DIVISION OF GERM CELL BIOLOGY

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Mammalian testes produce numerous sperm for a long period. This property that is fundamental for continuity of life across generations is supported by the robust stem cell system, which is defined by the ability of proliferation that is associated with self-renewal (maintenance of their own undifferentiated population) and differentiation (production of different cell type(s)),

The Division of Germ Cell Biology, which was launched in 2008, aims to fully understand the mouse sperm stem cell system *in vivo* under the context of tissue architecture of testis. So far, we have revealed some characteristics of this potent and interesting stem cell system. First, differentiating germ cells that had been believed to be irreversibly committed to differentiation still retain the self-renewing potential and can contribute to stem cell pool maintenance ("potential stem cells"). Secondly, "reversion" from potential stem cells occurs at a higher frequency when testicular tissue is damaged and regeneration is induced. Thirdly, the undifferentiated spermatogonia population that includes both "actual" and "potential" stem cells localized to the vasculature (vascular- associated niche). We also discovered that stem cells turn over in an unexpectedly frequent and mathematically stochastic manner under steady-state situations that continuously produce sperm. (Nakagawa et al., Dev. Cell 2007, Yoshida et al., Science 2007; Nakagawa et al., Science 2010; Klein et al., Cell Stem Cell 2010).

In 2012, we have extended these investigations for the deeper understanding of this stem cell system. Firstly, we have investigated the nature and the behavior of the stem cells at a single cell resolution. Secondly, we have challenged the cellular and molecular nature of the vascular-associated niche. Thirdly, a mechanism underlying the coordinated spatio-temporal regulation of stem cells has been proposed.

I. Stem cell behavior at a single cell resolution in undisturbed testicular tissue

For the purpose of function-based investigation of stem cells, it is essential to analyze the behavior of cells over time. We have previously established experimental systems to study stem cell behavior in mouse spermatogenesis over time, without affecting the tissue architecture, namely live-imaging and pulse-labeling experiments. For live-imaging, taking advantage of the fluorescent protein-labeling, we have achieved continual filming of particular subsets of spermatogonia in the testes (Figure 1: Yoshida et al., Science 2007). For pulse-labeling, we have also established irreversible labeling of a particular spermatogonial population using tamoxifen-dependent activation of cre recombinase fused with a domain of the mutated human estrogen receptor (Figure 2: Yoshdia et al., Development 2006, Nakagawa et al., Dev. Cell 2007, Nakagawa Science 2010).

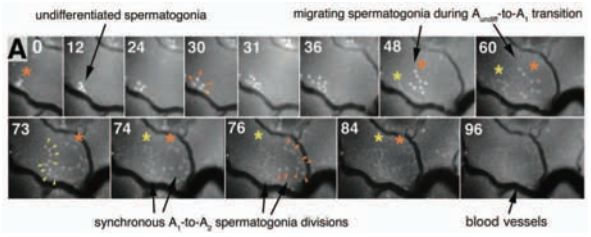

Figure 1. Live-imaging of fluorescence-labeled cells. An example of live-imaging observation of spermatogonia in a living testis. Spermatogonia labeled with GFP under Ngn3 promoter (white) were continuously filmed, shown cell division (30h and 76h) and active migration. A part of selected frames are shown. Numerals indicate the elapsed time in hours. (From Yoshida et al. Science 2007)

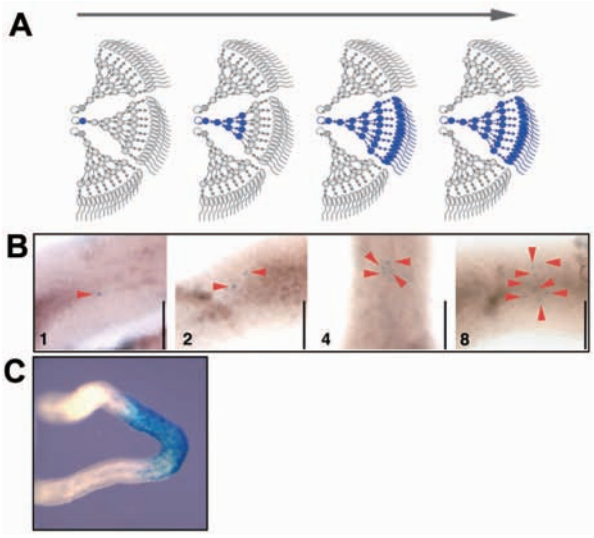

Figure 2. Pulse-labeling of subset spermatogonia with marker expression. (A) Principle of pulse-label of a stem cell. When a self-renewing stem cell is pulse-labeled, its descendants (shown in blue here) include both stem and differentiating cells. Then, the clone will persist in the tissue, as shown in the actual experiments in the following panels. (B) Spermatogonia labeled with LacZ expression (colored in blue) observed 2 days after induction of labeling with Tamoxifen administration. A_{single} (1), A_{pair} (2), $A_{aligned\text{-}4}$ (4), $A_{aligned\text{-}8}$ (8) are shown. (C) Three months after the pulse, labeled cells that are descendant of the induced spermatogonia form patches occupying a continual segment of the tubules including stem and differentiating cells. This reflects the stem cell function of the originally labeled cell. (From Yoshida Reproduction 2012 and Nakagawa et al., Dev. Cell 2007)

In addition, our results strongly suggest that within the undifferentiated group of spermatogonia consisting of A_{single}, A_{paired} and $A_{aligned}$ cells, a small subset expressing GFRα1 (glial cell line-derived neurotropic factor receptor alpha 1) is responsible for the stem cell functions, while those expressing Ngn3 (neurogenin 3, a bHLH transcription factor) are destined for differentiation while maintaining their potential to get back into being GFRα1-positive and self-renew (Nakagawa et al., 2010).

