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Overview of our research

Production of numerous sperm over a long period in the testis is fundamental for continuity of life across generations. The Division of Germ Cell Biology aims to understand the key processes of germ cell development that lead to robust sperm formation.

Our emphasis has specifically been on the regulation and functional behavior of spermatogenic stem cells (SSCs) in the testes of mice. SSCs play a crucial role, not only as the origin of long-term sperm production ensuring the continuity of life, but also as the crucible of genetic and potentially epigenetic mutations, the underpinning of evolution as well as congenital disease mechanisms. The principal and signature strategy of our research is to directly *observe* the behavior of individual germ cells *in vivo* in their natural (*i.e.* physiological) state and take advantage of intravital live-imaging, lineage analysis, and biophysical analysis.

Regarding the study of SSCs, first, we found that SSCs include a functional hierarchy, comprised of an "actual" stem cell compartment that is able to self-renew, and a differentiation-primed, "potential" stem cell compartment. We have been also investigating the cellular identity of "actual" SSCs, their *in vivo* behavior at a single-cell resolution, and the underlying mathematical principles, leading to the discovery of the dynamics of "population asymmetry" and subsequent "neutral competition" between SSCs. We are also highly interested in the area of "potential stem cells". In steady-state spermatogenesis, these largely contribute as transient amplifying cells and differentiate rather than self-renew. However, when tissues are insulted or testicular cells are transplanted into host testes, their probability of self-renewal increases and they effectively replenish the lost "actual" stem cells. Such flexible and probabilistic features of stem cells have been found to be paradigmatic for many other stem cell-supported tissues.

Our past key references related to these studies include Nakagawa *et al*., Dev. Cell 2007; Yoshida *et al*., Science 2007; Nakagawa *et al*., Science 2010; Klein *et al*., Cell Stem Cell 2010; Hara *et al*., Cell Stem Cell 2014, Ikami *et al*., Development 2015, and Tokue *et al*., Stem Cell Reports 2017.

Mechanism of "mitogen competition" regulates the stem cell density homeostasis in an "open" niche

In 2018, we developed a novel hypothesis regarding the mechanism of SSC maintenance in the mouse testis (Kitadate *et al*., Cell Stem Cell 2019). In contrast to better-investigated tissues such as *Drosophila* testes and ovaries, as well as mammalian intestinal crypts, where physical contact made by stem cells with an anatomically-defined niche provides a platform to maintain homeostasis, the question of how stem cell homeostasis is achieved in the mouse testis remains unknown. In the mouse testes' seminiferous tubules, the place where spermatogenesis proceeds, SSCs are motile and dispersed among their progeny, thus representing a class of microenvironment designated as an "open niche" (Yoshida, Dev. Growth. Diff. 2008). Intriguingly, the density of SSCs (defined as GFRα1$^+$ cells) is strikingly constant when averaged over a certain length (*e.g.*, >1cm), despite local fluctuations. This suggests a presence of an unknown mechanism that regulates the SSC homeostasis in a manner that stabilizes their average density.

I. FGF5 expression near the vasculature and its mitogenic function on GFRα1$^+$ spermatogonia

In order to find the key factors regulating the GFRα1$^+$ cell density, we screened the genes expressed in the tubule area

