DEPARTMENT OF DEVELOPMENTAL BIOLOGY

Chairman: Yoshitaka Nagahama

The Department is composed of three divisions and one adjunct division. Department members conduct molecular analysis on various aspects of developmental phenomena including: (1) differentiation and maturation of germ cells, (2) gene regulation in cell differentiation and growth, and (3) molecular basis of body plans.

DIVISION OF REPRODUCTIVE BIOLOGY

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The pituitary-gonadal axis plays an important role in regulating gametogenesis in vertebrates. Gonadotropins typically act through the biosynthesis of gonadal steroid hormones which in turn mediate various stages of gametogenesis. Their effects are particularly profound in teleost fishes which provide several excellent models for investigating the basic hormonal mechanisms regulating gonadal sex differentiation and gametogenesis (spermatogenesis, sperm maturation, oocyte growth and oocyte maturation). Our research focuses on (1) the identification of steroidal mediators involved in gonadal sex differentiation and gametogenesis, and (2) the mechanisms of synthesis and action of these mediators.

I. Endocrine regulation of gonadal sex differentiation

Sex determination and gonadal development vary considerably in fish. In addition to gonochorism, several types of hermaphroditism (protandry, protogyny and synchronous hermaphroditism) are found in fish. Nile tilapia, *Oreochromis niloticus*, is an excellent example of the precise nature of steroidogenic actions during gonadal sex differentiation. In this fish, 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. Fertilized eggs hatch after 4 days at 26°C. On the day of hatching, primordial germ cells (PGCs), which are morphologically distinguishable from somatic cells, are located in the outer layer of the lateral plate mesoderm around the hind gut. At 3 days post-hatching, PGCs are located in the gonadal anlagen after the formation of the coelomic cavity in the lateral plate mesoderm rather than through active migration.

The gene *vasa* encodes a DEAD (Asp-Glu-Ala-Asp) family of putative RNA helicase and is present in the germ line of several animal species. *Vas* (a *Drosophila vasa* homologue) gene expression pattern in germ cells during oogenesis and spermatogenesis has bee examined using all genetic females and males of tilapia. In the ovary, *vas* is expressed strongly in oogonia to diplotene oocytes and becomes localized as patches in auxocytes and then strong signals are uniformly distributed in the cytoplasm of previtellogenic oocytes, follwed by a decrease from vitellogenic to postvitellogenic oocytes. In the testis, *vas* signals are strong in spermatogonia and decrease in early primary spermatocytes. No *vas* RNA expression is evident in either diplotene primary spermatocytes, secondary spermatocytes, spermatids or spermatozoa. The observed differences in *vas* RNA expression suggest a differential function of *vas* in the regulation of meiotic progression of female and male germ cells. We have also generated medaka (*Oryzias latipes*) transgenic lines with green fluorescent protein (GFP) fluorescence controlled by the regulatory regions of the *olvas* gene in the germ cells (Fig. 1). The intensity of GFP fluorescence increases dramatically in PGCs located in the ventrolateral region of the posterior intestine around stage 25 (the onset of brood circulation). Whole-mount *in situ* hybridization and monitoring of ectopically located cells by GFP fluorescence suggest that 1) the increase in zygotic *olvas* expression occurs after PGC specification and 2) PGCs can maintain their cell characteristics ectopically after stages 20-25. The GFP expression persists throughout the later stages in the mature ovary and testis.

