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Overview of our research

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 function of "stem cells", which both maintain the undifferentiated cell pool, while generating differentiation-destined cells in a well balanced manner. The Division of Germ Cell Biology aims to fully understand the mouse sperm stem cell system under the context of *in vivo* testicular tissue. Our particular interests have been laid on the "undifferentiated spermatogonia", which are responsible for the stem cell functions. Our study has revealed several key properties of this interesting population.

First, we found that this stem cell system includes a functional hierarchy. It is comprised of an "actual" stem cell compartment that is prone to self-renew, and a differentiation-primed, "potential" stem cell compartment. Regarding the "actual" stem cells, we have been investigating their cellular identity, their *in vivo* behavior at a single-cell resolution, and the underlying mathematical principles. This lead to the discovery of "neutral competition" between the stem cells. We are currently investigating the molecular mechanism underlying their control of self-renewal and differentiation, and their connection to tissue environment. "Potential stem cells" are also of our enthusiastic interest: In undisturbed, steady-state spermatogenesis, they largely contribute as transient amplifying cells and differentiate rather than self-renew. However, when tissues are insulted or testicular cells are transplanted to a host testes, their probability of self-renewal jumps up and they effectively replenish the lost "actual" stem cells. Such a flexible, and probabilistic, feature of stem cell dynamics has been found paradigmatic for many other stem cell-supported tissues.

Key references from this division related to these studies that are currently public include Nakagawa et al., Dev. Cell 2007; Yoshida et al., Science 2007; Nakagawa et al., Science 2010; Klein et al., Cell Stem Cell 2010; and Hara et al., Cell Stem Cell 2014, Ikami et al., Development 2015, and Tokue et al., Stem Cell Reports 2017.

I. The identity of spermatogenic stem cells and their dynamics

Morphologically, the population of A_{undiff} includes 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 mitotic division process with which cytokinesis does not complete and the cytoplasmic connection between daughter cells persists via intercellular bridges. The prevailing stem cell theory proposed in 1971 states that stem cell activity is restricted to the population of A_s cells, 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), known as the "A_s model".

Figure 1 represents our latest proposed model for the functional structure of the spermatogenic stem cell system, which indeed proposes an alternative for the "A_s model". This is the simplest interpretation of the results of our functional analyses of GFRα1$^+$ spermatogonia, which act as the "actual" stem cells. These include intravital live-imaging experiments, clonal fate analysis of pulse-labeled cells, and biophysical modeling analysis of the results.

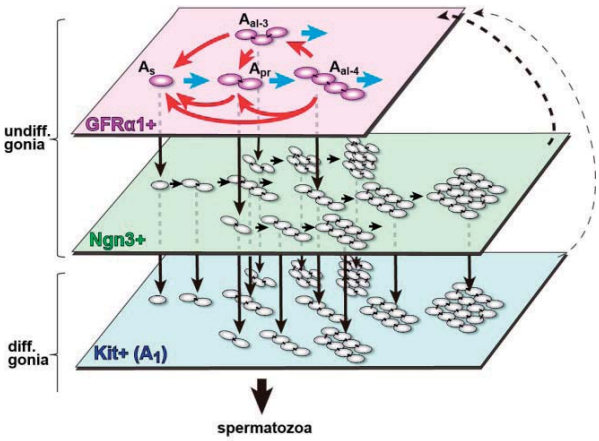

Figure 1. A proposed stem cell dynamics. On the top of the differentiation hierarchy, GFRα1$^+$ spermatogonia comprise a single stem cell pool, in which cells continually and reversibly interconvert between states of A_s, A_{pr} and A_{al} spermatogonia through incomplete cell division (blue arrows) 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). (Reprinted from Hara et al., Cell Stem Cell 2014.)

