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Fish exhibit a range of gonadal forms from gonochorism to several types of hermaphroditism, thus providing an excellent animal model to study the molecular mechanisms of sex determination, gonadal sex differentiation and gametogenesis in vertebrates. Our research focuses on (1) the identification of regulators involved in sex determination, gonadal sex differentiation, sexual plasticity, and gametogenesis (oocyte maturation and ovulation), and (2) the mechanisms of synthesis and action of these regulators.

I. Molecular mechanisms of sex determination, gonadal sex differentiation and sex change

We identified DMY (DM-domain gene on the Y chromosome) as the sex-determining gene of the medaka (*Oryzias latipes*), the first in non-mammalian vertebrates. Recently, we have developed a simple, cost effective and gene-specific transgenic RNAi technology for understanding the roles of the zygotic gene products in medaka. Knockdown of DMY in XY gonads induced down-regulation of the genes associated with testicular differentiation and up-regulation of the genes associated with ovarian differentiation, resulting in a complete male-to-female sex-reversal in adult XY medaka (Figure 1). Importantly, we were able to continue a trans-generational knockdown effect of DMY until at least the F3 generation. Since the RNAi effect is long lasting and inheritable, this will provide a powerful tool for the analysis of not only embryos, but also phenotypic consequences that develop over longer periods of time.

A search for the target genes of DMY led to the identification of gonadal soma derived factor (GSDF), a member of the transforming growth factor-beta superfamily. An XY-specific up-regulation was detected in the expression levels of GSDF in the whole embryos of medaka at 6 days post fertilization, coincident with the initiation of DMY expression in XY gonads. Conversely, the expression of GSDF was found to be very weak in XX gonads during embryogenesis. Importantly, GSDF and DMY were found to be co-localized in the same cell type in XY gonads. When the XY embryos were treated with estradiol-17 β (E2), in

order to reverse their phenotypic sex, a decline was observed in the expression of GSDF. These results suggest that GSDF plays an important role in testis differentiation in medaka, probably down stream of DMY.

The molecular control of ovarian development in medaka is less understood. Recently, we cloned three estrogen receptor (ER) subtypes (ER α , β 1 and β 2) from medaka and examined whether these ERs were involved in ovarian differentiation. The expression of ER β 2, but not ER α nor ER β 1, was up-regulated in XX embryos, but not XY embryos, collected during sex determination/differentiation. It is particularly important to note that the expression of ER β 2 was markedly increased at 6-8 dpf with a distinct peak at 7 dpf. This correlates well with the initiation of proliferative mitosis in female medaka. *In situ* hybridization revealed ER β 2 signals in XX gonads collected at 0 dph (8 dpf). This stage-specific expression in females is consistent with the notion that ER β 2 plays an important role in ovarian differentiation in medaka. We also examined the possible involvement of R-Spondin 1 (RSPO-1), a novel regulator of the Wnt/ β -catenin signaling pathway, in ovarian differentiation in medaka. RSPO-1 is expressed in XX gonads from as early as 0 day after hatching to the adult stage, while the expression was barely detected in XY gonads. When the XY embryos were treated with E2, a marked increase was observed in the expression of RSPO-1. Knockdown of RSPO-1 in XX gonads induced female-to-male sex-reversal, while overexpression of RSPO-1 in XY gonads induced male-to-female sex-reversal. These results indicate that RSPO-1 is critical to initiate the ovary pathway in medaka.

In the Nile tilapia (*Oreochromis niloticus*), we identified that GSDF/DMRT1 in XY gonads and *Cyp19a1/Foxl2* in XX gonads during early gonadal differentiation are critical for indifferent gonads to differentiate into either the testis or ovary. XX tilapia carrying extra copies of tilapia DMRT1 as a transgene induced various degrees of gonadal changes including complete sex change to testis, indicating that DMRT1 plays an important role in testicular differentiation. The critical role of Foxl2 in ovarian differentiation was confirmed by male sex reversal of XX transgenic tilapia carrying a dominant-negative mutant of *Foxl2*.

