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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 goal of the Division of Germ Cell Biology is to fully understand the mammalian sperm stem cell system under the context of *in vivo* tissue architecture. We have successfully revealed some characteristics of this interesting stem cell system. First, differentiating germ cells that had been believed to be irreversibly committed for differentiation still retain their 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 populations including both "actual" and "potential" stem cells are preferentially localized near vasculature (vasculature-associated niche). Lastly, stem cells turn over at an unexpectedly 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 stem cell function is restricted to the singly isolated spermatogonia (A_s cells).

I. Background: Testicular architecture and sperm stem cell theories

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.

The process of spermatogenesis takes place in seminiferous tubules of the testis (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}) or more (A_{al}) 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.

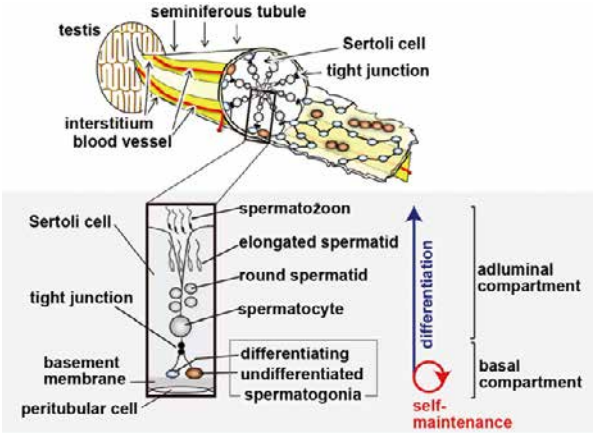

Figure 1. Spermatogenesis in seminiferous tubules. Spermatogenesis progresses from the basal- to adluminal compartment. Stem cells are thought to reside primarily within undifferentiated spermatogonia (brown) which are distributed sparsely on the basement membrane. Modified from Hara *et al*., Cell Stem Cell (2014).

The prevailing stem cell model, called the "A_s model", was proposed in 1971, suggesting that stem cell activity is 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).

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. In undisturbed steady-state spermatogenesis, the GFRα1+ subpopulation (mainly A_s, A_{pr}, and fewer A_{al}) 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 in undisturbed situations.

Seminiferous tubules represent a typical "open" stem cell niche. This makes a stark contrast to the well-investigated "closed" niche-supported tissues, such as *Drosophila* gonads or mammalian small intestine. The "closed" niche is a definitive, specified region of a tissue which typically tethers the stem cells, while cells that exit this niche region will differentiate. In contrast, seminiferous tubules do not harbor such a defined structure, but the stem cells (GFRα1+ spermatogonia) are scattered in the basal compartment with some, but significant, preference to the vasculature between the tubules. It remains an important unsolved question how

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2014. The former title is indicated by an asterisk (*).

stem cell fate is regulated (whether differentiated or remaining undifferentiated) in an open niche system.

In 2014, we questioned the cellular identity and the dynamics of the stem cells, as well as the mechanisms regulating stem cell fate in the recently proposed open niche model, as described below.

II. Cellular identity and the dynamics of spermatogenic stem cells

The aforementioned studies illustrate that the dynamics of GFRα1+ spermatogonia are an essential problem for a full understanding of the identity and behavior of mouse sperm stem cells. To address this, we performed single-cell-level analyses of *in vivo* dynamics of GFRα1+ spermatogonia, taking advantage of pulse-labeling and live-imaging studies, combined with biophysical modeling, in collaboration with Dr. Ben Simons of the University of Cambridge, UK..

GFRα1+ spermatogonia lie scattered unevenly on the basement membrane of seminiferous tubules (Figure 3), but their local density over a prolonged tubule length is remarkably constant in adult mice. By pulse-labeling the GFRα1+ spermatogonia with GFP, we obtained 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.

