Laboratory of Molecular Genetics for Reproduction

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Laboratory scope

Reproduction is a universal and fundamental system for organisms to produce generations. To accomplish this purpose efficiently, organisms develop sexual reproduction. Vertebrates, however, exhibit a variety of reproductive systems. The many modes of sex differentiation is one of the main components that contribute to variety. Our laboratory aims to reveal the fundamental mechanisms underlying reproduction, especially focusing on the mechanism of sex differentiation, and to understand how the mechanisms are related to a variety of reproductive systems.

We use medaka fish (*Oryzias latipes*) and 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 mechanism that produce a variety of reproductive systems.

I. Critical contribution of germ cells to direct sex differentiation

In gonochoristic vertebrates such as medaka and humans, a gene on the sex chromosome is responsible for the determination of sex. Once the process of sex determination is triggered by the gene, the animal begins to develop into either female or male and does not change the direction during its life cycle. The sex differentiation is unidirectional. On the other hand, it has been described that sex is a consequence of balancing between female and male process (biphasic process) because sex reversal is often reported even in gonochoristic vertebrates.

As the results of our previous studies, we have revealed that germ cells are critical for the biphasic process. In the absence of germ cells, we found that medaka exhibit complete male secondary characteristics at both endocrine and gene levels (Kurokawa *et al*., 2007 PNAS). This indicates that germ cells are essential for formation of ovaries. In addition, in the absence of germ cells, somatic cells are predisposed to male development even if the individuals do not have a Y chromosome. This view indicates that, other than sex determination genes, germ cells critically contribute to establishment of biphasic status.

Supporting this view, we have previously identified the gene responsible for this regulation, *amhrII* (anti-Müllerian hormone receptor). The ligand for this receptor is AMH (anti-Müllerian hormone), which is known to be secreted from male supporting cells (Sertoli cells) during mammalian sex differentiation, and is critical for Müllerian duct (female reproductive organ) regression in mammalian males. But in teleosts, there are no organs equivalent to the Müllerian duct. In addition, AMH belongs to a phylogenetically old and conserved type of TGFβ superfamily. These collectively suggest some conserved function other than Müllerian duct regression.

The mutant, called *hotei*, has a mutation in *amhrII* and exhibits a hypertrophic phenotype of germ cells and male to female sex reversal. This suggested that an AMH system regulates germ cell number and sex (Morinaga *et al*., 2007 PNAS).

Our analysis using the mutant indicates that an AMH system regulates germline stem cells. In the absence of the AMH signal, proliferation of a mitotically active type of germline stem cell is promoted in the gonads. The enhanced proliferation of germline stem cells, but not a direct effect of AMH impairment, causes sex reversal from male to female even if the medaka possesses a Y chromosome (Nakamura *et al*., 2010 Science, Nakamura *et al*., 2012 Development).

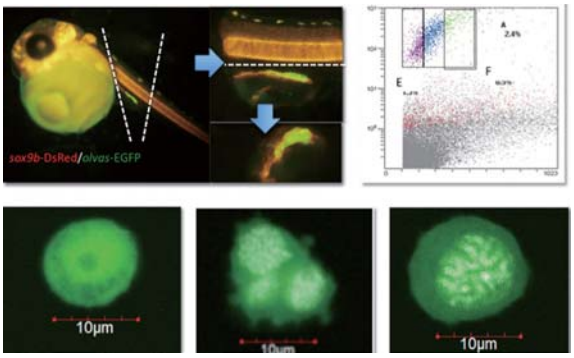

Figure 1. Isolation of different types of germ cells. Upper left; transgenic medaka with fluorescent germ cells. Upper right: Cell-sorting conditions that allow isolation of different germ cells. Bottom images (from left to right); stem-type germ cell, cystic germ cells (type II) and early diplotene germ cell.

II. The plasticity of germ cell sex

These results raise a still unaddressed fundamental issue of reproduction, the sex of germ cells. Before critical contribution of germ cells to sex differentiation, the sex of germ cells has to be determined.

The reciprocal transplant experiments between female and male by other groups show that the germline stem cells are sexually plastic or indifferent. This implies that the sex of germ cells may be determined at an early phase of gametogenesis. Our previous study indicates that germline

stem cells commit gametogenesis through synchronous and successive division before they enter meiosis (Saito *et al*., 2007 Dev. Biol.). This division is called typeII division. It is therefore possible that sex is determined in the process between the mitotically active type of germline stem cells and cystic germ cells undergoing typeII division.

With employment of transgenic medaka that visualize germ cells (Tanaka *et al*., 2001 PNAS), we have established cell-sorting conditions to isolate stem-like germ cells, cystic germ cells and germ cells at an early diplotene stage (Figure 1). Parallel with this establishment, we prepared medaka microarray input by compiling all the public medaka databases available. These enabled us to search the transcripts that feature in each stage of germ cells.