In tilapia, mitosis of germ cells begins around 10 days post-hatching in genetic females, but can not be

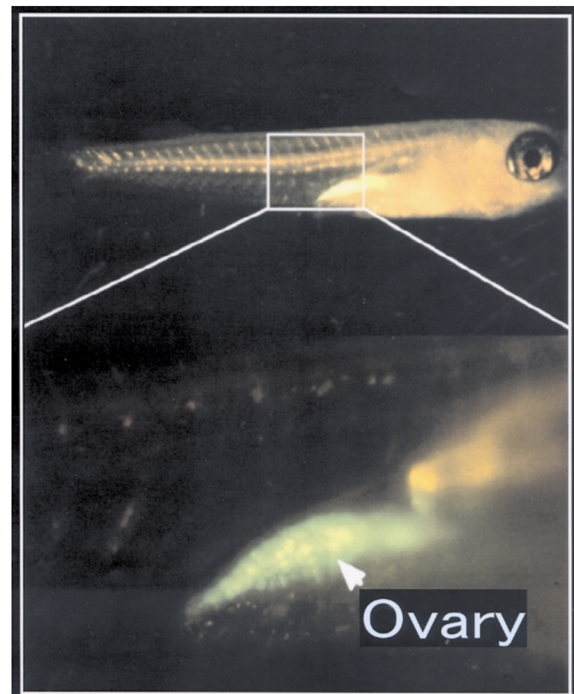

Fig. 1 GFP fluorescent germ cells in the ovary of female medaka, *Oryzias latipes*. The ovary contains numerous oocytes which can be seen as a green fluorescence.

confirmed until after sex differentiation in testes of genetic males. During the course of morphological sex differentiation, the behavior of somatic cells in the gonad is often sex-specific. In these cases, the sex of the gonad is easily distinguishable. In tilapia, gonadal sex is morphologically distinct at 20-25 days post-hatching. Ovarian differentiation is initially marked by storomal elongation of the gonad for the formation of the ovarian cavity. Testicular differentiation is characterized by the appearance of a narrow space in the storomal tissue representing the formation of the efferent duct. Steroid-producing cells in ovaries, but not testes, at the undifferentiated and differentiating stages express all of the steroidogenic enzymes required for estradiol-17β biosynthesis from cholesterol. These results strongly suggest that endogenous estrogens act as the natural inducers of ovarian differentiation in tilapia. This hypothesis is further supported by evidence of masculinization of genetic female tilapia by inhibition of estrogen synthesis using an inhibitor of cytochrome P450 aromatase. In contrast, the ability of steroid-producing cells to synthesize steroid hormones in the testes only appears at the time of testicular differentiation. Transcripts of estrogen receptors (ER) α and β first appear in both female and male gonads of fry 10-15 days post-hatching. ERα and ERβ exhibit different expression patterns suggesting differential roles of ERα and ERβ in estrogen action on gonadal sex differentiation. We have isolated two DM (*Doublesex/Mab-3* DNA-binding mitif)-domain cDNAs from tilapia testis and ovary, named *DMRT1* and *DMO*, respectively. *DMRT1* is expressed only in Sertoli cells and *DMO* is detected only in oocytes by *in situ* hybridization. The correlation between expression of *DMRT1* and testicular differentiation of both normal XY-male and sex reversed XX-males suggest that *DMRT1* is a candidate testis determining gene in tilapia. In contrast, abundant *DMO* expression in pre- and early vitellogenic oocytes in XX- and sex reversed XY-females indicates a relationship between *DMO* and oocyte growth.

II. Endocrine regulation of spermatogenesis

Spermatogenesis is an extended process of differentiation and maturation of germ cells resulting in haploid spermatozoa. The principal stimuli for vertebrate spermatogenesis are thought to be pituitary gonadotropins and androgens. However, the mechanisms of action of these hormones remain unresolved. Using an organ culture system for eel testes consisting of spermatogonia and inactive somatic cells, we have shown that the hormonal regulation of spermatogenesis in eel testes involves the gonadotropin stimulation of Leydig cells to produce 11-ketotestosterone (11-KT), a potent androgen in fish. In turn, 11-KT activates Sertoli cells to stimulate the production of activin B. Addition of recombinant eel activin B to the culture medium induced proliferation of spermatogonia, producing late type B spermatogonia, within 15 days in the same manner as did 11-KT. cDNAs encoding two androgen receptors (ARα and ARβ) have been cloned, for the first time in any vertebrates, from eel and tilapia testes. *In situ* hybridization reveals that although both AR mRNAs are present in eel testes prior to HCG injection, only ARα transcripts increase during HCG-induced spermatogenesis suggesting that ARα and ARβ play different roles in spermatogenesis. Activin B binds to activin type I and II receptors on spermatogonia to stimulate *de novo* synthesis of G1/S cyclins and CDKs leading to the initiation of mitosis. Interestingly, cyclin A1 transcripts are first detected in primary spermatocytes during HCG-induced spermatogenesiss in eel testes suggesting an important role for cyclin A1 in the progression to meiosis of male germ cells. Overexpression of GFP-labeled cyclins A and E in type A spermatogonia induces spermatogonial proliferation, followed by meiosis

