DIVISION OF REPRODUCTIVE BIOLOGY

Professor:	*NAGAHAMA, Yoshitaka*
Associate Professor:	*YOSHIKUNI, Michiyasu*
Research Associates:	*OKUBO, Kataaki*
	OHNO, Kaoru
Technical Staff:	*KOBAYASHI, Hiroko*
NIBB Research Fellow:	*BASU, Dipanjan*
PRESTO Researcher:	*MATSUDA, Masaru*
Postdoctoral Fellows:	*BASU, Shakuntala*
	BHANDARI, Ramji Kumar
	CHAUBE, Radha
	GUAN, Guijun
	IJIRI, Shigeho
	KANEKO, Hiroyo
	LAU, En-Lieng
	OHTA, Kohei
	PAUL, Bindhu
	SAKAI, Fumie
	SHIBATA, Yasushi
	SUZUKI, Aya
	USAMI, Takeshi
	WANG, De-shou
	YI, Mei-Sheng
	ZAHO, Haobin
Graduate Students:	*OHMURO-MATSUYAMA, Yuki*
	KURITA, Kayoko
	ZHOU, Linyan
Visiting Scientist:	*TANAKA, Masaki*
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 two fish species to investigate the molecular mechanisms of sex determination (medaka, *Oryzias latipes*) and gonadal sex differentiation (Nile tilapia, *Oreochromis niloticus*).

Medaka possesses 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. Our loss- (natural mutants analysis and knock-down) and gain- (over-expression using transgenic techniques) of-function studies indicate that *DMY* is the sex-determining gene of medaka. *DMY* provides the first example of a sex-determining gene in non-mammalian vertebrates.

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 gonadal anlargen 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 recently showed that Foxl2 and Ad4BP/SF-1 play important roles in the differential expression of aromatase in XX tilapia gonads during sex 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*. In XY 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 transformation 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, thus providing an excellent animal model to investigate molecular mechanisms of sex change. Aquarium experiments were carried out in the laboratory. Two males (M/M) or two females (F/F) were kept in separate tanks. In the M/M aquarium, the smaller male changes its sex to female. On the contrary, the larger female changes its sex to male in the F/F aquarium. Behavioral changes occurred within 30 minutes of social manipulation (Figure 1). After pairing, the larger male or female attacked the smaller fish, which fled and often hid in a nest. After 30 min., however, the larger fish began to court the smaller fish. These results suggest that the brain sex of a sequential hermaphrodite is determined independently of gonadal effect and is induced only by social cues.

Figure 1. Sexual behavior of *Trimma okinawae* immediately after pairing

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 the 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 2). 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.

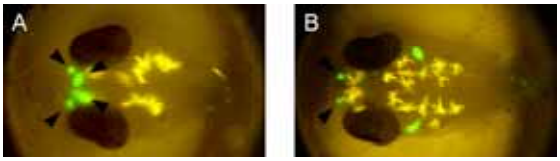

Figure 2. Knockdown of *kal1* in the transgenic medaka that express GFP in gnrh1 (panel A) and gnrh3 (panel B) neurons resulted in the deficient migration of GnRH neurons (arrowheads).

IV. Endocrine regulation of oocyte maturation

The process of oocyte maturation is the time period between the resumption of meiosis and the second meiotic metaphase, and thus, 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 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: GSS, 1-methyladenine (maturation-inducing hormone, MIH), and maturation-promoting factor (MPF) in starfish, and LH, 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) (MIH), and MPF in fish (Figure 3).

The primary hormone involved in starfish reproduction has been called the gonad-stimulating hormone (GSS), which is a peptide hormone produced in the radial nerves. Although the hormonal activity of GSS has been known for a long time, only recently has it been possible to determine its primary structure. GSS has been purified 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.

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 showed that the 26S proteasome initiates cyclin B degradation through the first cut of its NH_2 terminus at lysine 57.

An endocrine-disrupting chemical, diethylstilbestrol (DES), a nonsteroidal estrogen, triggers oocyte maturation in goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*). The morphology (the time course of the change in germinal vesicle breakdown) and an intracellular molecular event (the *de novo* synthesis of cyclin B) induced by DES are indistinguishable from those induced by 17α, 20β-DP. Both 17α, 20β-DP- and DES-induced oocyte maturation is inhibited by an antibody against the membrane progestin receptor. These results suggest that DES may act on the membrane progestin receptor as an agonist of 17α, 20β-DP.

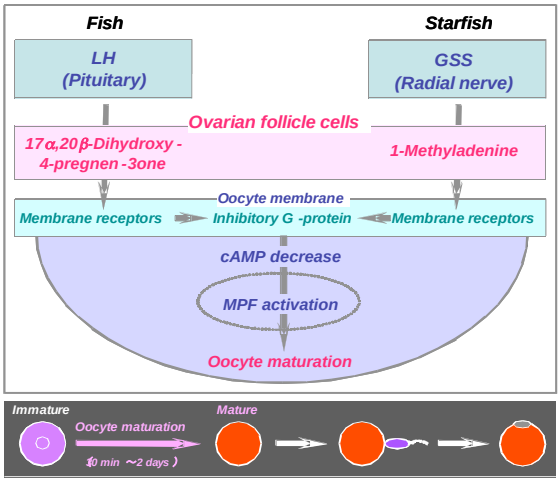

Figure 3. Regulation of oocyte maturation in fish and starfish

Publication List:

Original papers

Bhandari, R.K., Nakamura, M., Kobayashi, T., and Nagahama, Y. (2005). Suppression of steroidogenic enzyme expression during androgen-induced sex reversal in Nile tilapia (*Oreochromis niloticus*). Gen. Comp. Endocrinol., in press.

