Laboratory of Molecular Genetics for Reproduction †

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Laboratory scope

Reproduction is a universal and fundamental system for organisms to produce new generations. To accomplish this purpose, organisms have developed their own sexual strategies, which allow them to adapt to their environment, thereby progressing toward maximum efficiency of reproduction. During the embryo and larval terms, organisms develop many cell-lineages that have special and essential roles in each different process of reproduction. These lineages are mostly conserved among vertebrates.

Vertebrates, however, exhibit a variety of reproductive systems. The mechanisms of sex determination and sex differentiation are some of the components of the reproductive system which produce this variety. Actually, there are many modes of sex determination. Sex determination genes are different among vertebrates. Sex determination does not even have to be controlled genetically. This variety is allowed by the different employment and different emergence of the cell lineages during embryogenesis. Therefore, it is important to address the roles of each cell lineage for understanding the fundamental mechanism underlying a variety of reproductive systems. Currently, our lab focuses on the core mechanisms which are independent of sex determination genes and which produce and maintain the sex. The core mechanism can be referred to as cellular interaction between germ cells and surrounding somatic cells, wherein germ cells have the ability to feminize somatic cells while the surrounding somatic cells are predisposed to male development. These characters of each set of cells are totally independent of the sex determination gene on the medaka Y chromosome. We are addressing the details of this core mechanism by analyzing each cell lineage in the context of sex differentiation.

To accomplish the purpose of our study, we use medaka fish (*Oryzias latipes*). We have been generating transgenic medaka enabling us to analyze how different cell lineages are involved in the process of gonad formation and sex differentiation *in vivo*. Additionally, in order to identify the genes essential for reproduction, we carried out a mutational screening of medaka with defective phenotypes and disrupted several candidate genes. With these two unique analytical methods (visualizing cells, and mutants), we are attempting to unveil both the fundamental mechanisms and the specific mechanisms that produce a variety of reproductive systems.

Through these analyses, we have been revealing the presence of germline stem cells in the ovary. This was the first proof of this system in vertebrates (Nakamura et al 2010 Science). The fluorescently labeled germline stem cells keep producing eggs with fluorescence during the entire period of medaka reproduction, which is a conclusive indication of the presence of germline stem cells. The tricks employed in this experiment are transgenic medaka that allow heat-inducible gene expression.

I. An issue of sex determination of germ cells

In the core mechanism of sex, germ cells are responsible for feminization and somatic cells are for masculinization. An important thing here is that we can determine the sex intentionally, if the core mechanism is to be modified, without any effect from the sex determination gene. Then a big issue is how the sex of germ cells, in other words, the fate decision of germ cells to become sperm or eggs, is determined. Few people have addressed this issue in vertebrates.

One way of looking at this issue is that there is no germ cell-intrinsic mechanism of sex. Somatic cells might send a signal(s) to germ cells during each developmental stage of spermatogenesis/oogenesis. In this case, it would be difficult to discern the germ cell-intrinsic mechanism of sex. Alternatively, we might assume that the sexual fate of germ cells is determined at the initial commitment into gametogenesis. Somatic cells may only regulate the timing and/or supply an essential material(s) for germ cells to develop into the next stage of gametogenesis. In this case, once the somatic cells give a cue to germ cells for which way to go, the path toward oogenesis or spermatogenesis is already determined. But nobody has shown which way of understanding is right in germ cell sex determination.

Since transplanted germ cells from testis into females develop into eggs and the germ cells from ovaries become sperm in testis, It is generally accepted that germline stem cells are sexually indifferent or unfixed. No morphological difference between germ cells from females or those from males exist during early stages of gametogenesis, until they reach the pachytene stage in meiosis. At the pachytene stage, female germ cells are larger than male germ cells. These observations have collectively suggested that germ cell-sex determination occurs at some point between the germline stem cell stage and that of the pachytene stage (figure 1).

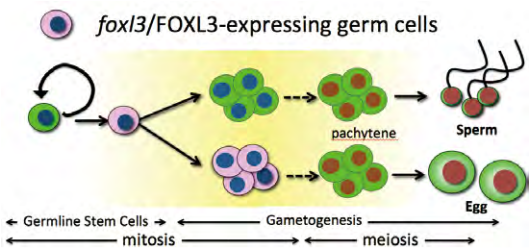

Figure 1. Simplified cellular processes of gametogenesis. Given that an intrinsic mechanism of sex determination is present in germ cells, it should be between the stage of germline stem cells and that of pachytene in meiosis (yellow graded area). *foxl3* is found to be expressed only in female germ cells (pink germ cells) in the yellow graded term.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2015. The former title is indicated by an asterisk (*).
†: This laboratory was closed on 31 May, 2016.

