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. Celluar 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 differentation 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) while mutants with an excess number of germ cells cause complete feminization (Morinaga et al., 2007 PNAS). The sex reversal to female is the secondary effect of the over-proliferation of germ cells due to the impairment of an ancient type of the TGFβ signal, the anti-Müllerian hormone (AMH) system (manuscript submitted). Importantly these sex reversals are independent of presence or absence of the sex determination gene on the Y chromosome. This means that the balancing between germ cells and somatic cells is essential for proper manifestation of sex directed by the sex determination gene. We have therefore proposed that the germ cells critically contribute to the biphasic process of sex in medaka.

We have also identified the niche structure (called germinal cradle) that harbors germline stem cells in the medaka ovary for the first time in vertebrates (Nakamura et al., 2010 Science). Since the cells that constitute the cradle express the AMH ligand and receptor, the cradles are the place not only for regulation of proper and continuous production of eggs but also for the biphasic process of manifestation of sex (manuscript submitted). The cradles are also characterized with the expression of *sox9b*, which is specifically expressed in male developing gonads in mammals and is essential for mammalian testis formation (figure 2).

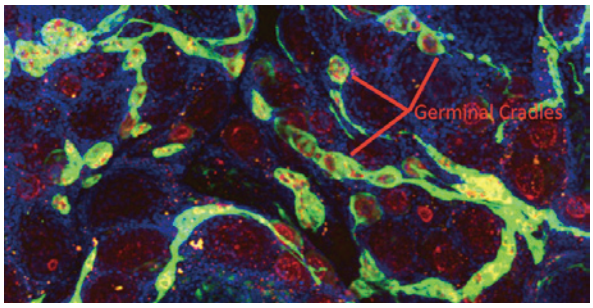

Figure 1. Red bars indicate location of ovarian niche (germinal cradles) expressing the *sox9b* gene. The germ cells are labeled with red.

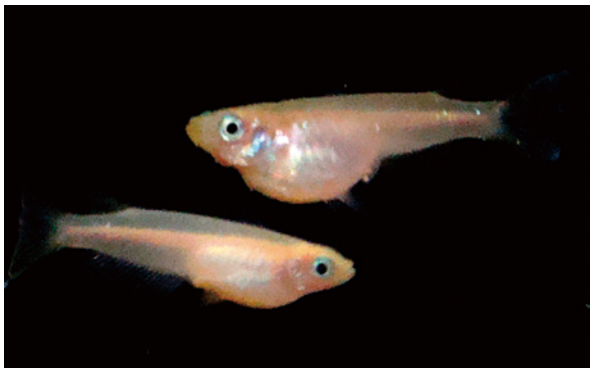

Figure 2. Hetrozygous *sox9b*-mutant medaka have a normal appearance but some exhibits female to male sex reversal.

II. *Sox9b* is essential for germ cell maintenance but not critically contribute to testis determination.

As mentioned above in mammals, *Sox9* is essential for initiation of testis formation and is under transcriptional regulation of mammalian testis determining gene, *Sry*, on the Y chromosome. Consistent with the function of mammalian *sox9* gene, there are several reports on the upregulation of *sox9* expression in testis of other vertebrates. These reports may collectively suggest the conserved role of *sox9* in testis determination across the vertebrate species. However, in medaka, *sox9* is expressed not only in Sertoli cells of the testis but also in germinal cradles with germline stem cells. This puts into doubt the conventional story of a conserved role of *Sox9*. Therefore we have isolated two *sox9b* mutant medaka with different alleles.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2011. The former title is indicated by an asterisk (*).

The phylogenetic and syntenic analyses show that *sox9b* is one of the co-orphologues of mammalian *Sox9* in medaka. We have found that only *sox9b* is expressed in the developing somatic (supporting) cells. This indicates that *sox9b* is the functional homologue of mammalian *Sox9* in medaka gonads.

The detailed examination of developing gonads shows that the number of germ cells are reduced in the *sox9b* mutant. This is consistent with a decreasing level of mitotic activity and an increasing level of apoptosis of germ cells in the mutant. The reduced levels parallel the mutant allele number, suggesting the dose-dependent effect of *sox9b* function in the phenotype. Very interestingly, even in the homozygous mutant, the gonad is formed, indicating that identity of the supporting cells to develop into the gonadal somatic cells is retained with the lack of *sox9b* function. In addition to the decreasing germ cell phenotype, the heterozygous mutant with XX chromosomes (genetically female) exhibits masculinization. This is a completely opposite phenotype of sex reversal (male to female) from that observed in mammals

We have found that the extracellular matrix (ECM) that separates interstitial regions from germ cells and supporting cells is largely disorganized in the mutant gonads. The germ cells often protrude blebs from the discontinuous ECM, suggesting that cellular association is impaired in the mutant. Chimeric analyses between the wild type and the mutant clearly demonstrates that mutant cells, which have the ability to develop into the gonads, are inclined to be expelled from the chimeric gonads (figure 3). The degree of contribution is dependent on the number of functional *sox9b* alleles. From these results, sox9b functions to maintain the germ cells through the regulation of ECM (Nakamura et al., 2012 PLoS ONE) .