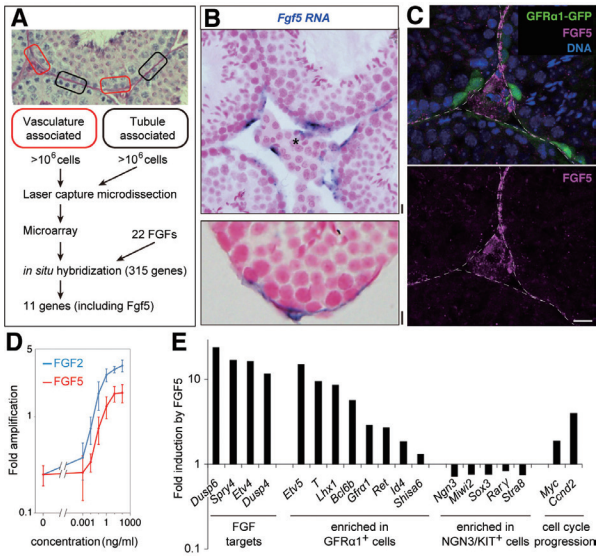

Figure 1. (A) Outline of the screening for genes preferentially expressed in the vasculature-associated region. (B) Representative ISH images for *Fgf5* (blue) in testis sections, counterstained with nuclear fast red. (C) Representative image of an inter-tubular region of a GFRα1-GFP mouse testis stained for GFP (green), FGF5 (magenta) and DNA (blue). Scale bars, 10 μm. (D) Mitogenic effect of FGF5 (red) or FGF2 (blue) on cultured spermatogonia. Fold increase in the number of GS cells cultured with indicated concentration of FGF5 for 8 days. Shown in average ± SEM (n = 3 independent experiments). (E) Effects of FGF5 on gene expression. GS cells depleted of FGF2 and GDNF for 3 days were supplemented with or without FGF5 (100 ng/ml) for 24 hours, followed by cDNA microarray analyses. * intertubular arterioles/venules All figures are reproduced from modifications taken from Kitadate *et al*., Cell Stem Cell (2019).

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2018. The formertitle is indicated by an asterisk (*).

that were facing the interstitium and vasculature, where GFRα1⁺ cells preferentially locate (Figure 1A). From this screening, we focused on *Fgf5*, since it is located in peritubularly located large flattened cells (termed lymphatic endothelial cells) near the interstitium (Figures 1B and C). By using an *in vitro* spermatogonial culture, we found that FGF5 showed mitogenic and anti-differentiation effects (Figures 1D and E).

II. FGF5 controls GFRα1⁺ cell density in a linear dosage-dependent manner

We then investigated the *in vivo* role of FGF5 in mice carrying a null allele (*Fgf5*⁻) or an extra copy of BAC-mediated transgene (*BAC-Fgf5*^{Tg}). In mutant testes, the average density of GFRα1⁺ spermatogonia showed a strikingly linear correlation with *Fgf5* dosage (Figure 2A). These mutants notably sustained steady-state spermatogenesis with different density *set points* of GFRα1⁺ cells that correlate in a manner that depends linearly on *Fgf5* dosage during adulthood (Figure 2B).

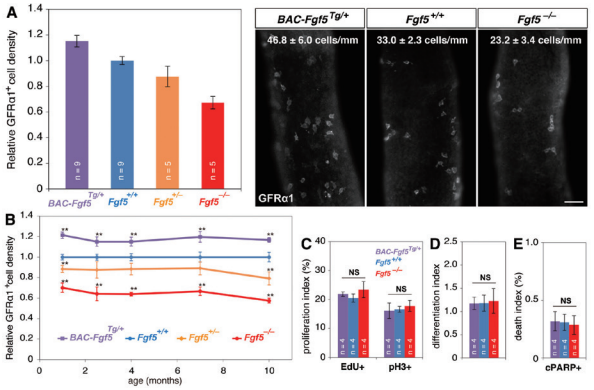

Figure 2. (A) Average densities ± SEM of GFRα1⁺ cells and representative IF images of whole-mount seminiferous tubules for GFRα1 (right) of 2.5 month-old mice with indicated genotypes. Scale bar, 50μm. (B) Average densities ± SEM of GFRα1⁺ cells in mice with indicated genotypes and ages. (C–E) Indexes of proliferation (EdU⁺ and pH3⁺ fractions), differentiation [quantified as the RARγ⁺/KIT⁻ (≈NGN3⁺) over GFRα1⁺ cell ratio], and death (cPARP⁺ fraction) in GFRα1⁺ cells of indicated mice at 2.5 months of age.

III. Each GFRα1⁺ cell receives an unchanged level of FGF signal in *Fgf* mutants

Given the mitogenic and differentiation-inhibiting functions of FGF5, we first considered whether GFRα1⁺ cells receive altered levels of FGF signal in mutants, which in turn changes their fate resulting in altered densities. However, the rates of proliferation, differentiation and death of GFRα1⁺ cells, as well as their gene expression profile, surprisingly were not different between *Fgf5*$^{-/-}$, *BAC-Fgf5*$^{Tg/+}$ and WT mice (Figures 2C–E; not shown), indicating conserved fate behavior of GFRa1⁺ cells between mutants.