II. Sexual plasticity in the adult gonochoristic fish

We treated females of two species of adult gonochoristic fish, the Nile tilapia and medaka, with aromatase inhibitors (AI) for up to five months to block the conversion of androgens to estrogens. In both species, suppression of E2 production via AI treatment caused a rapid degeneration of ovarian tissues, leading to the differentiation and development of testicular tissues. Sex-changed fish show a typical male pattern of estrogen and androgen levels, secondary sex characteristics, producing fertile sperm in the newly formed testes. The control ovaries of adult tilapia and medaka contained some isolated cysts adjacent to the ovarian cavity. These cysts of medaka generally contained a single PGC-like *vasa*-positive germ cell which was surrounded by a few somatic cells. AI treatment induced proliferation of these germ cells in the exposed medaka ovaries, indicating that the

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PGC-like germ cells along the germinal epithelium underwent *de novo* differentiation in the absence of estrogen, giving rise to the testicular germ cells in the AI-treated gonads. These findings indicate that the cysts on the dorsal side of the adult ovaries are the origin of germ cells in the newly formed testicular tissue. Our results also indicate that gonochoristic fish maintain their sexual plasticity to adulthood and that estrogens play a critical role in maintaining the female phenotype.

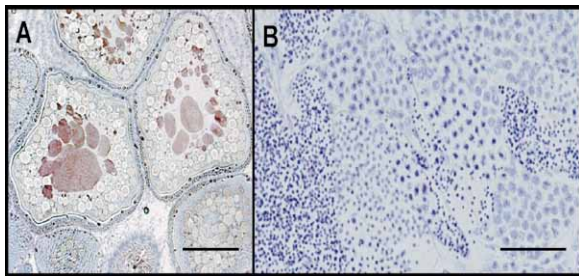


Figure 1. A, Ovary of control XX medaka. B, Gonad of AI-treated XX medaka having testicular tissue with various stages of spermatogenesis. Scale bars are 200 μ m (A) and 80 μ m (B).

III. Regulation of oocyte maturation and ovulation

Our studies using vertebrate (fish) and invertebrate (starfish) models have revealed that the basic mechanisms involved in oocyte maturation are the same in these two species despite the differing chemical nature of the hormonal agents involved. In both species, three major mediators have been shown to be involved (*Three step model*): a gonad-stimulating substance (GSS), 1-methyladenine (maturation-inducing hormone, MIH), and a maturation-promoting factor (MPF) in starfish, and gonadotropin (LH), 17α , 20β -dihydroxy-4-pregnen-3-one (DHP) (MIH), and MPF in fish. Importantly, these actions of MIHs have been shown to be mediated through the membrane receptors. More recently, DHP has also been shown to be involved in ovulation (follicle rupture). Interestingly, this action of DHP is mediated through nuclear progesterin receptors in the granulosa cells.

We recently purified GSS from the radial nerves of starfish (*Asterina pectinifera*) and the complete amino acid sequence was determined. Phylogenetic analyses revealed that starfish GSS was a relaxin-like peptide. Chemically synthesized GSS induced not only oocyte maturation and ovulation in isolated ovarian fragments, but also unique spawning behavior followed by the release of gametes shortly after injection. Thus, this study represents the first evidence of a relaxin system in invertebrates and points towards a novel reproductive role for this peptide in starfish. This work was done in collaboration with Drs. M. Mita and M. Yoshikuni.

Publication List

[Original papers]

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- Charkraborty, T., Shibata, Y., Zhou, L.Y., Katsu, Y., Iguchi, T., and Nagahama, Y. (2010). Differential expression of three estrogen receptor subtype mRNAs in gonads and liver from embryos to adults of the medaka, *Oryzias latipes*. *Mol. Cell. Endocrinol.* 2010 Dec 10.
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[Review Article]

- Paul-Prasanth, B., Nakamura, M., and Nagahama, Y. (2010). Sex determination in Fishes. In *Hormones and Reproduction of Vertebrates*, Vol. 1, D.O. Norris, and K.H. Lopez eds. (Academic Press), pp. 1-14.