II. Identification of a sexually different gene in early germ cells (Nishimura et al 2015 Science)

We have prepared all XX or all XY medaka larva and have purified germ cells during early gametogenesis. Different stages of germ cells were successfully purified using fluorescent activated cell sorter (FACS) and the transcriptomes of different stages and different sexes of germ cells were analyzed by next-generation sequencing.

One gene that attracted us was *foxl3*, a gene encoding a transcriptional factor with the forkhead domain. The gene is initially expressed in both female and male developing gonads but male developing gonads lose its expression while *foxl3* expression persists in female germ cells. Interestingly, some populations of germline stem cells express this gene and, as oogenesis proceeds, expression in germ cells diminishes by the time they enter the pachytene stage. The expression pattern matched the theoretically expected expression pattern of the sex determination gene in germ cells (figure 1).

III. *foxl3* represses initiation of spermatogenesis in germ cells

To understand the function of *foxl3*/FOXL3, we generated *foxl3* mutant medaka using the TALEN-method.

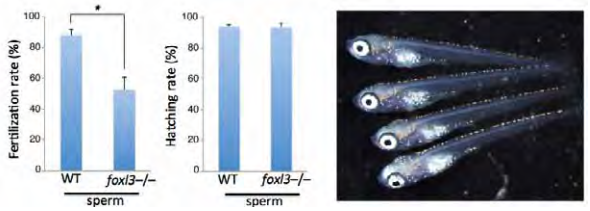

Figure 2. Although the rate is relatively low compared with normal sperm, sperm from mutant ovaries have the ability to fertilize eggs (left graph). Once the eggs are fertilized with sperm from the mutant ovary, they develop to hatch as efficiently as normal embryos (middle graph). Hatched larva grow into fertile medaka. The picture on the right shows medaka larva hatched from eggs fertilized with sperm from a mutant ovary.

Very interestingly, homozygous female medaka initiate development of sperm in the early larval stage. Together with the fact that *foxl3* is expressed specifically in female germ cells, this indicates that *foxl3* represses initiation of spermatogenesis in germ cells in female gonads.

IV. Functional sperm develop in the ovary

We artificially inseminated sperm from female mutants into normal eggs, and found that the fertilized eggs developed into normal embryos. The embryos hatched and grew into fertile adult medaka. This clearly demonstrates that sperm in the female mutants is functional (figure 2).

Then, we analyzed the structure of the gonads in the female mutants. The gonads display a typical ovarian structure with an ovarian cavity separated from the stromal compartment by a germinal epithelium. In normal ovaries, the germline stem cells with ovarian niches, Germinal Cradles, are located within the germinal epithelium and, as described, germline stem cells express *foxl3*. In the female *foxl3* mutants, sperm is developed in the germinal epithelium. Probably because there is no exit for the sperm to leave the germinal epithelium, the germinal epithelium with functional sperm expands towards the stromal compartment. Therefore the mutant ovary looks as if sperm fully fills an entire ovary (figure 3).

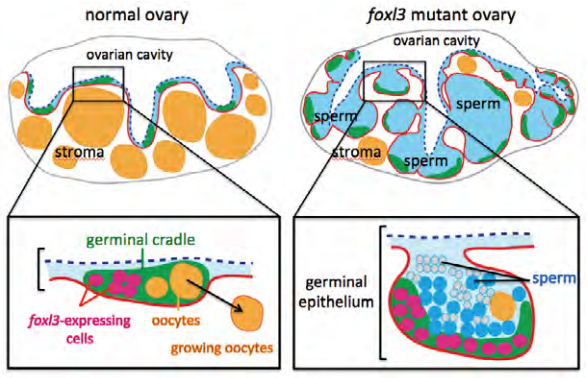

Figure 3. Schematic comparison of normal ovary with ovary producing sperm in the mutant female. Sperm develop in the germinal cradles in the germinal epithelium in the *foxl3* female mutant

These results reveal several important points:

1. An intrinsic mechanism of sex determination is present in germ cells. This mechanism can function against the direction of the sex determination gene and direct germ cells to develop to a sex opposite from the somatic sex.
2. Repression of initiation of spermatogenesis is a main component of the sexual switch of germ cells (the fate decision to develop into eggs or sperm) and involves *foxl3*.
3. Spermatogenesis does not require the testicular environment. It can occur in the ovarian structure and environment. This suggests that the testicular environment is required for the coordinated regulation of spermatogenesis with other parts of the body. This also suggests that germline stem cells with *foxl3* are ready for spermatogenesis.

Publication List:

〔Original paper〕

● Nishimura, T., Sato, T., Yamamoto, Y., Watakabe, I., Ohkawa, Y., Suyama, M., Nakamura, S., Saito, T.L., Yoshimura, J., Morishita, S., Kobayashi, S., and Tanaka, M. (2015). *foxl3* is a germ cell-intrinsic factor involved in sperm-egg fate decision in medaka. Science *349*, 328-331.