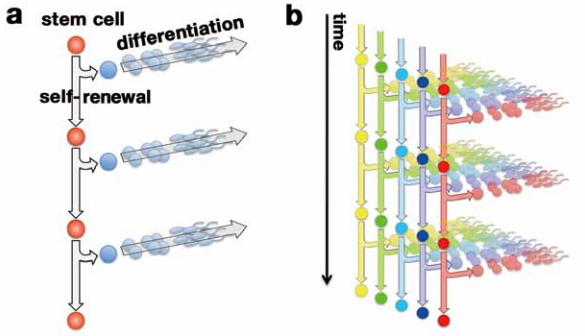

Figure 5. A classical view of stem cell behavior based on the idea of asymmetric division. (a) A single stem cell undergoes a sequence of asymmetric division that produces one self-renewing and one differentiating daughter cell. (b) Given that all the stem cells act as in (a), the stem cell repertoire and each stem cell's differentiating progeny (shown in different colors) will be stable over time in the tissue.

We analyzed the long-term behavior of steady-state mouse sperm stem cells that were pulse-labeled using an inducible cre-loxP system for over a year after pulse (Klein et al., Cell Stem Cell 2010; Nakagawa et al., Dev. Cell 2007), in collaboration with Ben Simons (Cambridge University).

If we suppose that the individual stem cells are preserved as a result of repeated asymmetric divisions, the stem cell repertoire should be constant and the number and the size of the stem cell cohorts will also be constant (Figure 5b). However, the number of the observed stem cell-derived clones decreased while the surviving clones expanded in size (Figure 6a). Mathematical analyses indicate that stem cells disappear frequently and stochastically with a surprisingly short average longevity of less than two weeks, and that the lost stem cells are replenished by the progeny of the neighboring stem cells (Figure 6b).

These findings represent a new idea for the functionality of a stem cell compartment, where cells replace each other and maintain themselves and supply differentiating progeny as a population.

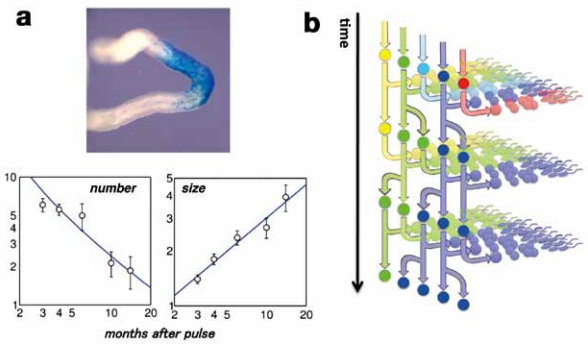

Figure 6. Actual behaviors of pulse-labeled sperm stem cells in seminiferous tubules. (a) Upper panel, a LacZ-labeled single sperm stem cell-derived clonal patch in seminiferous tubules three months after pulse. Visualized by X-gal reaction. Lower panels: Number (left, per testis) and size (right, length in mm) of the surviving pulse-labeled patches indicated periods after pulse (shown in months). (b) A schematic of stem cell behavior in steady state spermatogenesis. Stem cells show frequent replacement by their neighbors, making the stem cell repertoire unstable.

III. Perspectives

We feel that the above findings elucidate novel and fundamental features of mouse sperm stem cells. Accordingly one of the next steps is to provide a molecular basis of the reversibility underlying stem cell differentiation and reversion, as well as the mutual replacement happening between the stem cells.

We will be also investigating aspects of the environmental control of the stem cell population. One is the spatial regulation: We have previously observed that undifferentiated spermatogonia preferentially localize to the vasculature-proximal region (Yoshida et al., Science 2007), while the details of the nature of these 'niche' regions is still unknown. Second is temporal regulation: The differentiation of undifferentiated spermatogonia does not occur randomly but shows a beautiful periodicity once every 8.6 days, representing the seminiferous epithelial cycle.

We hope these studies will shed light and lead to the better understanding the mouse sperm stem cell system.

Publication List

〔Original papers〕

- Kitadate, Y., and Kobayashi, S. (2010). Notch and Egfr signaling act antagonistically to regulate germ-line stem cell niche formation in *Drosophila* male embryonic gonads. Proc. Natl. Acad. Sci. USA *107*, 14241-14246.
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- Matson, C.K., Murphy, M.W., Griswold, M.D., Yoshida, S., Bardwell, V.J., and Zarkower, D. (2010). The mammalian doublesex homolog DMRT1 is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells. Dev. Cell *19*, 612-624.
- Nakagawa, T., Sharma, M., Nabeshima, Y., Braun, R.E., and Yoshida, S. (2010). Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science *328*, 62-67.
- Nakane, Y., Ikegami, K., Ono, H., Yamamoto, N., Yoshida, S., Hirunagi, K., Ebihara, S., Kubo, Y., and Yoshimura, T. (2010). A mammalian neural tissue opsin (Opsin 5) is a deep brain photoreceptor in birds. Proc. Natl. Acad. Sci. USA *107*, 15264-15268.
- Uemura, M., Hara, K., Shitara, H., Ishii, R., Tsunekawa, N., Miura, Y., Kurohmaru, M., Taya, C., Yonekawa, H., Kanai-Azuma, M., and Kanai, Y. (2010). Expression and function of mouse Sox17 gene in the specification of gallbladder/bile-duct progenitors during early foregut morphogenesis. Biochem. Biophys. Res. Commun. *391*, 357-363.

〔Review Article〕

- Yoshida, S. (2010). Stem cells in mammalian spermatogenesis. Develop. Growth Differ. *52*, 311-317.