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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/differentiation and gametogenesis in vertebrates. Our research using several teleost fishes focuses on (1) the identification of regulators and steroidal mediators involved in sex determination, gonadal sex differentiation and gametogenesis, and (2) the mechanisms of synthesis and action of these mediators.

Ⅰ. Molecular mechanisms of sex determination and gonadal sex differentiation

We have been using medaka (*Oryzias latipes*) to investigate the molecular mechanisms of sex determination and Nile tilapia (*Oreochromis niloticus*) to investigate gonadal sex differentiation. Medaka possess a stable genetic XX/XY sex determining system. We identified *DMY* as the sex-determining gene of medaka. *DMY* encodes a protein of 267 amino acids including a DNA-binding motif, the DM domain, found in other genes involved in sexual development. A genomic DNA fragment carrying *DMY* was sufficient to induce testis differentiation and subsequent male development, producing fertile sperm (Figure 1). It is important to note that *DMY* transgenic XX medaka are fully functional and fertile males, whereas *Sry* transgenic mice are sterile (Koopman *et al*., 1991). Thus, medaka is the first

Figure 1. *DMY* transgenic adult medaka (XX) with white body color (A) having testis (B)

transgenic vertebrate shown to undergo complete sex reversal. Interestingly, *DMY* is a homolog of *DMRT1*, another DM domain gene that is involved in male development, and appears to be derived from a duplicated copy of autosomal *DMRT1*. *DMY* is also found in *O. curvinotus*, which is most closely related to medaka, but is not found in other *Oryzias* species or other fishes. These findings clearly illustrate the vast diversity of sex-determining genes in fish.

In tilapia, all genetic female (XX) or male (XY) broods are available. Through cDNA subtraction between XX and XY gonads during sex differentiation and microarray hybridization followed by gene expression analyses by RT-PCR and *in situ* hybridization, we have concluded that in tilapia, *Foxl2/Cyp19a1* plays a crucial role in ovarian differentiation and *DMRT1* in testicular differentiation. The transcripts of Foxl2 and aromatase (there are two forms of aromatase in teleost fishes: the ovarian form (Cyp19a1) and brain form (Cyp19a2)) were expressed only in XX gonads at 5 days after hatching (dah), with a marked elevation in expression during the next two days. 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*. In XY tilapia fry, *DMRT1* gene is expressed male-specifically in testicular Sertoli cells prior to and during sex differentiation. XX tilapia carrying extra copies of tilapia *DMRT1* as a transgene induced various degrees of gonadal changes including complete sex change to testis. It is of great interest to note that some of the sex reversed XX tilapia produced sperm with extremely high motility (Wang *et al*., unpublished).

We also investigated the expression pattern of a novel type of P450c17 (P450c17-Ⅱ) lacking the lyase activity (see below) in gonads during sex differentiation. The results on tilapia and medaka (for example, its first appearance at 10-20 dah in XX tilapia and 70 dah in XY tilapia) suggest that P450c17-Ⅱ might be involved in the initiation of meiosis during early sex differentiation in these fishes. Further studies on these lines are expected to reveal whether 17α,20 β-DP or another C-21 steroid is involved in the initiation of meiosis and, consequently, sex differentiation.

Ⅱ. Molecular mechanisms of sex change

The gobiid fish, *Trimma okinawae*, possesses ovarian and testicular tissues simultaneously in its gonad and is able to change sex repeatedly in both directions depending on its social surroundings. We examined the involvement of gonadotropins in sex change by determining the changes in gonadotropin (FSH and LH) receptor gene expression in gonads during the onset of sex changes from female to male and male to female. The expression of the *GtHRs* was found

to be confined to the active gonad of the corresponding sexual phase. When the sex change was occurring from female to male, the ovary initially had high levels of *FSHR* and *LHR*, which eventually went up in the testicular tissue once the fish had realized the fact that it was bigger than the other fish in the aquarium. The opposite of this scenario was observed if another fish bigger than the newly sex-changed male was introduced into the aquarium. Swapping of the gonads started with switching of the *GtHR* expression that was discernible within 8-24 hrs of the visual cue. Further *in vitro* culture of the transitional gonads with a supply of exogenous gonadotropin (hCG) revealed that the to-be-active gonad acquired the ability to produce the corresponding sex hormone within one day of the activation of GtHR. Conversely, the to-be-regressed gonad did not respond to the exogenous gonadotropin, demonstrating the absence of *GtHR* expression. A successive sex changing fish like *T. okinawae* is an excellent animal model to elucidate the mysterious role of the brain in bringing out the sexuality of an individual and also for the depiction of sexual plasticity at the organismal level (Figure 2).

