DIVISION OF REPRODUCTIVE BIOLOGY

Professor:	*NAGAHAMA, Yoshitaka*
Associate Professor:	*YOSHIKUNI, Michiyasu*
Research Associates:	*OKUBO, Kataaki*
	OHNO, Kaoru
NIBB Research Fellow:	*SUZUKI, Aya*
Postdoctoral Fellows:	*CUI, Jianzhou*
	GUAN, Guijun
	KANEKO, Hiroyo
	LAU, En-Lieng
	LI, Jianzhong
	MATSUDA, Masaru
	OHTA, Kohei
	PAUL, Bindhu
	SAKAI, Fumie
	SHIBATA, Yasushi
	USAMI, Takeshi
	WANG, De-shou
	ZHAO, Haobin
Graduate Students:	*KURITA, Kayoko*
	ZHOU, Linyan
Technical Assistants:	*HARA, Ikuyo*
	HAYAKAWA, Rie
	HIRAKAWA, Eri
	SHIBATA, Emiko
	TAKAKI, Chikako
Secretary:	*SHIMADA, Yu*

Our research 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

Fish have a range of gonadal differentiation types including gonochoristic species as well as hermaphroditic species. 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. Using positional cloning and detailed sequence analysis of BAC clones by shotgun sequencing, we identified *DMY* (DM domain gene on the Y chromosome) as a strong candidate for the sex-determining gene of medaka. *DMY* encodes a protein of 267 amino acids including the highly conserved DM domain. The involvement of *DMY* in the process of sex-determination was first confirmed by the advent of two naturally occurring sex-reversed mutants, in which *DMY* was either truncated or expressed at reduced levels. More recently, we performed over-expression experiments using the *DMY* genomic region or *DMY* cDNA, which can induce testis development in genetic females (XX) (Figure 1). A 117-kb genomic DNA fragment carrying *DMY* was able to induce testis differentiation and subsequent male development in XX medaka. In addition, over-expression of *DMY* cDNA under the control of the CMV promoter also caused XX sex reversal. These results demonstrate that *DMY* is sufficient for male development in medaka, and suggest that the functional difference between the X and Y chromosomes in medaka is a single gene. These data indicate that *DMY* is an additional sex-determining gene in vertebrates.

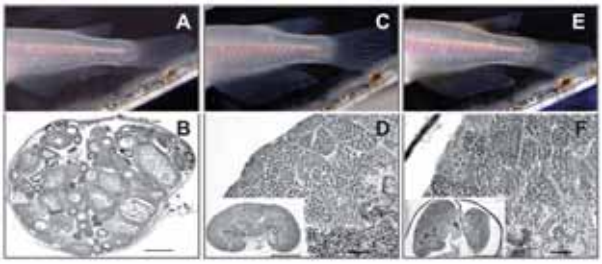

Figure 1. Phenotypic analyses of *DMY* transgenic adult medaka. Transgenic medaka include: a white (XX) female (A) with an ovary (B), a white (XX) male (C) with testes (D), and an orange-red (XY) male (E) with testes (F).

We also used two fundamentally different ways of sex reversal, *DMY* knock down and estradiol-17β (E2) treatment, to determine the possible function of DMY during early gonadal sex differentiation in XY medaka. Our findings revealed that the mitotic and meiotic activities of the germ cells in *DMY* knock-down XY larvae (the day of hatching) were identical to that of the normal XX larvae, suggesting the microenvironment of these XY gonads to be similar to that of the normal XX gonad, where *DMY* is naturally absent. Conversely, E2 treatment failed to initiate mitosis in the XY gonad, possibly due to an active *DMY*, even though it could initiate meiosis. The present study is the first to prove that the germ cells in the XY gonad can resume the mitotic activity, if *DMY* was knocked-down.

In tilapia, all genetic female (XX) or male (XY) broods can be obtained by artificial fertilization of normal eggs (XX) and sex-reversed, pseudo male sperm (XX), or normal eggs (XX) and super male sperm (YY), respectively. Steroidal enzymes P450scc, 3β-HSD, and P450c17 are found at high levels in female gonads of tilapia at 7-10 days posthatching, but are only seen weakly in males and not until 30 days posthatching. Further, the enzyme aromatase is only detected in ovaries. Treatment of XX fry with fadrozole (aromatase inhibitor) or tamoxifen (estrogen receptor antagonist) caused complete sex reversal to functional males. These results suggest that endogenous estrogens are critical for directing initial ovarian differentiation in tilapia.