The above findings lead to a hypothesis that FGF supply is a limiting factor that is competed for among the GFRα1⁺ cells. In this case, the levels of FGF signal received by each GFRα1⁺ cell would be equalized among *Fgf* mutants harboring different densities of GFRα1⁺ cells.

IV. GFRα1⁺ spermatogonia consume FGF5

To further develop this hypothesis, we then examined whether GFRα1⁺ cells consume the extracellular FGF5 when they receive its signal *in vivo*, as this was probably the simplest form of competition consistent with the general mechanism of FGF signal reception by target cells. We detected speckled FGF5 signals inside GFRα1⁺ cells (Figure 3A). Significant portions of FGF5 cytoplasmic signals were co-localized with SDC4 as cytoplasmic puncta on LAMP1⁺ lysosomes (Figures 3B). These observations indicated that GFRα1⁺ cells consume extracellular FGF5, supporting the idea that GFRα1⁺ cells compete for extracellular FGF.

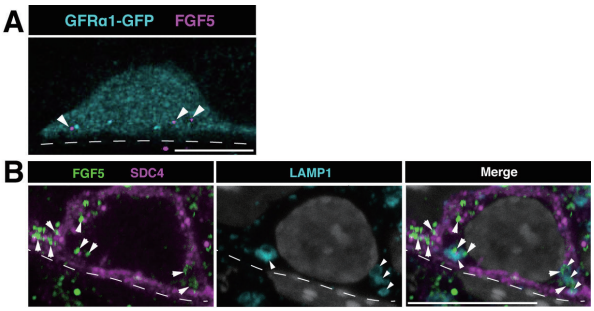

Figure 3. (A) Representative images of GFRα1-GFP⁺ (cyan) cells exhibiting the speckled cytoplasmic staining of FGF5 (magenta, white arrowheads). (B) Representative images of SDC4⁺ (magenta) cells co-stained for FGF5 (green) with LAMP1 (cyan). FGF5⁺ speckles (arrows) were often co-localized with LAMP1⁺ (arrowheads) foci in SDC4⁺ cytoplasmic clamp. Scale bars, 10 μm.

V. Homeostatic stem cell density regulation follows from a model of "mitogen competition"

To understand the mechanism of density regulation, we developed a minimal theoretical model, in which GFRα1⁺ cells are exposed to a steady supply of FGF from the lymphatic endothelial cells, which in turn elicits the concentration-dependent mitogenic and differentiation-inhibiting activities, while also being consumed by, the GFRα1⁺ cells.

This model was found to be capable of capturing the counterintuitive observation that the fate behavior of GFRα1⁺ cells does not change in *Fgf* mutants (Figures 2C–E), as a consequence of the steady-state FGF concentration being always pinned and at which the increase (renewal) and decrease (differentiation) of GFRα1⁺ cells is balanced. Given that GFRα1⁺ cells effectively compete with each other for the limited supply of FGFs, we refer to this mechanism as the "mitogen competition ".

We then questioned whether the model could also predict quantitatively the dynamics of stem cells, if the sytem is strongly perturbed from a steady state. Analysis of the model predicted that the recovery of GFRα1⁺ cell density following a strong perturbation from its steady-state (viz. uninjured) value would show decaying oscillations. To test this prediction, we examined the kinetics during regeneration following the reduction of GFRα1⁺ cells by injecting a cytotoxic reagent, busulfan. Strikingly, decaying oscillations that converged into the steady-state value over several months were indeed observed, with a profile that quantitatively matched with our theory (Figure 4).