In addition, in collaboration with Professor Benjamin Simons, a theoretical physicist of Cambridge University, we have shown that the spermatogenic stem cells show a stochastic and continuous turnover between neighbors (Nakagawa et al., Dev. Cell 2007, Klein et al., Cell Stem Cell 2010): Instead of the 'text book' type of asymmetric divisions that always give rise to one self-renewing and one differentiating daughter, fate selection for stem cells either to remain undifferentiated or to differentiate appears to be stochastic. As a result, fates of stem cell-derived cohorts are highly variable and show neutral competition between stem cell clones.

Based on these preceding findings, we began investigating the behavior of GFRα1+ spermatogonia in undisturbed testis using live-imaging and pulse-labeling at a single cell resolution. These have allowed us to directly demonstrate for the first time that this tiny subset of spermatogonia indeed acts as the stem cell pool supporting the steady-state turnover of spermatogenic cells. We are now intensively challenging the problem of how to link the actual single-cell-level behavior (such as cell division, fragmentation of spermatogonial syncytia, fate selection, whether to differentiate or remain undifferentiated, and death) of the GFRα1+ spermatogonia and the overall robustness of the stem cell system at a level of population, with the aid of mathematical modeling.

II. Nature of the stem cell niche microenvironment in the mouse testis

It is generally believed that some specialized microenvironment within tissue is involved in the stem cell system as an indispensable element that regulates the stem cell behavior, designated as the "stem cell niche". In contrast to well-defined examples of the stem cell systems accompanying anatomically defined niches where stem cells always settle and differentiation accompanies their exit from the niche region, such as *Drosophila* gonads, mammalian testis do not exhibit such a defined substructure. Therefore it has long been a mystery how stem cell behavior is regulated in the context of this tissue, namely the seminiferous tubules (Figure 3). Regarding this issue, it has been shown that, within the 'basal compartment' of seminiferous tubules, the undifferentiated spermatogonia show a preferential localization to the area adjacent to blood vessels that make a network running through the interstitial space between the seminiferous tubules (Chiarini-Garcia, Biology of Reproduction 2001, Yoshida et al., Science 2007). However, again, lacking a discrete niche structure, this example of a stem cell niche has been considered to stand on a facultative nature.

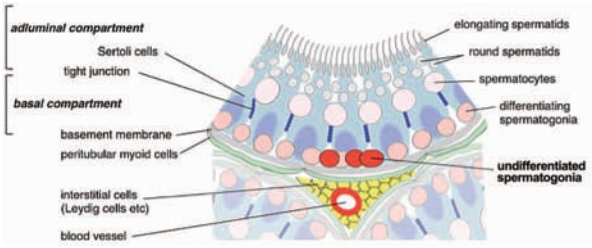

Figure 3. Architecture of seminiferous tubules, the spermatogenic center in the testis. Schema of seminiferous tubules. In basal compartment between the basement membrane and tight junction between Sertoli cells, undifferentiated spermatogonia (the population responsible for the stem cell pool) show a preferential localization to blood vessels running between the tubules. The differentiating progeny of the stem cells stratify into four layers, each of which is comprised of mitotic spermatogonia, meiotic spermatocytes, and haploid round and elongating spermatids. (Modified from Spradling et al, 2011)

Based on these preceding observations of our own and others, we hypothesize that the blood vessel-adjacent area is specialized and optimal for harboring and regulating the stem spermatogonia, and that this feature is reflected by the specialized gene expression in this area. We therefore have been challenging this issue by searching for genes showing localized expression to such a presumptive niche area. We first collected the area of interest using microdissection techniques on sectioned testis specimens, followed by cDNA microarray and *in situ* hybridization analyses to search for genes showing preferential expression. We have been studying these genes with regard to the spatial relationship between the sites of expression of these genes with undifferentiated (and stem) spermatogonia, as well as the possible function of these genes in stem cell regulation. These findings will provide basic and fundamental information on stem cell regulation in tissues harboring facultative niche.

III. Spatio-temporal stem cell regulation by retinoic acid metabolism

In seminiferous tubules, differentiation of Ngn3-positive undifferentiated spermatogonia into Kit-positive stage occurs periodically with an interval of 8.6 days, which is followed by programmed differentiation process toward spermatozoa that takes 35 days. This temporal regulation is essential for the integrity of the tissue and continuity of spermatogenesis in that this process supports the regular stratification of germ cells in the wall of seminiferous tubules from stem cells to mature testis (Figure 3).

