Division of Germ Cell Biology

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Overview of our research

The production of copious amounts of sperm over a long period in the testis is fundamental for the continuity of life across generations. The Division of Germ Cell Biology aims to understand the key processes of germ cell development, that lead to the robust production of sperm and the accurate transmission of genetic information.

In particular, our emphasis has been placed 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, 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,

Our lab has figured out a number of key properties of mouse SSCs. One of these is that they 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 investigating the cellular identity of "actual" SSCs, their *in vivo* behavior at a single-cell resolution, and their underlying mathematical principles, leading to the discovery of the dynamics of "population asymmetry" and subsequent "neutral competition" between SSCs. We have proposed that the aforementioned type of competition observed between SSCs could be the result of consumption of limited supply of self-renewal promoting extracellular ligands. The "potential stem cells" have also fascinated us. During steady-state spermatogenesis, these cells are largely active as transient amplifying cells and differentiate rather than self-renew. However, when tissues are insulted or testicular cells are transplanted into host testes, the probability of their self-renewal increases greatly. 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, Tokue *et al.*, Stem Cell Reports 2017, and Kitadate *et al.*, Cell Stem Cell 2019.

An important achievement we accomplished in 2020 was the revelation of SSC behavior in the context of post-transplantation reconstruction of spermatogenesis at a clonal resolution, and the reporting of a novel strategy to enhance the transplantation efficiency by tuning the post-transplantation fate of SSCs (Nakamura *et al.*, Cell Stem Cell 2021).

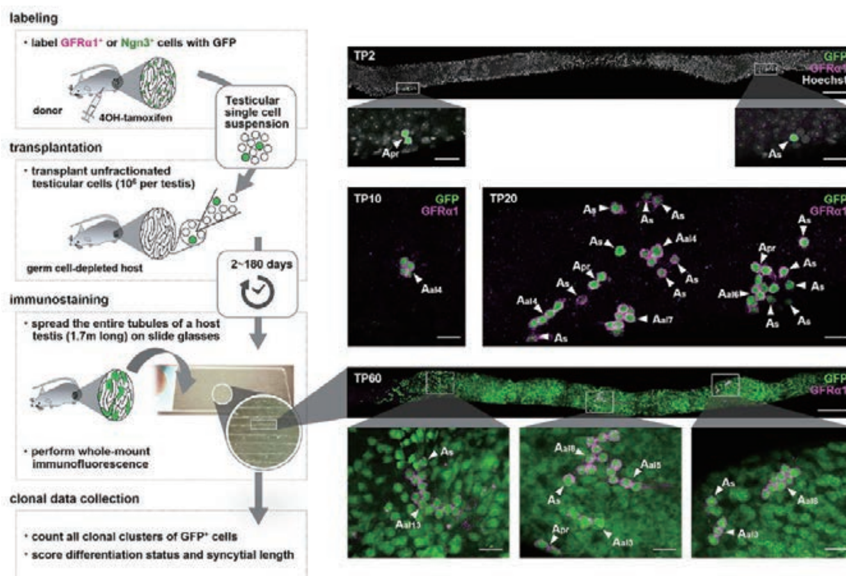

Figure 1. The "pulse-transplantation" experiment
In the donor mice, actual (GFRα1+) and potential (Ngn3+) SSCs are pulse-labelled, whose testes were dissociated into single cell suspension for transplantation to germ cell-depleted hosts. After certain times, the entire testes of the hosts were recovered, untangled seminiferous tubules were processed for immunostaining. *All* the pulse-labelled (GFP+) clones in the host tetes were scored for statistical analyses. Adopted from Cell Stem Cell *5*, 1443-1456 (2021).

Post-transplantation regeneration: a remarkable feature of stem cells promising a wealth of applications

Tissue stem cells can restore the impaired structure and function of host tissues following transplantation. In blood, hematopoietic stem cell transplantation has been established as a radical treatment for leukemia, while trials to test the viability of stem cell transplantation in mesenchymal and epithelial tissues are ongoing. In male germline, SSC transplantation was established by Brinster and their colleagues in 1994 using mice, which promised a wealth of applications such as restoration of fertility to cancer patients after chemotherapy and the preservation of genetic diversity. However, the inefficiency of this technology currently rules out its practical application.

Indeed, our knowledge about the fate behavior of individual SSCs and their progenies after transplantation remains poorly understood, thus limiting the potential to develop new strategies to increase the currently low transplantation efficiencies.