As crystalized in this model, our results suggest that the GFRα1$^+$ sub-population of A_{undff} spermatogonia, which include both A_s cells and syncytia (A_{pr} and A_{al}) comprises a single stem cell pool, in which cells continually interconvert between these morphologically heterogeneous states through stochastic incomplete division and fragmentation of syncytia. The incomplete division and syncytial fragmentation causes the expansion of this population, while the excess cells over

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2017. The former title is indicated by an asterisk (*).

a particular "quota" would overflow to become the NGN3$^+$ state of A_{undff}, which then further differentiate into KIT$^+$ "differentiating" spermatogonia that are largely devoid of self-renewing potential.

Currently, we are investigating the mechanism that determines the quota or tissue capacity of GFRα1$^+$ cells, as well as the detailed nature of the internal heterogeneity of the population of GFRα1$^+$ cells. Further, it is also becoming apparent that the GFRα1$^+$ population is not totally homogeneous but associated with some heterogeneity in their gene expression and fate behavior, as described by our group (see below) and others. Therefore, our next challenges include understanding the overall scheme of the stem/progenitor cell dynamics in which short-term heterogeneity and long-term equipotency (randomness) are both observed in a compatible manner.

2. Mechanisms underlying the balanced differentiation and self-renewal of stem cells in an "open" stem cell niche

In general, stem cells support tissue homeostasis through continual production of differentiating progeny from a pool of undifferentiated cells. This is traditionally thought to be dependent on a couple of paradigmatic mechanisms: 1) "asymmetric cell division", which always gives rise to one self-renewing cell and one differentiating cell; 2) control by "an anatomically defined niche", inside of which stem cells remain undifferentiated, but outside of which they differentiate (Fuller and Spradling, 2007; Morrison and Spradling, 2008). However, the mouse spermatogenic stem cell system appears to not show a defined facultative niche environment and does not rely on asymmetric division; their fates are found to be stochastic (Hara et al., 2014; Klein et al., 2010; Klein and Simons, 2011).

In 2017, we challenged a fundamental question: How sperm stem cells follow different fates (to differentiate or to self-renew) in response to homogeneously distributed extracellular signals, in a facultative (or open) niche environment? The result is published in Tokue et al., Stem Cell Reports (2017).

2-1. Wnt/β-catenin signaling promotes differentiation of GFRα1$^+$ to NGN3$^+$ cells

To unveil the signal that promotes the GFRα1$^+$ to NGN3$^+$ transition. We first compared the gene expression profiles between GFRα1$^+$ and NGN3$^+$ cells by cDNA microarray. From the microarray data, we focused on Wnt signaling. In cultured spermatogonia (viz. GS cell), Wnt/β-catenin signaling induced *Ngn3* expression (Figure 2A). Moreover, mice carrying a gain-of-function mutation of *β-catenin* showed spermatogenesis defects, in which the number of GFRα1$^+$ cells was reduced (Figure 2B), whereas no significant reduction in retinoic acid receptor gamma (RARγ)$^+$ cells (largely corresponding to NGN3$^+$ cells) was observed. Consequently, the RARγ$^+$ cell-to-GFRα1$^+$ cell ratio increased, consistent with the idea that GFRα1$^+$ to NGN3$^+$ differentiation increased.

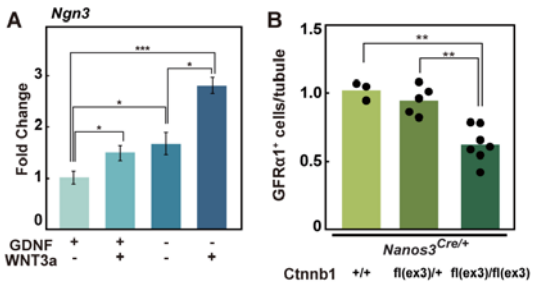

Figure 2. Wnt/β-catenin signal promotes the differentiation of GFRα1$^+$ spermatogonia *in vitro* and *in vivo*. (A) Expression of *Ngn3* mRNA in GS cells in the presence or absence of GDNF or WNT3a. GS cells were stimulated by the indicated conditions and analyzed by RT-qPCR for the *Ngn3* mRNA level. (B) Average numbers of GFRα1$^+$ cells per tubule, observed in mice with indicated genotypes. Number of GFRα1$^+$ cells was reduced in *Nanos3*$^{Cre/+}$;*Ctnnb1* $^{fl(ex3)/fl(ex3)}$ *mice*, in which β-catenin was activated in a germ cell specific manner. Modified from Tokue et al., Stem Cell Reports (2017),

