

DIVISION OF GERM CELL BIOLOGY



Professor
YOSHIDA, Shosei

- Assistant Professor:** KITADATE, Yu
 NAKAGAWA, Toshinori
- Technical Staff:** MIZUGUCHI, Hiroko
- JSPS Researcher:** IKEDA, Tatsuro
- Postdoctoral Fellow:** HIRA, Seiji
- SOKENDAI Graduate Student:** HIRANO, Kodai
 WANG, Zhe
- Visiting Graduate Student:** SATO, Toshiyuki
- Visiting Undergraduate:** BABAGUCHI, Hirotaka
- Technical Assistant:** KON, Yayoi
 MARUYAMA, Ayumi
 FUJITA, Miyako
- Secretary:** KUBOKI, Yuko

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

We have 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 GFR α 1⁺ 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 GFR α 1⁺ spermatogonia

In order to find the key factors regulating the GFR α 1⁺ cell density, we screened the genes expressed in the tubule area that were facing the interstitium and vasculature, where GFR α 1⁺ cells preferentially locate (Figure 1A). From this screening, we focused on *Fgf5*, since it is located in peritubularly located large flattened cells (termed lymphatic endo-

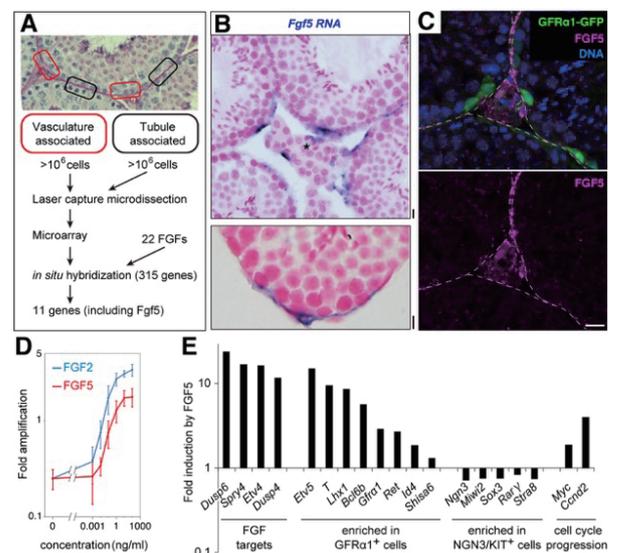


Figure 1. (A) Outline of the screening for genes preferentially expressed in the vasculature-associated region. (B) Representative ISH images for *Fgf5* (blue) in testis sections, counterstained with nuclear fast red. (C) Representative image of an inter-tubular region of a GFR α 1-GFP mouse testis stained for GFP (green), FGF5 (magenta) and DNA (blue). Scale bars, 10 μ m. (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 \pm 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).

thelial 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 GFR α 1⁺ 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 GFR α 1⁺ spermatogonia showed a strikingly linear correlation with *Fgf5* dosage (Figure 2A). These mutants notably sustained steady-state spermatogenesis with different density *set points* of GFR α 1⁺ cells that correlate in a manner that depends linearly on *Fgf5* dosage during adulthood (Figure 2B).

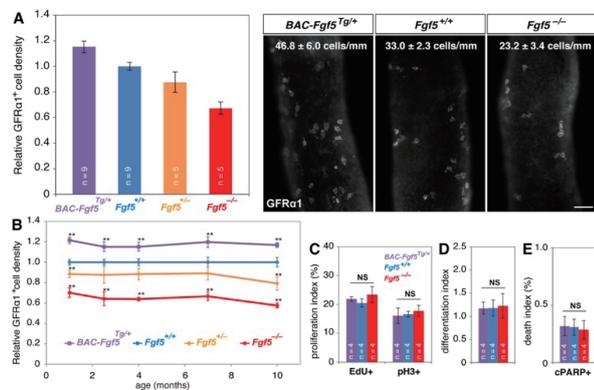


Figure 2. (A) Average densities \pm SEM of GFR α 1⁺ cells and representative IF images of whole-mount seminiferous tubules for GFR α 1 (right) of 2.5 month-old mice with indicated genotypes. Scale bar, 50 μ m. (B) Average densities \pm SEM of GFR α 1⁺ cells in mice with indicated genotypes and ages. (C–E) Indexes of proliferation (Edu⁺ and pH3⁺ fractions), differentiation [quantified as the RAR γ ⁺/KIT⁻ (\approx NGN3⁺) over GFR α 1⁺ cell ratio], and death (cPARP⁺ fraction) in GFR α 1⁺ cells of indicated mice at 2.5 months of age.

III. Each GFR α 1⁺ cell receives an unchanged level of FGF signal in *Fgf* mutants

Given the mitogenic and differentiation-inhibiting functions of FGF5, we first considered whether GFR α 1⁺ 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 GFR α 1⁺ 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 GFR α 1⁺ cells between mutants.

The above findings lead to a hypothesis that FGF supply is a limiting factor that is competed for among the GFR α 1⁺ cells. In this case, the levels of FGF signal received by each GFR α 1⁺ cell would be equalized among *Fgf* mutants harboring different densities of GFR α 1⁺ cells.