Clonal fate analysis of actual and potential stem cells following transplantation

A remarkable feature of SSCs that our group has discovered is the presence of "potential stem cells", which significantly contributes to the post-transplantation regeneration in a manner similar to "actual stem cells" (Nakagawa *et al*., 2007). Therefore, based on an experimental setting of pulse-labeling actual and potential SSCs using the specific expression of GFRα1 and Ngn3 in these cells, respectively, we first investigated their detailed clonal fate following transplantation in the host testes (Figure 1). *All* the clones that had been derived from labelled SSCs and resided in the host testes were counted and scored for the number of constituent SSCs and differentiated cells over a broad time line spanning from 2 days to 180 days after transplantation, when single donor cell-derived clones formed robust repopulating *colonies*.

Actual and potential SSCs follow indistinguishable clonal fates with comparable repopulation efficiencies

It was clear that single SSC-derived clones follow highly divergent fates, showing varying clone sizes (*e.g.*, the number of SSCs in individual clones, or physical extension within the tissue), rather than showing a stereotypical pattern. The clonal fate data derived from actual (GFRα1$^+$) and potential (Ngn3$^+$) SSCs first show that these cells form long-term repopulating colonies at comparable, albeit not equal, efficiencies. When Ngn3$^+$ cells were transplanted, a significant fraction of them were quickly converted into GFRα1$^+$ cells within 2 days; an observation consistent with the results of the preceding studies. Interestingly, GFRα1$^+$ cell- and Ngn3$^+$ cell-derived clones evolve parallel to indistinguishable size distributions and kinetics over time. Thus, actual and potential SSCs are equipotent in terms of clonal evolution during post-transplantation repopulation, irrespective of the original state on transplantation.

Most of the initially settled SSCs are lost through stochastic differentiation and cell death

We subsequently looked in detail into the clonal fates of donor SSCs. We unexpectedly found that the number of SSCs that initially resided in the tissue was considerably larger than those which eventually formed colonies over the long term (*i.e.*, after couple of months). After being attached to the host tissue and moved to the location in which SSCs are normally found (on the basement membrane of seminiferous tubules), the vast majority of SSCs were lost through differentiation and cell death, with only a small fraction (one out of tens) contributing to long-term repopulation.

Furthermore, in collaboration with the Ben Simon Lab at the University of Cambridge, we asked whether this variable and intricate fate behavior can be explained by a minimal mathematical model, relying on an assumption that all SSCs are equivalent, and select their fates probabilistically at defined rates reliant only on their current state (*i.e*., unrelated to the past history). Significantly, such simple models were found to be capable of predicting the broad range of clonal fate data with high accuracy, supporting the aforementioned model assumption that all SSCs are equipotent. This insight is in stark contrast with the prevailing thought that only a small fraction of GFRα1$^+$ and/or Ngn3$^+$ cells are definitive SSCs that form colonies at high probabilities (presumptively, 100%).

Post-transplantation fate of SSCs can be modified through differentiation inhibition

The suggestion by the modeling analysis that all SSCs are equivalent motivated us to test whether SSC fate can be altered to increase repopulation efficiency. In particular,

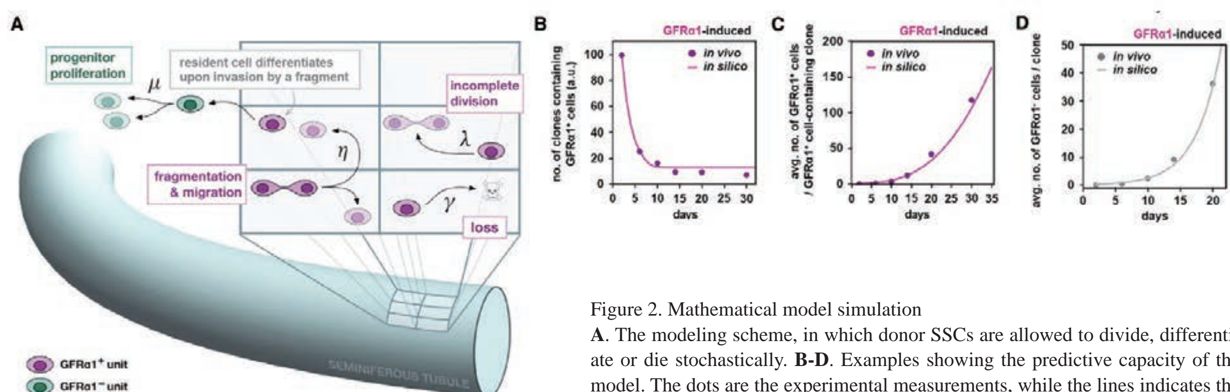

Figure 2. Mathematical model simulation
A. The modeling scheme, in which donor SSCs are allowed to divide, differentiate or die stochastically. **B-D**. Examples showing the predictive capacity of the model. The dots are the experimental measurements, while the lines indicates the simulation. Adopted from Cell Stem Cell *5*, 1443-1456 (2021).

given that irreversible commitment of SSCs to differentiate and lose their self-renewing potential is induced by, and dependent on, retinoic acid (RA) signaling, we asked if the administration of a chemical inhibitor of retinoic acid (RA) synthesis, WIN18,446, can affect the behavior of transplanted SSCs. We found that WIN18,446 treatment can, in an *in vivo* context, not only inhibit differentiation of donor SSCs, but also incline them toward self-renewal and significantly increase the number of self-renewing pools of surviving SSCs. Such an effect was observed more dramatically in Ngn3+ cells that were primed for differentiation, and effectively recruited to self-renewing pool by WIN18,446 treatment.