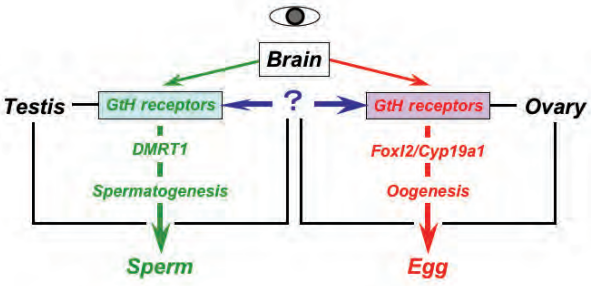

Figure 2. Gonadal sex change in *T. okinawae* may be triggered by visual/brain-stimulated switching of gonadotropin (GtH) receptor gene expression in gonads.

Ⅲ. Embryonic development of gonadotropin-releasing hormone (GnRH) neurons

Appropriate development of GnRH neurons is essential for reproductive competence. The mechanisms underlying this process are, however, poorly defined because of the unavailability of an *in vivo* animal model. We have generated transgenic medaka that express GFP under the control of the GnRH gene promoters and shown that they could provide a useful model for the study of the GnRH neuronal development, including human disorders of GnRH deficiency. It has recently been shown that mice deficient for p73, a newly described homolog of the tumor suppressor gene p53, are not interested in mating, although its mechanism is unknown. Using the transgenic medaka, we here examined a possible role for p73 in the development of GnRH neurons. Antisense-mediated ablation of p73 led to the fusion of the bilateral GnRH neuronal clusters in the terminal nerve ganglion and an inappropriate increase in the number of these neurons. This result identified p73 as a novel factor involving the development of GnRH neurons and would account for the reproductive and behavioral defects in p73-deficient mice.

Ⅳ. Endocrine regulation of oocyte maturation and ovulation

A period of oocyte growth is followed by a process called oocyte maturation (the resumption of meiosis) which occurs prior to ovulation and is a prerequisite for successful fertilization. Oocyte maturation has been studied in a variety of vertebrates and invertebrates including mammals, amphibians, fishes, and starfishes, but the endocrine regulation of oocyte maturation has been investigated most extensively in fishes. 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 (Figure 3). 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 (17α,20β-DP) (MIH), and MPF in fish.

We recently purified GSS from the radial nerves of starfish (*Asterina pectinifera*), and the complete amino acid sequence was determined. GSS is a heterodimeric peptide with a molecular weight of 4737, consisting of A and B chains; the A chain contains 24 residues and the B chain 19 residues. Chemically synthesized GSS is as active as native GSS in the homologous *in vitro* GVBD assay (M. Mita, M. Yoshikuni *et al*., unpublished).

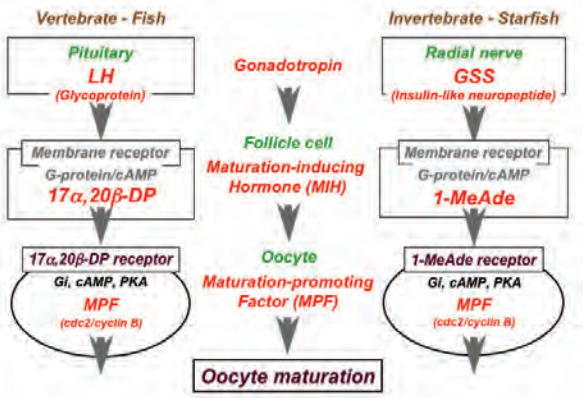

Figure 3. Hormonal control of oocyte maturation in fish and starfish – *Three step model*

