DIVISION OF GERM CELL BIOLOGY

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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 stem cell system.

The Division of Germ Cell Biology aims to fully understand the mouse sperm stem cell system under the context of *in vivo* tissue architecture. 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 for 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 including both "actual" and "potential" stem cells are preferentially localized near vasculature (vasculature-associated niche). We also discovered that stem cells turn over at an unexpected frequent and stochastic manner under a steady-state situation that continuously produces sperm (Nakagawa *et al*., Dev. Cell 2007; Yoshida *et al*., Science 2007; Nakagawa *et al*., Science 2010; Klein *et al*., Cell Stem Cell 2010). These observations have thrown doubt on the prevailing stem cell model, the "A_s model", postulating that the stem cell function is restricted to the singly isolated spermatogonia (A_s cells).

In 2013, we performed single-cell-level analyses of a tiny spermatogonial population (viz. GFRα1+) taking advantage of pulse-labeling and live-imaging studies, combined with biophysical modeling. The results indicate that the entirety of GFRα1+ spermatogonia (including both A_s and syncytia) comprise a single equipotent stem cell pool and cells continually interconvert between A_s and syncytial states. This novel paradigm would replace the "A_s model" that was proposed over 40 years ago (Hara *et al*., Cell Stem Cell *in press*).

I. Background: Testicular cells and stem cell models

In mammalian testis, a huge number of sperm are continuously produced over the reproduction period (1-2 years in mice), which relies on stem cell activity. For decades, the cellular identity and behavior of spermatogenic stem cells has been argued as a fundamental question in the field of spermatogenesis. However, cellular identity and behaviors of stem cells remain poorly understood in mouse spermatogenesis.

The process of spermatogenesis takes place in seminiferous tubules (Figure 1). In mice, mitotic stages of spermatogenic cells, called "spermatogonia", are divided into "undifferentiated" and "differentiating" populations. "Undifferentiated spermatogonia" are found as singly isolated cells (A_s), or syncytia consisting mainly of 2 (A_{pr}), 4 ($A_{al\text{-}4}$), 8 ($A_{al\text{-}8}$), or 16 ($A_{al\text{-}16}$) 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.

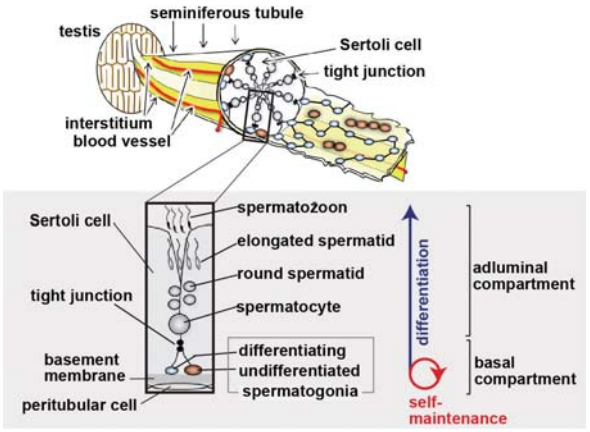

Figure 1. Spermatogenesis in seminiferous tubules. Spermatogenesis progresses from the basal- to adluminal compartment. Stem cells are thought to reside primarily within undifferentiated spermatogonia (brown) which are distributed sparsely on the basement membrane. Figures are modified from Cell Stem Cell (DOI: 10.1016/j.stem.2014.01.019) with permission.

Based on the detailed morphological analyses of fixed specimens, it was proposed in 1971 that stem cell activity may be restricted to the population of A_s spermatogonia, 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). This hypothesis is known as the "A_s model". On the other hand, over the last decade, studies from some groups, including ours, have shown that the population of undifferentiated spermatogonia can be divided on the basis of their heterogeneous gene expression. To summarize, In undisturbed steady-state spermatogenesis, the GFRα1+ subpopulation (mainly A_s, A_{pr} and fewer A_{al}, shown later in Figure 3) is thought to reside on the top of the hierarchy: As well as maintaining their own population, GFRα1+ cells also give rise to the second, Ngn3+, subpopulation of undifferentiated spermatogonia (comprised of more A_{al} and fewer A_s and A_{pr}). Ngn3+ cells, although retaining an ability to revert into GFRα1+ cells that becomes apparent during regeneration after tissue insult, mostly differentiate into differentiating spermatogonia and then meiotic

spermatocytes and haploid spermatids.

These studies indicate that the dynamics of GFRα1+ spermatogonia is the essential question to fully understand identity and behavior of the mouse sperm stem cells. In our previous study, inspired by the A_s model, GFRα1+ A_s spermatogonia were hypothesized to be the primary population of stem cells (Figure 2; Nakagawa *et al*., Science 2010). However, this idea warrants experimental evaluation.