Transient suppression of donor SSC differentiation boosts long-term repopulation and restores host fertility

Finally, we examined if the overall long-term repopulation efficiency could be increased, after this type of differentiation inhibition is terminated so as to allow the SSCs to proceed with spermatogenesis. Remarkably, we observed a dramatic increase in long-term repopulation efficiency, (*i.e.*, by 5-10 times) which was capable of restoring the fertility of a host that could not sire offspring through natural mating. Pups obtained from the donor SSC-derived sperm generated in the WIN18,446-treated host grew into healthy and fertile adults without any apparent abnormalities.

These results not only support the thesis that all SSCs are equipotent, but also offer a novel strategy to increase the transplantation efficiency through tuning the donor cell fate by changing the hosts' niche environment. While WIN18,446 treatment is effective, in theory, this efficiency could be further increased another ten times if all transplanted SSCs successfully repopulate, Technologically, the use of WIN18,446 and/or other reagents modifying the SSC fate shows promise in the field of SSC transplantation across broader applications, from restoring fertility to cancer patient after therapy to the preservation of genetic diversity.

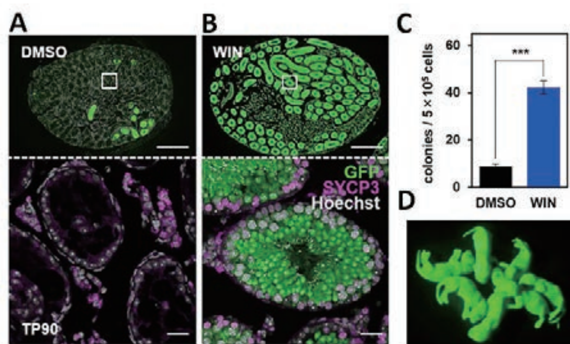

Figure 3. Restoration of host fertility using WIN18,446
A-B. Representative appearance of the host testes 90 days after transplantation of GFP+ donor testicular cells, in control (DMSO) and WIN18,446-treated hosts. Note the dramatic increase of the repopulation efficiency. **C.** Quantification of the repopulation efficiency based on the number of repopulating colonies. **D.** Offspring sired by the WIN18,446-treated host through natural mating with wild-type female. The green fluorescence indicates that the pups were originated from GFP+ donor testicular cells developed into functional sperm in the host testes. Adopted from Cell Stem Cell *5*, 1443-1456 (2021).

Publication List:

〔Original Papers〕

- Rezende-Melo, C.A., Caldeira-Brant, A.L., Drumond-Bock A. L. and Buchold, G.M., Shetty, G., Almeida, F.R.C.L., Matzuk, M.M., Hara, K., Yoshida, S., Meistrich, M.L., and Chiarini-Garcia, H. (2020). Spermatogonial asynchrony in Tex14 mutant mice lacking intercellular bridges. Reproduction *160*, 205–215. DOI: 10.1530/REP-20-0118
- Sznurkowska, M.K., Hannezo, E., Azzarelli R., Chatzeli, L., Ikeda, T., Yoshida, S., Philpott, A., and Simons, B.D. (2020). Tracing the cellular basis of islet specification in mouse pancreas. Nat. Commun. *11*. DOI: 10.1038/s41467-020-18837-3
- Yeh, Y.-H., Hu, M., Nakagawa, T., Sakashita, A., Yoshida, S., Maezawa, S., and Namekawa, S.H. (2021). Isolation of murine spermatogenic cells using a violet-excited cell-permeable DNA binding dye. JOVE-J. Vis. Exp. DOI: 10.3791/61666
- Jörg, D.J., Kitadate, Y., Yoshida, S., and Simons, B.D. (2021). Stem Cell Populations as Self-Renewing Many-Particle Systems. Annu. Rev. Conden. Ma. P. *12*, 135-153. DOI: 10.1146/annurev-conmatphys-041720-125707

〔Review Article〕

- Yoshida, S. (2020). Mouse spermatogenesis reflects the unity and diversity of tissue stem cell niche systems. Cold Spring Harb. Perspect. Med. *10*. DOI: 10.1101/cshperspect.a036186