IV. GFR α 1⁺ spermatogonia consume FGF5

To further develop this hypothesis, we then examined whether GFR α 1⁺ 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 GFR α 1⁺ 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 GFR α 1⁺ cells consume extracellular FGF5, supporting the idea that GFR α 1⁺ cells compete for extracellular FGF.

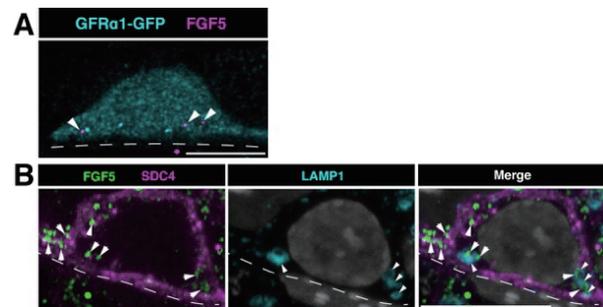


Figure 3. (A) Representative images of GFR α 1-GFP⁺ (cyan) cells exhibiting the speckled cytoplasmic staining of FGF5 (magenta, white arrowheads). (B) Representative images of SDC4⁺ (magenta) cells co-stained for FGF5 (green) with LAMP1 (cyan). FGF5⁺ speckles (arrows) were often co-localized with LAMP1⁺ (arrowheads) foci in SDC4⁺ cytoplasmic clamp. Scale bars, 10 μ m.

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 GFR α 1⁺ 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 GFR α 1⁺ cells.

This model was found to be capable of capturing the counterintuitive observation that the fate behavior of GFR α 1⁺ 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 GFR α 1⁺ cells is balanced. Given that GFR α 1⁺ 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 system is strongly perturbed from a steady state. Analysis of the model predicted that the recovery of GFR α 1⁺ 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 GFR α 1⁺ 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).

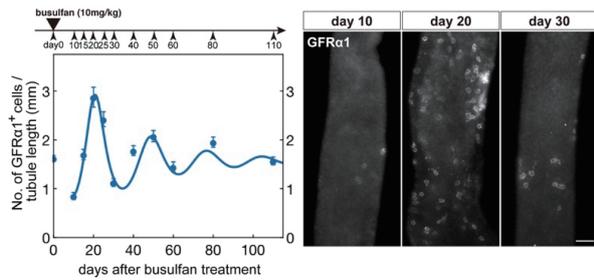


Figure 4. Observed kinetics of the $GFR\alpha 1^+$ cell density following busulfan treatment in WT (left), and examples of IF images of whole-mount seminiferous tubules stained for $GFR\alpha 1$ (right). Model results (curves) compared to experimental measurements (dots) of the average $GFR\alpha 1^+$ cell density following busulfan treatment.

To summarize, our results show that the *in vivo* fate behavior of $GFR\alpha 1^+$ 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.

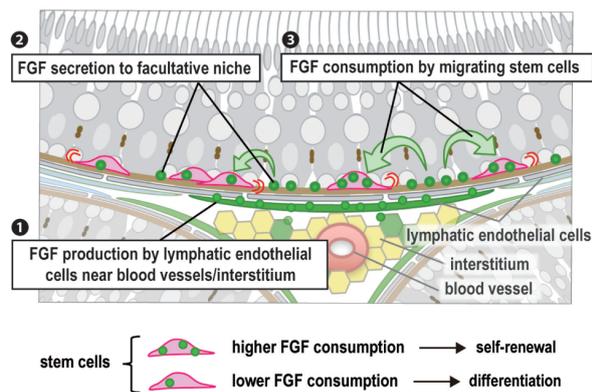


Figure 5. A conceptual diagram of mitogen competition leading to stem cell density homeostasis. Limited supply of FGF from a subset of lymphatic endothelial cells in proximity to vasculature plays a key role in stem cell density homeostasis.

Publication List:

[Original Papers]

- 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. (2019). Competition for mitogens regulates spermatogenic stem cell homeostasis in an open niche. *Cell Stem Cell* 24, 79-92. doi: 10.1016/j.stem.2018.11.013
- Nakamura, S., Hira, S., Fujiwara, M., Miyagata, N., Tsuji, T., Kondo, A., Kimura, H., Shinozuka, Y., Hayashi, M., Kobayashi, S., and Mukai, M. (2019). A truncated form of a transcription factor Mamo activates *vasa* in *Drosophila* embryos. *Commun. Biol.* 2, 422. doi: 10.1038/s42003-019-0663-4
- Shinozuka, T., Takada, R., Yoshida, S., Yonemura, S., and Takada, S. (2019). Wnt produced by stretched roof-plate cells is required for the promotion of cell proliferation around the central canal of the spinal cord. *Development* 146, dev159343. doi: 10.1242/dev.159343

[Review Articles]

- Yoshida, S. (2019). Heterogeneous, dynamic, and stochastic nature of mammalian spermatogenic stem cells. *Current Topics in Developmental Biology* 135, 245-285. doi: 10.1016/bs.ctdb.2019.04.008
- Yoshida, S. (2020). Mouse spermatogenesis reflects the unity and diversity of tissue stem cell niche systems. *Cold Spring Harbor Perspectives in Biology*, a036186. doi: 10.1101/cshperspect.a036186