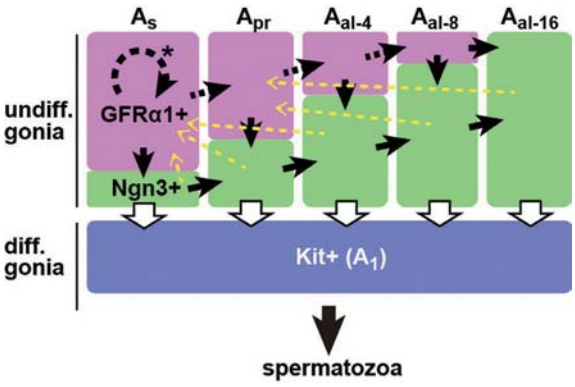

Figure 2. Extended A_s model. A previously proposed hierarchy of GFRα1+ and Ngn3+ subpopulations of undifferentiated spermatogonia, as well as Kit+ differentiating spermatogonia (modified from Nakagawa *et al*., 2010). Black and white solid arrows indicate processes that had been directly observed, whereas the black broken arrows represent a presumptive dynamics of GFRα1+ cells, in which only GFRα1+ A_s self-renew (asterisk). Yellow broken arrows indicate the processes of "reversion", which occur infrequently in steady state.

Following the aforementioned background, we have been challenging to understand the identity and dynamics of the stem cells by analyzing the fate behaviors of GFRα1+ spermatogonia at a single-cell-resolution in undisturbed mouse testis. To achieve this, we took advantage of originally established approaches of *in vivo* live-imaging and pulse-labeling studies, combined with biophysical modeling analyses that were performed in collaboration with Dr. Ben Simons of the University of Cambridge, UK.

II. Population asymmetric stem cell maintenance

The population of GFRα1+ spermatogonia lies scattered unevenly on the basement membrane of seminiferous tubules (Figure 3), but their local density over a prolonged tubule length was found to be remarkably constant in adult mice. To trace the fate behavior of GFRα1+ spermatogonia, we established *GFRα1-CreER^{T2}; CAG-CAT-GFP* mice that can irreversibly label GFRα1+ spermatogonia with GFP by administrating 4OH-tamoxifen into the abdomen, in collaboration with Dr. Hideki Enomoto of Kobe University, Japan. Using this model, we obtained the clonal fate data of GFRα1+ spermatogonia from several days to over a year, and showed that, although the entirety of GFRα1+ spermatogonia maintained their own population while giving rise to differentiating progeny, the individual GFRα1+ spermatogonia followed highly variable and intricate fate behaviors. These findings suggest that the maintenance of GFRα1+ spermatogonia is achieved by "population asymmetry", in which balanced stem cell maintenance and production of differentiating progeny are achieved at a population-level. This paradigm is a recently emerged concept in the stem cell biology field, marking a stark contrast to the classical "division asymmetry", in which every cell division provides asymmetrically fated daughter cells (one self-renewing and one differentiating).

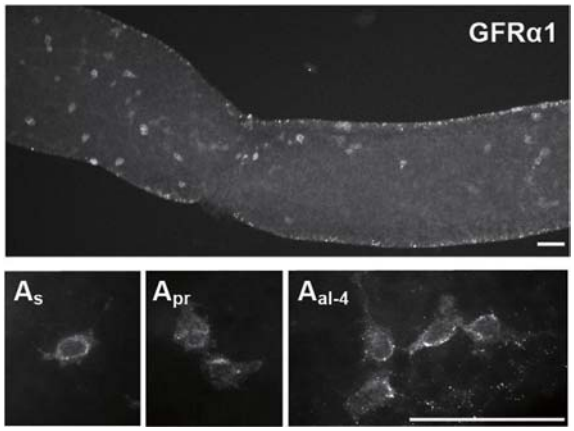

Figure 3. Morphology of GFRα1+ spermatogonia. Immunofluorescence for GFRα1 in a whole-mount seminiferous tubule specimen. Lower, higher magnification of GFRα1+ A_s, A_{pr}, and A_{al-4}.Bars, 50μm.

III. Interconversion of stem cells between different morphological states

We next investigated the behavior of GFRα1+ spermatogonia, by means of *in vivo* live-imaging of *GFRα1-GFP* mouse testis up to 3 days, again collaborating with Dr. Hideki Enomoto. From the numerous 3-day-long observations that altogether effectively cover one year of spermatogonial behavior, it is indicated that GFRα1+ cells continually change their morphological states between A_s, A_{pr} and A_{al} spermatogonia through a combination of "incomplete cell division (leading to syncytial extension)" and "syncytial fragmentation (through breakage of intercellular bridges)", while giving rise to differentiating progeny from all of these morphological states. The live-imaging study further provides quantitative insight into the dynamics of GFRα1+ cells: incomplete cell division and syncytial fragmentation of GFRα1+ spermatogonia appear to occur at constant rates, independent of the unit length.