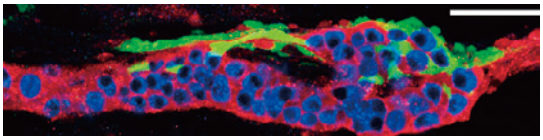

Figure 3. Chimeric gonad between wild type (red) and *sox9b*-mutant (green) cells. The mutant cells have the ability to develop into gonadal somatic cells but contribute less to the gonad than the wild type cells. Blue cells are germ cells.

Next we addressed the possible involvement of *sox9b* in the sex determination process. As mentioned above, the loss of sox9b function results in development into male gonads. The expression analysis demonstrates that the gonads in the XX mutant express male-specific genes, showing that in the XX mutants testicular development proceeds normally as in wild-type XY medaka. The sex reversal phenotype is rescued by one copy of a functional *sox9b* gene, Unlike in mammals, transgenic medaka with three copies of functional *sox9b* do not show any sign of masculinization. From these data, we have concluded that *sox9b* is not a critical contributor of male sex determination.

Then the next question is how the sex reversal to male occurs in the *sox9b* XX mutant. We have suspected that the loss of germ cells might cause the sex reversal. To confirm this hypothesis, we have tried to recover the number of germ cells in the *sox9b* mutant. Since the heterozygous *hotei* mutant possesses increasing mitotic activity, a compound mutant with *hotei* mutant allele and *sox9b* mutant allele were generated and analyzed. The heterozygous compound mutant had the normal number of germ cells and did not display sex reversal. This demonstrates that the sex reversal is primarily due to the loss of germ cells (Nakamura et al., 2012 PLoS ONE).

III. Functional divergence of *sox9* explains the different configuration of ovaries between medaka and mammals.

In medaka, *sox9b* contributes to the maintenance of germ cells including germline stem cells. Our results show that the testis-determining function of mammalian sox9 appends to the conserved role of sox9 in germ cell maintenance as neofunctionalization. With the acquisition of male determining function, sox9 expression was lost in the mammalian developing ovary. This is very well consistent with the loss of, or low number of, germline stem cells in the mammalian ovary. (figure 4) .

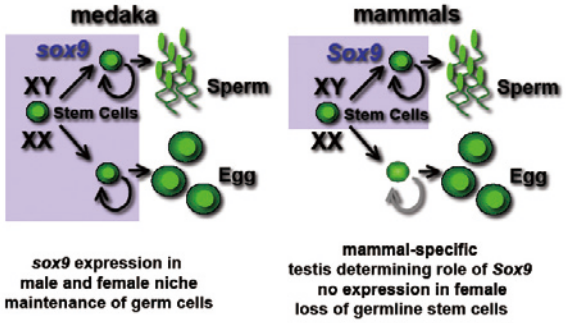

Figure 4. As sox9 acquired male determining functionality its expression was lost in the mammalian ovary.

Publication List

〔Original paper〕

- Hano, T., Oshima, Y., Kinoshita, M., Tanaka, M., Mishima, N., Wakamatsu, Y., Ozato, K., Shimasaki, Y., and Honjo, T. (2011). Evaluation of the effects of ethinylestradiol on sexual differentiation in the olvas-GFP/STII-YI medaka (transgenic *Oryzias latipes*) strain as estimated by proliferative activity of germ cells. Aquatic Toxicol. *104*, 177-184.

〔Review papers〕

- Nakamura, S., Kobayashi, K., Nishimura, T., and Tanaka, M. (2011). Ovarian germline stem cells in the teleost fish, medaka (*Oryzias latipes*). Int. J. Biol. Sci. *7*, 403-409.
- Naruse, K., Tanaka, M., and Takeda, H. (eds). (2011). "Medaka" a model for organogenesis, human disease, and evolution. total 387 pages. Springer-Verlag, Tokyo ISBN 978-4-431-92690-7.