2-2. SHISA6, a cell-autonomous Wnt inhibitor, is expressed in a subset of GFRα1$^+$ cells

We found that *Wnt6* is expressed in Sertoli cells, a supporting somatic cell type, in a spatially ubiquitous manner, suggesting that WNT6 is participating in the differentiation-promotion in seminiferous tubules, and that GFRα1$^+$ cells are uniformly exposed to WNT6 signals. These raised a next question: How do stem cells persist without exhaustion if they are equally bathed with Wnt ligand(s)?

To address this issue, we hypothesized that a Wnt inhibitor(s) may confer resistance to Wnt/β-catenin signaling in some fraction of GFRα1$^+$ cells. In our microarray data, we found *Shisa6* an interesting candidate, because *Shisa6* was highly enriched in the GFRα1$^+$ fraction and some of the other *Shisa* family members have been reported as Wnt inhibitors. Experiments using *Xenopus laevis* embryos and luciferase assays in HEK293T cells showed that SHISA6 is a novel Wnt inhibitor that acts autonomously (Figure 3A). Moreover, *Shisa6* expression was restricted in about 30% of the GFRα1$^+$ population *in vivo* (Figure 3B).

2-3. SHISA6$^+$ spermatogonia show stem cell characters and contribute to maintenance of GFRα1$^+$ cells

We found that, although *Shisa6* KO or stabilized *β-catenin* heterozygous mutants show no apparent phenotype in the pool of GFRα1$^+$ cells, synthetic heterozygotes for these alleles showed a reduced GFRα1$^+$ cell pool and spermatogenesis defects. Thus, SHISA6 plays a role in the maintenance of the stem cell pool by suppressing Wnt/β-catenin signaling in a cell-autonomous manner (Figure 4A).

We then addressed the behavior of SHISA6$^+$ cells conjectured from that of *T* (*Brachyury*)$^+$ cells, which were found to largely overlap with SHISA6$^+$ cells. A pulse-label and chase experiment showed that T$^+$ cells have the ability to continually produce progeny differentiating to sperm for at least 6 months. Together, these results suggest that T$^+$ (and probably SHISA6$^+$) cells have stem cell-related characteristics (Figure 4B)

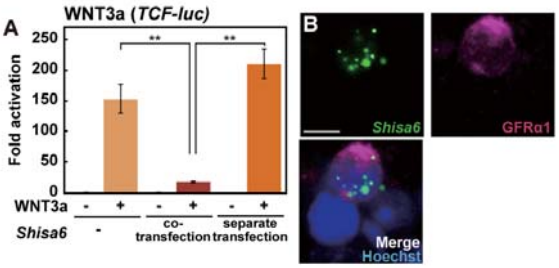

Figure 3. Function and expression of SHISA6 (A) Cell-autonomous inhibition of Wnt/β-catenin signaling by SHISA6. HEK293T cells were transfected with a Wnt reporter luciferase construct and a *Shisa6* expression plasmid either simultaneously (co-transfection), or separately (transfected cells were mixed afterword), followed by stimulation of Wnt/β-catenin signal. The Wnt-dependent Luciferase activity was suppressed only when Shisa6 was co-transfected. (B) Representative image of dissociated testicular cells of adult mice doubly stained for *Shisa6* by FISH and for GFR (B) Representative image. Note the overlapped expression of *Shisa6* and GFRsa is modified from Tokue et al., Stem Cell Reports (2017),

