Laboratory of Molecular Genetics for Reproduction

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Laboratory scope

Our laboratory aims to reveal the molecular mechanisms of the formation of the gonads and sex differentiation. We use medaka fish (*Oryzias latipes*) for these purposes and have been generating transgenic medaka (Figure 1) enabling us to identify different cell lineages by fluorescence and to analyze the process of gonad formation and sex differentiation in vivo. Additionally, in order to identify the genes essential for gonadogenesis, we carried out a mutational screening of medaka with defective gonads and are performing a positional cloning. With these two unique analytical methods (visualizing cells and mutants), we are attempting to unveil the fundamental mechanisms of sex differentiation and plasticity common to many organisms.

I. Cellular biphasic process critical for manifestation of the sex

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.

This view was also supported by our recent mutant analysis (Nakamura et al., 2012 PLoS ONE). Medaka with a mutation in the *sox9b* gene show a female to male sex reversal. In the mutant, an initial pathway to determine the sex functions and the gonad normally is formed. But we found that *sox9b* mutant does not maintain the germ cells. The extracellular matrix produced by supporting cells surrounding germ cells is largely disorganized in the mutant (Figure 1). Therefore it was expected that loss of germ cells but not impairment of the initial male pathway might cause a female to male sex reversal. In order to prove this hypothesis, we tried recovering the number of germ cells using *sox9b* and *amhrII* heterozygous compound mutant. In the compound mutant, germ cells come back to the normal number and no sex reversal was observed, supporting our idea that germ cells are important for the proper manifestation of sex

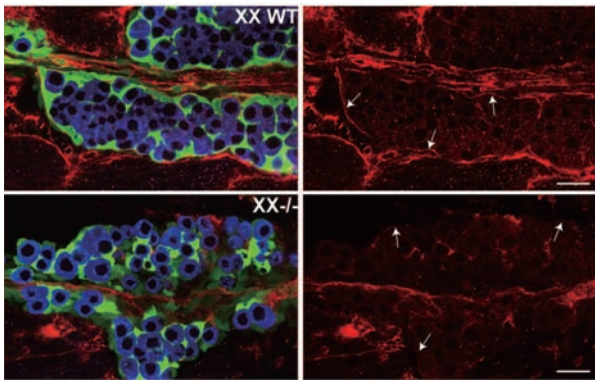

Figure 1. Extracellular matrix (laminin: red) in the *sox9b* mutant gonad (bottom panels) and in the wild-type gonad (upper panels). Distribution is impaired in the mutant. Green; *sox9b*-expressing supporting cells. Blue; germ cells (from PLoS ONE 2012).

II. How the number of germ cells are regulated during the course of early sex determination process

As mentioned above we can say that regulation of germ cell number is an important component of proper sex differentiation. We have previously identified the gene responsible for this regulation, *amhrII* (anti-Mullerian 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 males. But in teleosts, there are no organs found that are 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 a male to female sex reversal. This suggested that an AMH system regulates germ cell number and sex.

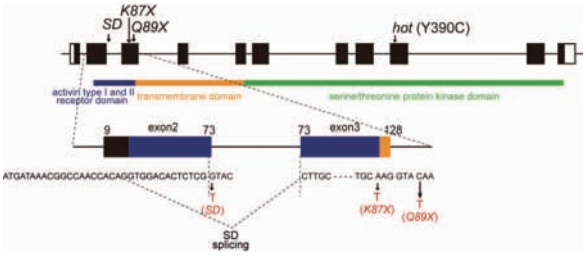

Figure 2. Hetrozygous *sox9b*-mutant medaka have a normal appearance but some exhibit female to male sex reversal.

First we isolated other alleles of *amhrII*, which causes a stop codon at the very last N terminal region and lacks important domains. Therefore it is very likely that these alleles are a functional null. Consistent with this, the mutated AMHRIIs fail to transmit an intercellular signal in the cultured cells. These allelic mutants displayed the same phenotype as the *hotei* mutant, excessive number of germ cells and sex reversal, indicating that a *amhrII* mutation in *hotei* also causes a functional null phenotype.