Bhandari, R.K., Ashraful, M.A., Higa, M., Soyano, K., and Nakamura, M. (2005). Evidence that estrogen regulates the sex change of honeycomb grouper (*Epinephelus merra*), a protogynous hermaphrodite fish. J. Exp. Zool. *303A*, 497-503.

Chang, X.T., Kobayashi, T., Senthilkumaran, B., Kobayashi-Kajiura, H., Sudhakumari, C.C., and Nagahama, Y. (2005). Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia. Gen. Comp. Endocrinol. *141*, 101-115.

Horiguchi, R., Tokumoto, M., Nagahama, Y., and Tokumoto, T. (2005). Molecular cloning and expression of cDNA coding four spliced isoforms of casein kinase Iα in goldfish oocytes. Biochim. Biophys. Acta *1727*, 75-80.

Horiguchi, R., Yoshikuni, M., Tokumoto, M., Nagahama, Y., and Tokumoto, T. (2005). Identification of a protein kinase which phosphorylates α subunit of the 26S proteasome and changes in its activity during meiotic cell cycle in goldfish oocytes. Cell. Signal. *17*, 205-215.

Kobayashi-Kajiura, H., Kobayashi, T., and Nagahama, Y. (2005). Cloning of cDNAs and the differential expression of A-type cyclins and Dmc1 during spermatogenesis in the Japanese eel, a teleost fish. Dev. Dyn. *232*, 1115-1123.

Kobayashi, Y., Sunobe, T., Kobayashi, T., Nagahama, Y., and Nakamura, M. (2005). Gonadal structure of the serial-sex changing fish. Dev. Growth Differ. *47*, 7-13.

Kobayashi, Y., Sunobe, T., Kobayashi, T., Nakamura, M., Suzuki, N., and Nagahama, Y. (2005). Molecular cloning and expression of *Ad4BP/SF*-1 in the serial sex changing gobiid fish *Trimma okinawae*. Biochem. Biophys. Res. Comm. *332*, 1073-1080.

Nakamoto, M., Suzuki, A., Matsuda, M., Nagahama, Y., and Shibata, N. (2005). Testicular type *Sox9* is not involved in sex determination, but might be in the development of testicular structures in the medaka, *Oryzias latipes*. Biochem. Biophys. Res. Comm. *333*, 729-736.

Nakamura, I., Evans, J.C., Kusakabe, M., Nagahama, Y., and Young, G. (2005). Changes in steroidogenic enzyme and steroidogenic acute regulatory protein messenger RNAs in ovarian follicles during ovarian development of rainbow trout. Gen. Comp. Endocrinol. *144*, 224-231.

Ohmuro-Matsuyama, Y., Okubo, K., Matsuda, M., Ijiri, S., Wang, D.S., Guan, G.J., Suzuki, T., Matsuyama, M., Morohashi, K., and Nagahama, Y. (2005). Liver receptor homologue-1 activates brain aromatase promoter of medaka, *Oryzias latipes*. Mol. Reprod. Develop., in press.

Okubo, K., Sakai, F., Lau, E.L. Yoshizaki, G., Takeuchi, Y. Naruse, K., Aida, K., and Nagahama, Y. (2005). Forebrain gonadotropin-releasing hormone neuronal development: Insights from transgenic medaka and the relevance to X-linked Kallmann syndrome. Endocrinology, in press.

Oshima, Y., Kato, T., Wang, D.S., Murakami, T., Matsuda, Y., Nagahama, Y., and Nakamura, M. (2005). Promoter activity and chromosomal location of the *Rana rugosa* P450 aromatase (CYP19) gene. Zool. Sci., in press.

Sunobe, T., Nakamura, M., Kobayashi, Y., Kobayashi, T., and Nagahama, Y. (2005). Aromatase immunoreactivity and the role of enzymes in steroid pathways for inducing sex change in the hermaphrodite gobiid fish *Trimma Okinawae*. Comp. Biochem. Physiol. Part A *141*, 54-59.

Sunobe, T., Nakamura, M., Kobayashi, Y., Kobayashi, T., and Nagahama, Y. (2005). Gonadal structure and P450scc and 3β-HSD-like immunoreactivity in the gobiid fish *Trimma okinawae* during bidirectional sex change. Ichyiol. Res. *52*, 27-32.

Tokumoto, M., Nagahama, Y., Thomas, P., and Tokumoto, T. (2005). Cloning and identification of a membrane progestin receptor in goldfish ovaries and evidence it is an intermediary in oocyte meiotic maturation. Gen. Comp. Endocrinol., in press.

Wayne, N.L., Kuwahara, K., Aida, K., Nagahama, Y., and Okubo, K. (2005). Whole-cell electrophysiology of gonadotropin releasing hormone (GnRH) neurons that express green fluorescent protein (GFP) in the terminal nerve of transgenic medaka (*Oryzias latipes*). Biol. Reprod., in press.

Zhou, L.Y., Wang, D.S., Senthilkumaran, S., Yoshikuni, M., Shibata, Y., Kobayashi, T., Sudhakumari, C.C., and Nagahama, Y. (2005). Cloning, expression and characterization of three types of 17β-hydroxysteroid dehydrogenases from the Nile tilapia, *Oreochromis niloticus*. J. Mol. Endocrinol. *35*, 103-116.

Review articles

Matsuda, M. (2005). Sex determination in the teleost medaka, *Oryzias latipes*. Ann. Rev. Genet. *39*, 293-307.

Shibata, Y. (2005). Modification of egg envelope proteins during fertilization in teleost fish. In New Impact on Protein Modification in the Regulation of Reproductive System, T. Tokumoto, ed. (Kerala, Research Signpost), pp.115-134.