Based on the classic finding that retinoids (whose prominent active form is retinoic acid) are essential for the timed differentiation of undifferentiated spermatogonia, we investigated how the differentiation of spermatogonia is regulated by retinoic acid signaling, taking advantage of experimental modulation of retinoid supply and examination of retinoid metabolism-related enzyme expression patterns (Sugimoto et al., Mech. Dev. 2012). It has been demonstrated that a series of enzymes involved in retinoic acid metabolism at different steps are expressed separately but in a beautifully

orchestrated manner among multiple differentiating germ cell types (i.e., meiotic and haploid cells) and somatic Sertoli cells (which nourish all the steps of germ cells in seminiferous tubules), suggesting strongly that RA concentration changes in a 8.6 day cyclic manner, and that it increases at the same timing with that of differentiation of spermatogonia into Kit-positive. In addition, expression of RA metabolism-related genes in Sertoli cells are under regulation of RA signaling, forming positive and negative regulatory loops, leading us to propose that it is RA itself that coordinates the orchestration of metabolism between neighboring cell types that constitute the seminiferous tubules (Figure 4).

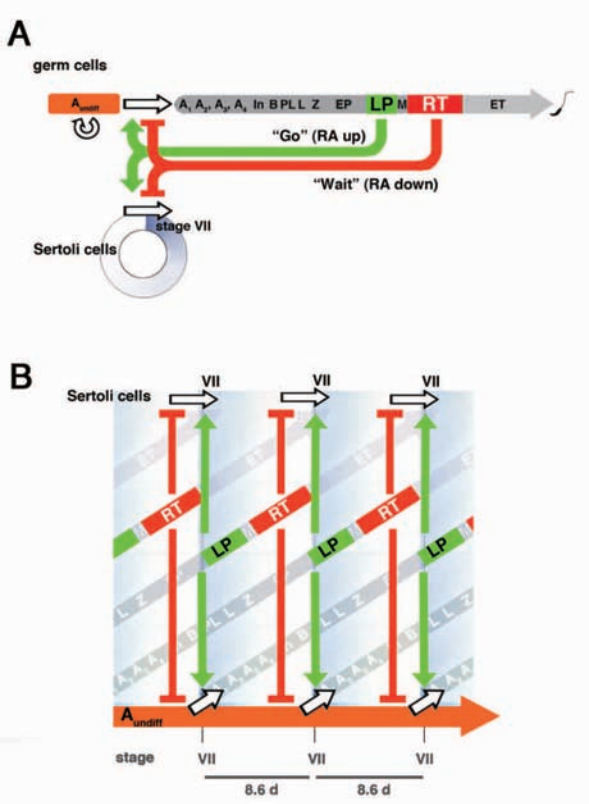

Figure 4. Proposed model for coordination of periodic differentiation of germ cells linked by retinoid metabolism. (A) Particular stages of meiotic cells (LP) and haploid cells (RT) send the *'go'* and *'wait'* signals to A_{undff} and Sertoli cells, by regulating the local RA metabolism to increase and decrease the RA concentration, respectively. (B) These signals will occur reciprocally and periodically to maintain the seminiferous epithelial cycle. (From Sugimoto et al., Mech. Dev. 2012)

IV. Perspectives

As described above, we have been tackling the robust stem cell system that supports mouse spermatogenesis from different aspects and scales. Once this research is achieved it will be quite intriguing to ask how these events are linked with each other and integrated into the robustness of the whole stem cell system. For that purpose, it is clear that we still need to know about molecules (and genes that encode these factors) that play essential roles but have not been identified. As well, mathematical analyses and modeling will be quite powerful to link the observations obtained in different scales.

Publication List

〔Original papers〕

- Koyanagi, S., Hamasaki, H., Sekiguchi, S., Hara, K., Ishii, Y., Kyuwa, S., and Yoshikawa, Y. (2012). Effects of ubiquitin C-terminal hydrolase L1 deficiency on mouse ova. Reproduction *143*, 271-279.
- Nakamura, Y., Usui, F., Miyahara, D., Mori, T., Ono, T., Kagami, H., Takeda, K., Nirasawa, K., and Tagami, T. (2012). X-irradiation removes endogenous primordial germ cells (PGCs) and increases germline transmission of donor PGCs in chimeric chickens. J. Reprod. Dev. *58*, 432-437.
- Sato, T., Yokonishi, T., Komeya, M., Katagiri, K., Kubota, Y., Matoba, S., Ogonuki, N., Ogura, A., Yoshida, S., and Ogawa, T. (2012). Testis tissue explantation cures spermatogenic failure in c-Kit ligand mutant mice. Proc. Natl. Acad. Sci. USA *109*, 16934-16938.
- Sugimoto, R., Nabeshima, Y., and Yoshida, S. (2012). Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of Sertoli cells in mouse seminiferous epithelium. Mech. Dev. *128*, 610-624.

〔Review article〕

- Yoshida, S. (2012). Elucidating the identity and behavior of spermatogenic stem cells in the mouse testis. Reproduction *144*, 293-302.