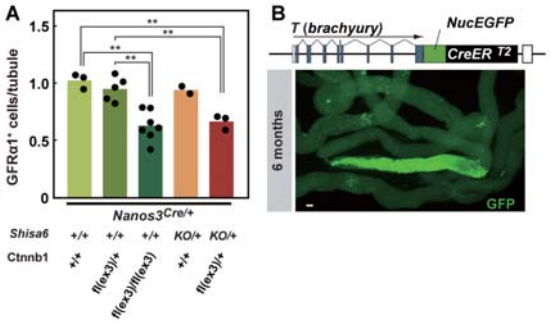

Figure 4. SHISA6 and spermatogenic stem cells (A) SHISA6 maintains the GFR S+ cell pool through inhibiting the Wnt/l pool through i. Average number of GFRve+ cells per tubule section in testes with the indicated genotypes, based on double IF for GFR microtesticular sections. (B) (Upper) A schematic of the structure of the *T*$^{hEGFP\text{-}CreERT2}$ allele, which enables pulse-labeling of T+ cells using CreER^{T2}. (Lower) A result of pulse-labelling of T+ cells with 6 months of chase. Note that GFP (the lineage reporter) positive cells form a prominent patch in a particular segment of seminiferous tubules. Modified from Tokue et al., Stem Cell Reports (2017),

2-4. SHISA6 protects the stem cells from differentiation promoting Wnt/β-catenin signaling: A proposal of a generic mechanism of stem cell regulation in facultative niches

To conclude, we propose a generic mechanism underlying the heterogeneous stem cell fates in facultative niche environments. Different levels of cell-autonomous inhibitor (SHISA6, in this case) may confer heterogeneous resistance to uniformly distributed extracellular signaling that promotes differentiation (such as WNTs). Here, stem cells with higher levels of inhibitors would remain in the undifferentiated cell pool with higher probabilities, while those with lower levels of inhibitors are more inclined to differentiate (Figure 5).

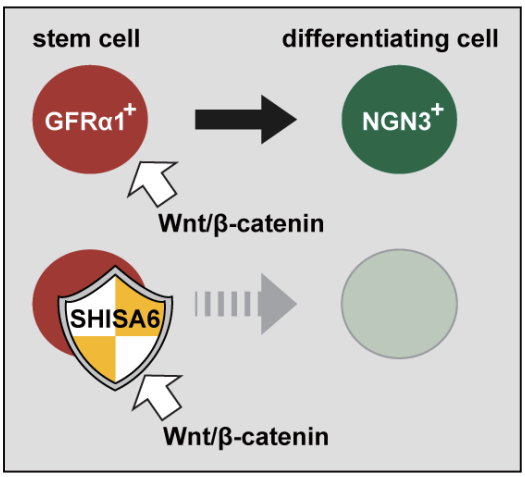

Figure 5. A conceptual diagram of differentiation promotion by Wnt/β-catenin signaling to become Ngn3+ and its cell-autonomous protection by SHISA6 in a subset of GFRd + cells. The heterogeneous expression of SHISA6 may confer different levels of differentiation probability in an open niche environment in which differentiation-promoting Wnt ligand appears to distribute uniformly. Modified from Tokue et al., Stem Cell Reports (2017),

Publication List:

〔Original Paper〕

- Tokue, M., Ikami, K., Mizuno, S., Takagi, C., Miyagi, A., Takada, R., Noda, C., Kitadate, Y., Hara, K., Mizuguchi, H., Sato, T., Taketo, M.M., Sugiyama, F., Ogawa, T., Kobayashi, S., Ueno, N., Takahashi, S., Takada, S., and Yoshida, S. (2017). SHISA6 confers resistance to differentiation-promoting Wnt/beta-catenin signaling in mouse spermatogenic stem cells. Stem Cell Rep. *8*, 561-575.