We then investigated the plausible role of Foxl2 in ovarian differentiation through transcriptional regulation of aromatase gene (*Cyp19a1*), using mono-sex tilapia fry. *Foxl2* expression, like that of *Cyp19a1*, is sexually dimorphic in gonads prior to the occurrence of morphological sex differentiation, co-localizing with *Cyp19a1* and *Ad4BP/SF-1* in the stromal cells and interstitial cells in gonads of normal XX and sex-reversed XY fish. Under *in vitro* conditions, Foxl2 binds to the sequence, ACAAATA in the promoter region of the *Cyp19a1* gene directly through its forkhead domain (FH),

and activates the transcription of *Cyp19a1* with its C-terminus. Foxl2 can also interact through the FH with the ligand binding domain of Ad4BP/SF-1 to form a heterodimer and enhance the Ad4BP/SF-1 mediated *Cyp19a1* transcription. Disruption of endogenous Foxl2 in XX tilapia by over-expression of its dominant negative mutant induces varying degrees of testicular development with occasional sex reversal from ovary to testis. Such fish display reduced expression of *Cyp19a1* as well as a drop in the serum levels of E2 and 11-ketotestosterone (11-KT) (Figure 2). Although the XY fish with wild type tilapia *Foxl2* over-expression never exhibited a complete sex reversal, there were significant structural changes, such as tissue degeneration, somatic cell proliferation and induction of aromatase, with increased serum levels of E2 and 11-KT. These results suggest that Foxl2 plays a decisive role in the ovarian differentiation of tilapia by regulating aromatase expression.

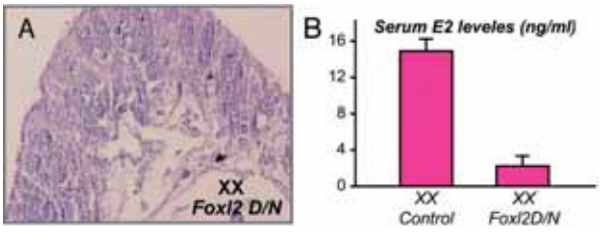

Figure 2. Transgenic XX tilapia with over-expression of Foxl2 dominant negative mutant (D/N) showing a complete sex change from ovary to testis (A). Serum E2 levels in XX tilapia with over-expression of Foxl2 D/N were 7 times lower than that of the XX control fish (B).

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. These results suggest an important role for DMRT1 in testicular differentiation in tilapia.

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. As sex change in both directions can be socially manipulated, *T. okinawae* provides an excellent animal model to investigate the molecular mechanisms of sex change.

The involvement of gonadotropins in sex change was examined 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. Expression appears to be related to sexual phase with quick location switching of the two genes after social manipulation to stimulate sex change. This differential expression of the two gonadotropin receptor genes is an earlier event occurring in gonads after pairing and plays a critical role in the sex change.

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

Neurons that synthesize and release GnRH are essential for the central regulation of reproduction. X-linked Kallmann syndrome (X-KS), characterized by failed gonadal function, is caused by a mutation in *KAL1*, which is suggested to regulate the development of GnRH neurons. Since rodents lack *Kal1* in their genome, the pathogenesis of X-KS has been difficult to study. We identified a *KAL1* ortholog in medaka. Antisense knockdown of the *KAL1* ortholog in the transgenic medaka in which GnRH neurons were visualized with GFP led us to observe the inappropriate accumulation of GnRH neurons in the olfactory compartment and loss of their ability to migrate into the forebrain (Figure 3). This result was consistent with that reported in a fetus with X-KS. Thus, our data demonstrate that X-KS can be phenocopied by antisense knockdown of *kal1* and can be directly monitored in the transgenic medaka. Taken together, the medaka system provides a useful *in vivo* model for studying disorders of GnRH deficiency including X-KS.

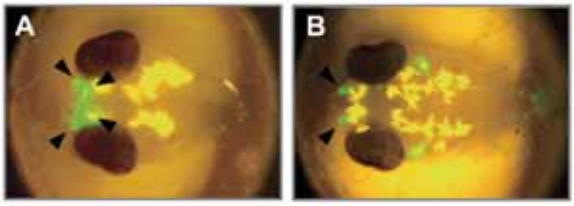

Figure 3. Knockdown of *kal1* in the transgenic medaka that express GFP in gnrh1 (A) and gnrh3 (B) neurons resulted in the deficient migration of GnRH neurons (arrowheads).

IV. Endocrine regulation of oocyte maturation

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. It has been well established that hormones play an important role in inducing oocyte maturation in invertebrates as well as in vertebrates. 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: gonad-stimulating substance (GSS), 1-methyladenine (maturation-inducing hormone, MIH), and 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 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).

In fish, LH acts on ovarian follicle cells to produce fish MIH (17α, 20β-DP). 17α, 20β-DP is synthesized by a two-step process involving two ovarian cell layers, the thecal and granulosa cells. 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. Upon egg activation, MPF is inactivated by degradation of cyclin B. We demonstrated that the 26S proteasome initiates cyclin B degradation through the first cut of its NH_2 terminus at lysine 57.

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Review articles

Nagahama, Y. (2005*). Molecular mechanisms of sex determination and gonadal sex differentiation in fish. Fish Physiol. Biochem. *31*, 105-109.

* papers published after the publication of ì2005 Annual Reportî