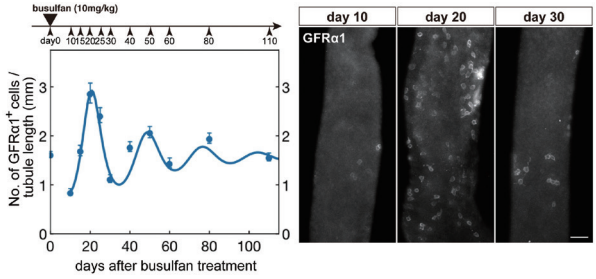

Figure 4. Observed kinetics of the GFRα1⁺ cell density following busulfan treatment in WT (left), and examples of IF images of whole-mount seminiferous tubules stained for GFRα1 (right). Model results (curves) compared to experimental measurements (dots) of the average GFRα1⁺ cell density following busulfan treatment.

To summarize, our results show that the *in vivo* fate behavior of GFRα1⁺ cells is regulated by the mitogenic and anti-differentiation effects of FGFs released in proximity to the vasculature (Figure 5). We propose that competition for mitogens might be a paradigmatic mechanism that can explain the regulation of stem cell homeostasis in a wide range of tissues.

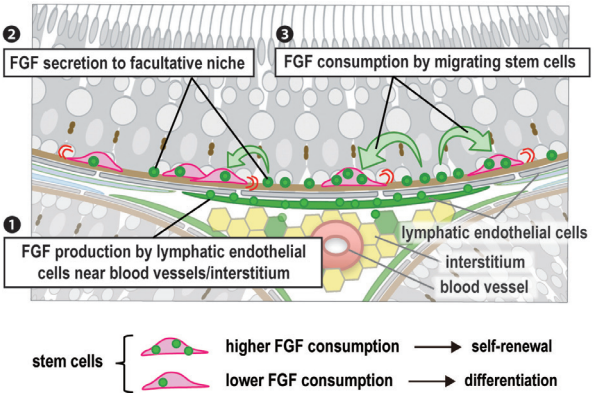

Figure 5. A conceptual diagram of mitogen competition leading to stem cell density homeostasis. Limited supply of FGF from a subset of lymphatic endothelial cells in proximity to vasculature plays a key role in stem cell density homeostasis.

Publication List:

〔Original Papers〕

- Sakamoto, S., Thumkeo, D., Ohta, H., Zhang, Z., Huang, S.R., Kanchenawong, P., Fuu, T., Watanabe, S., Shimada, K., Fujihara, Y., Yoshida, S., Ikawa, M., Watanabe, N., Saitou, M., and Narumiya, S. (2018). mDia1/3 generate cortical F-actin meshwork in Sertoli cells that is continuous with contractile F-actin bundles and indispensable for spermatogenesis and male fertility. PLoS Biol. *16*, e2004874.
- Shawki, H.H., Oishi, H., Usui, T., Kitadate, Y., Basha, W.A., Abdellatif, A.M., Hasegawa, K., Okada, R., Mochida, K., El-Shemy, H.A., Muratani, M., Ogura, A., Yoshida, S., and Takahashi, S. (2018). MAFB is dispensable for the fetal testis morphogenesis and the maintenance of spermatogenesis in adult mice. PLoS ONE *13*. e0190800.

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

- Kitadate, Y., Jörg, D.J., Tokue, M., Maruyama, A., Ichikawa, R., Tsuchiya, S., Segi-Nishida, E., Nakagawa T., Uchida, A., Kimura-Yoshida, C., Mizuno, S., Sugiyama, F., Azami, T., Ema, M., Noda, C., Kobayashi, S., Matsuo, I., Kanai, Y., Nagasawa, T., Sugimoto, Y., Takahashi S., *Simons, B.D., and *Yoshida, S. Competition for mitogens regulates spermatogenic stem cell homeostasis in an open niche. Cell Stem Cell. 2018 Dec 20. (*: Co-corresponding authors)

〔Review Articles〕

- Yoshida, S. (2018). Open niche regulation of mouse spermatogenic stem cells: Develop. Growth Differ. *60*, 542-552.
- Yoshida, S. (2018). Regulatory mechanism of spermatogenic stem cells in mice: their dynamic and context-dependent behavior, In Reproductive and Developmental Strategies, K. Kobayashi, T. Kitano, Y. Iwao, and M, Kondo, eds. (Springer Japan), pp. 47-67.