We next investigated the behavior of GFRα1+ spermatogonia, by means of *in vivo* live-imaging. 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 measurements indicate that the rates of "incomplete division" and "syncytial fragmentation" may be constant regardless of whether they are syncytia or A_s. We were then motivated to try to capture these cells using a biophysical modeling scheme, which essentially depends only on the rates of "incomplete division" and "syncytial fragmentation". Intriguingly, the model accurately predicted the wide range of intricate clonal fate behaviors in steady-state over wide time scales from several days to over a year, and the recovery from tissue insult observed in laboratory experiments.

The results together 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, while giving rise to Ngn3+ progeny (Figure 2). This novel paradigm would challenge the "A_s model" that was proposed over 40 years ago.

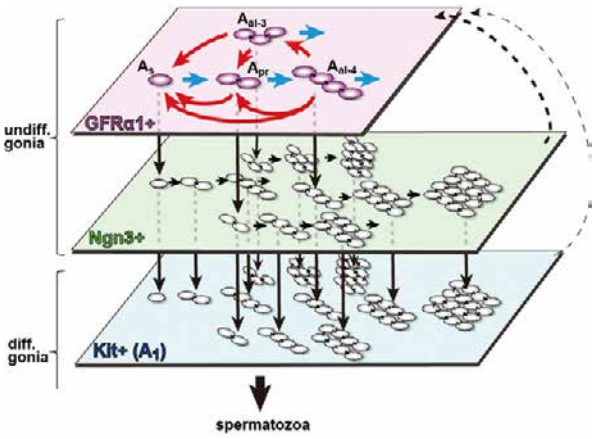

Figure 2. A proposed stem cell dynamics. GFRα1+ spermatogonia comprise a single stem cell pool, in which cells continually and reversibly interconvert between states of A_s, A_{pr} and A_{al} spermatogonia through incomplete cell division (blue arrows) and syncytial fragmentation (red arrows), while giving rise to Ngn3+ cells. Thus, 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, such as after tissue insult or during regeneration from damage etc. (broken arrows). Reproduced from Hara *et al*., Cell Stem Cell (2014).

II. Undifferentiated spermatogonia show heterogeneous differentiation competence in response to retinoic acid.

As described above, seminiferous tubules represent a typical open niche of stem cells. In particular, GFRα1+, Ngn3+, and Kit+ spermatogonial subpopulations are intermingled in the basal compartment (Figure 3). In addition, these are likely to be ubiquitously bathed with retinoic acid (RA), a strong differentiation-inducer, that occurs periodically along with the cycle of seminiferous epithelium (8.6 days per a cycle). So, a fundamental question is raised: Out of the entire population of undifferentiated spermatogonia with stem cell potential on the basis of the activity of post-transplantation colony formation, why do Ngn3+ cells preferentially differentiate and why do GFRα1+ cells maintain their own population (self-renewal)?

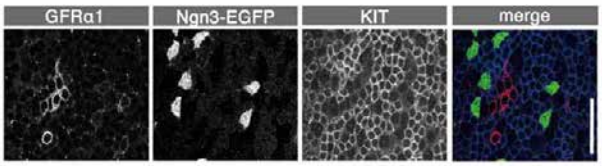

Figure 3. Localization of GFRα1+, Ngn3+, and KIT+ spermatogonia. Representative images of triple immuno-stained whole-mount seminiferous tubules of an *Ngn3-EGFP* mouse. Scale bars = 50 μm.

By pulse-labeling the Ngn3+ and GFRα1+ populations by means of the tamoxifen-inducible cre-loxP system, we tested the response of these cells to RA. The results first indicated

that Ngn3+ cells rapidly and efficiently differentiated into Kit+ cells in response to RA. In contrast, GFRα1+ cells did not show effective differentiation induced by RA, but they generated the Ngn3+ cells in an RA-independent manner. Thus, GFRα1+ and Ngn3+ cells show distinctive differentiation competence in response to RA.