In fish, LH acts on ovarian follicle cells to produce fish MIH (17α,20β-DP) immediately prior to oocyte maturation. There is a distinct shift in follicular steroidogenesis from estradiol-17β (E2) during oocyte growth (vitellogenesis) to 17α,20β-DP during oocyte maturation. This occurs in two stages, the first being the shift in the synthesis of precursor steroids in thecal cells, while the other is the shift in the final steroidal mediator production in granulosa cells. We have already shown that there is a distinct shift in the steroidogenic enzyme genes from ovarian aromatase (*Cyp19a1*) to 20β-hydroxysteroid dehydrogenase (*20β-HSD*), occurring in the granulosa cells of ovarian follicles prior to oocyte maturation. The triggering of the steroidogenic shift by GtHs in granulosa cells can be achieved by the differential actions of two transcription

factors, Ad4BP/SF-1 (aromatase) and CREB (20β-HSD).

The major remaining question was the differential availability of precursor steroid, 17α-hydroxyprogesterone. This was essentially because, until now, a single enzyme P450c17, possessing 17α-hydroxylase and 17, 20 lyase activities to mediate the production of estrogen and 17α,20β-DP, has been described among the vertebrates in general. Recently, we discovered a novel type of P450c17 (P450c17-Ⅱ) lacking the lyase activity in several teleost species, and showed that P450c17-II, but not P450c17-I, is responsible for the shift in precursor steroid from testosterone to 17α-hydroxyprogtesterone in both medaka and tilapia. Thus, our studies have resolved a long-standing question in the field of steroidogenesis with respect to oocyte maturation. As the novel type of P450c17 is found to have only the hydroxylase activity, we investigated whether this is the gene which is responsible for the cortisol production by analyzing its expression pattern in the head kidney of tilapia during different developmental stages. Interestingly, only P450c17-Ⅱ is found to be expressed in the interregnal cells of the head kidney from very early stages (5 dah) to adulthood (8 month old) (Figure 4). Since one of the most important physiological functions of the interrenal cell is to produce cortisol, our data suggests that only P450c17-Ⅱ is responsible for the cortisol production in the interrenal cells

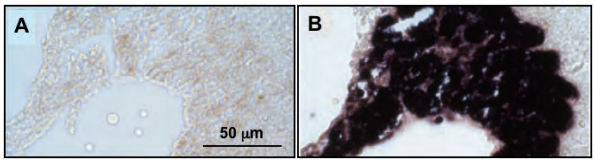

Figure 4. Expression of tilapia *P450c17-I* (A) and –Ⅱ (B) in the interrenal cells of the head kidney

Unlike other steroid hormones, 17α,20β-DP binds to a novel, G-protein-coupled membrane progestin receptor (non-genomic action), leading to the *de novo* synthesis of cyclin B, the regulatory component of MPF, which activates a preexisting 35-kDa cdc2 kinase via phosphorylation of its threonine 161 by a threonine kinase, thus producing the 34 kDa active cdc2.

Ovulation is a precisely timed process by which a mature oocyte is released from an ovarian follicle. This process is initiated by the pituitary surge of LH and is temporally associated with the transcriptional regulation of several genes. The molecular mechanisms that control the complex process of ovulation are not well understood in vertebrates. Our recent studies (Shibata *et al*., unpublished) on medaka have demonstrated that 17α,20β-DP can induce ovulation (follicle rupture) in the mature follicles *in vitro*. It is particularly important to note that this action of 17α,20β-DP is mediated through its nuclear progestin receptors (nPRs) expressed in the granulosa cells. We were able to find that nPR mRNA expression is induced by gonadotropin prior to ovulation. Thus, 17α,20β-DP is the key hormone for the induction of not only maturation (through its membrane receptors), but also ovulation (through its nuclear receptors).

Publication List

〔Original papers〕

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- Zhou, L.Y., Wang, D.S., Kobayashi, T., Yano, A., Paul-Prasanth, B., Suzuki, A., Sakai, F., and Nagahama, Y. (2007). A novel type of P450c17 lacking the lyase activity is responsible for C21-steroid biosynthesis in the fish ovary and head kidney. Endocrinology *148*, 4288-4291.
- Zhou, L.Y., Wang, D.S., Shibata, Y., Paul-Prasanth, B., Suzuki, A., and Nagahama, Y. (2007). Characterization, expression and transcriptional regulation of *P450c17-I* and -Ⅱ in the medaka, *Oryzias latipes*. Biochem. Biophys. Res. Comm. *362*, 619-624.