III. Endocrine regulation of oocyte maturation

Meiotic maturation of fish oocytes is induced by the action of maturation-inducing hormone (MIH). 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) has been identified as the MIHs of several fish species. The interaction of two ovarian follilce cell layers, the thecal and granulosa cell layers, is required for the synthesis of 17α,20β-DP. The theal layer produces 17α-hydroxyprogesterone that is converted to 17α,20β-DP in granulosa cells by the action of 20β-hydroxysteroid dehydrogenase (20β-HSD). The preovulatory surge of LH-like gonadotropin is responsible for the rapid expression of 20β-HSD mRNA transcripts in granulosa cells during oocyte maturation. Two types of 20β-HSD cDNAs are expressed in ovarian follicles of rainbow trout (*Oncorhynchus mykiss*). Recombinant proteins produced by expression in *E. coli in vitro* show that one (type A) has 20β-HSD activity but that the other (type B) does not. Among the three distinct residues between the protein products encoded by the two cDNAs, two residues (positions 15 and 27) are located in the N-terminal Rossmann fold, the coenzyme binding site. We have then generated mutants by site-directed mutagenesis at the following positions: MutA/I15T, MutB/T15I, and MutB/Q27K. Enzyme activity of wild-type A is abolished by substitution of Ile-15 by Thr (MutA/I15T). Conversely, enzyme activity is acquired by the replacement of Thr-15 with Ile in type B (MutB/T15I). MutB/T15I mutant shows properties similar to the wild-type A in every aspect tested. Mutation MutB/Q27K has only partial enzyme activity, indicating that Ile-15 plays an important role in enzyme binding of cofactor NADPH.

17α,20β-DP induces oocyte maturation by acting on a pertussis toxin-sensitive G-protein-coupled membrane receptor. The early steps of 17α,20β-DP action involve the formation of downstream mediator of this steroid, the maturation-promoting factor or metaphase-promoting factor (MPF) consisting of cdc2 kinase and cyclin B. 17α,20β-DP induces oocytes to synthesize cyclin B which activates a preexisting 35-kDa cdc2 kinase via phosphorylation of its threonine 161 by a threonine kinase (MO15), thus producing the 34 kDa

active cdc2. The function of the MOS/MAPK pathway during 17α,20β-DP-induced oocyte matration has been investigated using goldfish oocytes. Mos is absent in immature oocytes. It appears before the onset of germinal vesicle breakdown (GVBD), increases to a maximum in mature oocytes arrested at MII and disappears after fertilization. MAPK is activated after Mos synthesis but before MPF activation, and its activity reaches maximum at MII. Injection of either *Xenopus* or goldfish *c-mos* mRNA into one blastomere of 2-cell-stage *Xenopus* and goldfish embryos induces metaphase arrest, suggesting that goldfish Mos has a cytostatic factor (CSF) activity. Injection of constitutively active *Xenopus c-mos* mRNA into immature goldfish oocytes induced MAPK activation, but neither MPF activation nor GVBD occurs. Conversely, the injection of goldfish *c-mos* antisense RNA inhibits both Mos synthesis and MAPK activation in the 17α,20β-DP-treated oocytes, but these oocytes undergo GVBD. These results indicate that the Mos/MAPK pathway is not essential for initiating goldfish oocyte maturation despite its general function as a CSF.

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