We then questioned the molecular mechanism that caused such differential responses to RA. To this end, the transcriptomes of GFRα1+ and Ngn3+ fractions were compared by gene expression microarray analysis. Among a number of genes involved in RA signal reception, particularly, retinoic acid receptor gamma (*Rarγ*), expression was preferentially observed in Ngn3+ cells over GFRα1+ cells. This was also true for the RARγ protein (Figure 4). On the other hand, most of the other RA-related genes showed similar levels of expression.

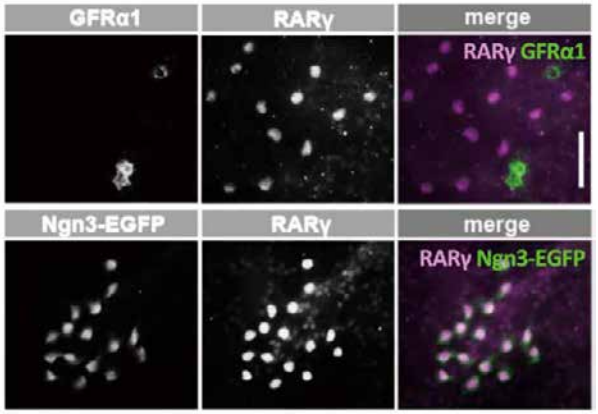

Figure 4. Expression of RARγ in Ngn3+, but not in GFRα1+, spermatogonia. Representative images of double immuno-stained whole-mount seminiferous tubules of an *Ngn3-EGFP* mouse. Scale bars = 50 μm. Modified from Ikami *et al*., Development *142*, 1-11 (2015).

We then addressed the roles of RARγ by ectopic expression in GFRα1+ cells that normally do not express this gene. Intriguingly, enforced expression of RARγ provided the GFRα1+ cells with differentiation competence in response to RA. This indicates the key role of RARγ in the heterogeneous differentiation competence and the subsequent fate selection of undifferentiated spermatogonia.

From these results, heterogeneous differentiation competence, combined with the periodically but ubiquitously distributed RA, appears to allocate the undifferentiated cells' fates for the continuity of spermatogenesis (Figure 5). We propose that heterogeneous differentiation competence combined with spatially ubiquitous distribution of differentiation-inducing signals would be paradigmatic for stem cell regulation in an opened niche based model.

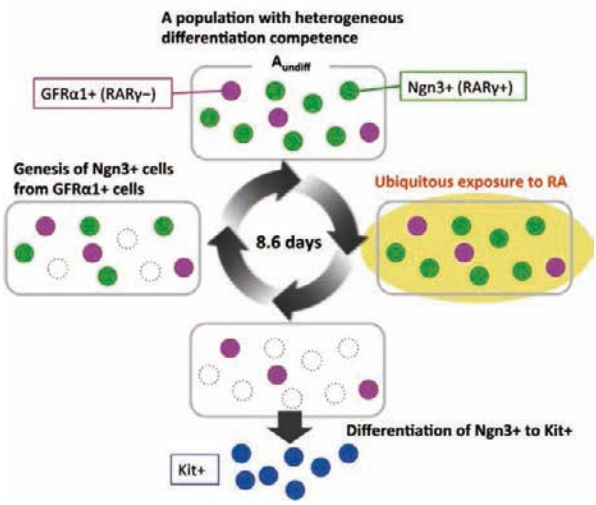

Figure 5. A scheme for the fate allocation of undifferentiated spermatogonia along the cycle of seminiferous tubules. See text for details.

Publication List

〔Original paper〕

- Hara, K., Nakagawa, T., Enomoto, H., Suzuki, M., Yamamoto, M., Simons, B.D., and Yoshida, S. (2014). Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. Cell Stem Cell *14*, 658-672.

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

- Ikegami, K., Atsumi, Y., Yorinaga, E., Ono, H., Murayama, I., Nakane, Y., Ota, W., Arai, N., Tega, A., Iigo, M., Darras, V.M., Tsutsui, K., Hayashi, Y., Yoshida, S., and Yoshimura, T. Low temperature-induced circulating triiodothyronine accelerates seasonal testicular regression. Endocrinology 2014 Nov 18.