From the live-imaging study, it was also apparent that GFRα1+ spermatogonia were in constant movement in the basal compartment without showing any systematic pattern. This contrasts with the behavior of Ngn3+ spermatogonia, which are less motile in the vasculature-associated region, before actively spreading over the basal compartment on transition into A_1 spermatogonia (Yoshida *et al*., Science 2007).

IV. Stem cell dynamics governed by the rates of incomplete cell division and syncytial fragmentation

The live-imaging measurements indicate that the rates of the fundamental processes of "incomplete division" and "syncytial fragmentation" may be constant, independent of unit length. We were then motivated, to try to capture the dynamics of GFRα1+ spermatogonia using a biophysical modeling scheme, which essentially depends only on the aforementioned two measured rates.

Intriguingly, the model could accurately predict the wide range of intricate clonal fate behaviors experimentally observed including that of pulse-labeled GFRα1+ cells in steady-state over wide time scales from several days to over a year. Moreover, the model also nicely predicted the fate behaviors of GFRα1+ spermatogonia during regeneration following tissue insult. Given the ability of such a simple model to predict the complex *in vivo* behavior from steady-state to regeneration, we believe that the principles that define the dynamics of the GFRα1+ compartment have been successfully resolved.

V. Conclusion: A proposed stem cell dynamics

Figure 4 illustrates the proposed stem cell dynamics, in which GFRα1+ units continuously extend via incomplete division and fragment via intercellular bridge breakdown, while giving rise to Ngn3+ progeny. Interestingly, no GFRα1+ cell in this scheme meets the generic strict definition of "a stem cell", a cell that repeats self-renewal (viz. production of one or more cell(s) identical to the parental state following proliferation). Rather, individual cells constantly change their morphological states reversibly between single cells and variable lengths of syncytia. Through this process, at the population level, the population of GFRα1+ cells is maintained, while producing differentiating progeny.

To conclude, in contrary to the long-held "A_s model", we propose that the entirety of GFRα1+ spermatogonia comprises a single "stem cell pool". In particular, the stem cells can be defined, not as a particular cell type, but as a heterogeneous population in which cells continually interconvert between different states. We believe such a concept of "*dynamic heterogeneity*" emerging in mouse spermatogenesis may provide a novel paradigm for other tissue stem cell systems.

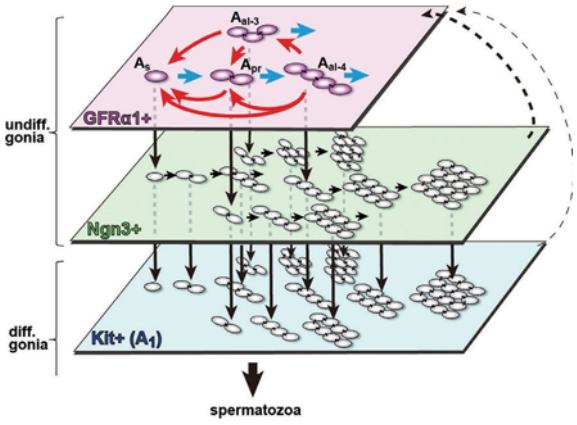

Figure 4. A proposed stem cell dynamics. On the top of the differentiation hierarchy, GFRα1+ spermatogonia comprise a single stem cell pool, in which cells continually and reversibly interconvert between states of A_s, A_{pr} and A_{al} spermatogonia through incomplete cell division (blue arrows) and 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).

Publication List

〔Original papers〕

- Nakamura, Y., Tasai, M., Takeda, K., Nirasawa, K., and Tagami, T. (2013). Production of functional gametes from cryopreserved primordial germ cells of the Japanese quail. J. Reprod. Dev. *59*, 580-587.
- Nonami, Y., Narita, K., Nakamura, H., Inoue, T., and Takeda, S. (2013). Developmental changes in ciliary motility on choroid plexus epithelial cells during the perinatal period. Cytoskeleton (Hoboken) *70*, 797-803.
- Shirakawa, T., Yaman-Deveci, R., Tomizawa, S., Kamizato, Y., Nakajima, K., Sone, H., Sato, Y., Sharif, J., Yamashita, A., Takada-Horisawa, Y., Yoshida, S., Ura, K., Muto, M., Koseki, H., Suda, T., and Ohbo, K. (2013). An epigenetic switch is crucial for spermatogonia to exit the undifferentiated state toward a Kit-positive identity. Development *140*, 3565-3576.

〔Review article〕

- Nakamura, Y., Kagami, H., and Tagami, T. (2013). Development, differentiation and manipulation of chicken germ cells. Dev. Growth Differ. *55*, 20-40.