Next we investigated the expression of both ligand and receptor. As expected from the expression in mammals, medaka *amh* is expressed in supporting cells that directly surround germ cells. Interestingly *amhrII* is also detected in supporting cells. This suggests that the AMH system functions in supporting cells in an autocrine manner. To further confirm this, chimeric gonads were generated with somatic cells from the *hotei* mutant and wild-type germ cells. These chimeric gonads showed an increasing number of germ cells. On the other hand, gonads with normal somatic cells, and germ cells from the *hotei* mutant did not exhibit any *hotei* phenotype. The result of chimeric analyses supports the expression analysis, whereby hyperactive germ cell proliferation is a result of loss of the AMH signal in supporting cells but is not due to a direct effect of AMH on germ cells.

III. The AMH system regulates mitotically active type I germ cells.

During sex differentiation in medaka gonads, there are found two different types of germ cells. In males, germ cells divide intermittently and the daughter germ cells are readily enclosed with surrounded supporting cells (type I division). In females, however, there is an additional type of germ cell, dividing synchronously and successively to form germ cell cysts (type II division). Therefore females have more germ cells than males. Our previous results using the *zenzai* mutant indicated that type I is a self-renewal type of division while type II division occurs in germ cells that are committed to gametogenesis (Saito et al., 2008 Dev. Biol.). In addition, we proved the presence of germline stem cells in mature medaka ovaries (Nakamura et al., 2010 Science).

A BrdU incorporation experiment revealed the presence of mitotically quiescent germ cells, a good indication of the stem character, in the developing gonads (Figure 3). In the *hotei* mutants, the number of the quiescent type I germ cells did not change, compared to that in wild-type. But the mutant has more type I germ cells totally than the wild type. This suggests that the AMH system does not regulate transition from a quiescent type of type I germ cells to mitotically active type I germ cells but promotes mitosis of typeI germ cells that had already been committed to the mitotic process (Nakamura et al., 2012 Development).

Collectively these analyses demonstrate that the AMH system acts in supporting cells autonomously and that this activity consequently regulates mitotically active type I germ cells. In the mutant, loss of AMH activity results in more germ cells, which then causes a male to female sex reversal.

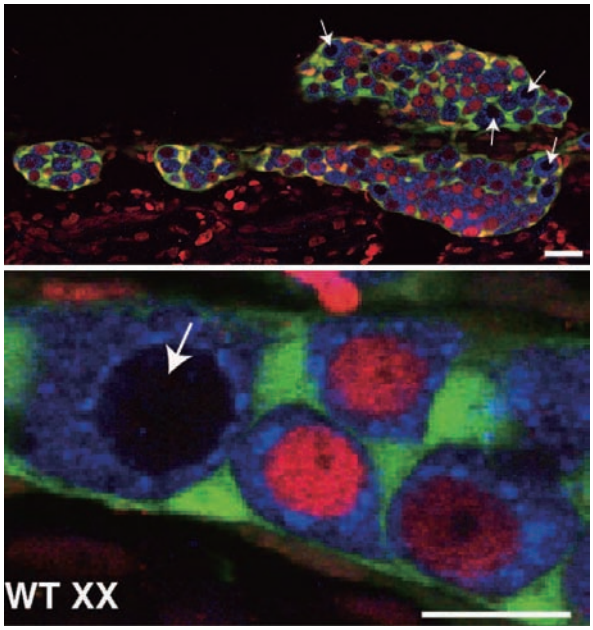

Figure 3. Presence of mitotically quiescent type I germ cells (arrows). Blue; germ cells, Red; BrdU, Green; supporting cells..

Publication List

〔Original papers〕

- Ichimura, K., Bubenshchikova, E., Powell, R., Fukuyo, Y., Nakamura, T., Tran, U., Oda, S., Tanaka, M., Wessely, O., Kurihara, H., Sakai, T., and Obara, T. (2012). A comparative analysis of glomerulus development in the pronephros of medaka and zebrafish. PLoS ONE *7*, e45286.
- Nakamura, S., Watanabe, I., Nishimura, T., Picard, J-Y., Toyoda, A., Taniguchi, Y., di Clemente, N., and Tanaka, M. (2012). Hyperproliferation of mitotically active germ cells dues to defective anti-Müllerian hormone signaling mediates sex reversal in medaka. Development *139*, 2283-2287.
- Nakamura, S., Watanabe, I., Nishimura, T., Toyoda, A., Taniguchi, Y., and Tanaka, M. (2012). Analysis of medaka *sox9* orthologue reveals a conserved role in germ cell maintenance. PLoS ONE *7*, e29982.

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

- Kobayashi, K., Kamei, K., Kinoshita, M., Czerny, T., and Tanaka, M. A heat-inducible cre/loxP gene induction system in medaka. Genesis 2012 Nov 3.