Interestingly, the analysis reveals the presence of sexually different transcripts in stem type germ cells. The sexually dimorphic expression can be also recognized in primordial germ cells at earlier stages (Figure 2).

First we addressed if the different expression occurred as a germ-cell autonomous event or is regulated by somatic cells. For this purpose, we generated chimeric medaka with different sex of germ cells (XX somatic cells vs XY germ cells or XY somatic cells vs XX germ cells). The expression of the transcripts is consistently enriched in XY germ cells when compared with XX germ cells, and this difference did not depend on the somatic sex at all. The results clearly demonstrate that the sexually different expression is regulated in a germ cell-autonomous manner.

It is generally accepted that sex determination gene is expressed in the somatic cells surrounding germ cells at the onset of gonadal formation. Our finding, therefore, made us suspect that sex determination gene is also expressed in primordial germ cells at stages earlier than gonadal formation and might cause the sexually different expression of the transcripts. We performed in situ hybridization and, as expected, detected the expression of the sex determination gene in the primordial germ cells of males. Then, the expression of the sex determination gene was knocked down by injection of grip-RNA. Unexpectedly, however, downregulation of sex determination gene does not affect any sexually different expression of the transcripts. This demonstrates that the Y chromosome-, but not the sex determination gene, dependent mechanism is involved in the sexually different expression of primordial germ cells.

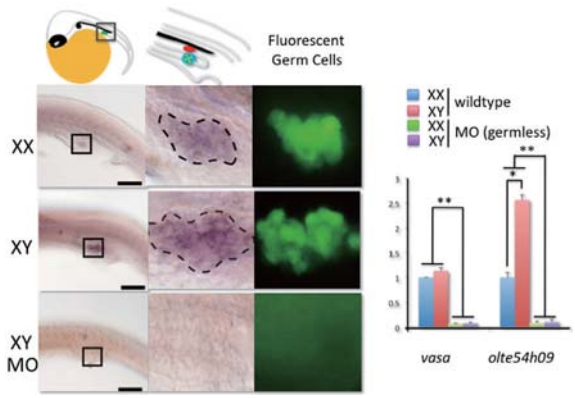

Figure 2. Presence of the transcripts that exhibit sexually different expression in the primordial germ cells.

The sexually dimorphic event is also manifested in the primordial germ cell behavior. We found that isolated primordial germ cells, before onset of sex determination genes in the somatic cells, exhibit the sexually different rate of proliferation in culture. Very interestingly, the sexually different expressing gene in the primordial germ cells affect the proliferation by the overexpression and knockdown experiments.

All the results mentioned above demonstrate the sexual plasticity of germ cells and several mechanisms, other than sex determination genes, that confer sexually different characters at cellular levels: importance of a Y chromosome. Actually we found the sexually different gene is mapped near the sex determination locus on the Y chromosome and that sex-specific SNPs are present in the promoter region of the sexually dimorphic gene. These results collectively suggest that the difference of the two sex chromosomes, but not sex determination gene, can contribute to manifestation of sexually different character at the cellular levels.

Publication List

〔Original papers〕

- Herpin, A., Adolfi, M.C., Nicol, B., Hinzmann, M., Schmidt, C., Klughammer, J., Engel, M., Tanaka, M., Guiguen, Y., and Schartl, M. (2013). Divergent expression regulation of gonad development genes in medaka shows incomplete conservation of the downstream regulatory network of vertebrate sex determination. Mol. Biol. Evol. *30*, 2328-2346.
- Ishikawa, T., Okada, T., Ishikawa-Fujisawa, T., Todo, T., Kamei, Y., Shigenobu, S., Tanaka, M., Saito, T.L., Yoshimura, J., Morishita, S., Toyoda, A., Sakaki, Y., Taniguchi, Y., Takeda, S., and Mori, K. (2013). ATF6a/b-mediated adjustment of ER chaperone levels is essential for development of the notochord in medaka fish Mol. Biol. Cell *24*, 1387-1395.
- Kobayashi, K., Kamei, K., Kinoshita, M., Czerny, T., and Tanaka, M. (2013). A heat-inducible cre/loxP gene induction system in medaka. Genesis *51*, 59-67.

〔Review articles〕

- Morohashi, K., Baba, T., and Tanaka, M. (2013). Steroid hormones and the development of reproductive organs. Sex. Dev. *7*, 61-79.
- Nishimura, T., and Tanaka, M. (2013). Function of germ cells in sex differentiation. In Sexual Plastility and Gametogenesis in Fishes, S. Subramanian, ed. (Nova Biomedical, New York), pp.291-304.
- Tanaka, M., and Capel, B. (2013). Forward to the special issue on sex determination. Dev. Dyn. *242*. (Editors of this Special Issue)