Chapter 4

Genetic Approaches to Understanding the Basic Mechanisms of Endocrine Disruptor Actions on Sex Determination/Differentiation in the Medaka, *Oryzias latipes*

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Summary

Various genetic and molecular approaches have been used to establish the madaka as a test animal model for studying the effects of endocrine disrupting chemicals. We identified, for the first time in any non-mammalian vertebrates, *DMY* as the sex-determining gene of medaka. The cloning and sequencing of a number of genes that are thought to be associated with gonadal sex differentiation have been performed and some are still in progress (Fig. 1). These molecular probes provide useful tools to understand not only the molecular mechanisms of sex determination and gonadal sex differentiation but also to determine the mode of action of various endocrine disruptors during early stages of development of medaka. Finally, approximately 25,000 EST clones were obtained from whole embryos and fry during sex determination and differentiation and adult ovary. These EST clones were 5'-end sequenced and those associated with early stages of sex differentiation were re-arrayed in a DNA chip to evaluate their functional changes during the processes of endocrine disruption.

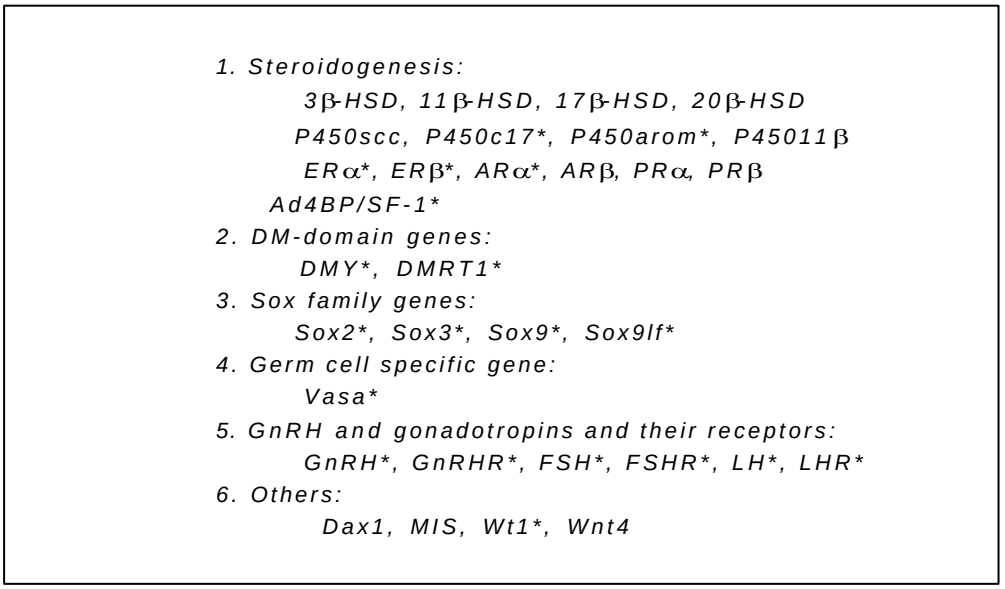

Fig. 1. Genes that are thought to be associated with sex determination and gonadal sex differentiation in vertebrates.

*, already cloned or cloning is in progress in medaka.

1. Introduction

A number of environmental pollutants have been shown to disrupt reproductive function in both wild populations and laboratory animals through interactions with different endocrine pathways. Primary targets for these endocrine active contaminants are steroid hormone receptors and steroidogenic enzymes. Recent evidence from studies on fish suggests that the periods of sex determination and gonadal sex differentiation are potential targets for endocrine disrupting chemicals present in the environment (Fig. 2). Treatments with exogenous sex hormones around the time of sex differentiation have profound effects on gonadal development including sex reversal, hermaphroditism and sterilization [1]. The gonads are, therefore, extremely sensitive to environmental stimuli around the time of sex differentiation, often with irreversible effects. As a result, environmental endocrine disruptors likely affect sex differentiation in wild fish. To determine causation and the mechanisms of disruption, it is critical to have a working knowledge of normal gonadal sex differentiation and its regulation. The medaka, *Oryzias latipes*, is an ideal model for studies related to endocrine disruption due to several reasons. This fish can spawn daily with regulated photo-thermal conditions, possess short time of maturation allowing the possibility to utilize both parental populations and progeny, and can be manipulated for transgenic studies. Here, I will review some of our recent attempts to establish medaka as a test organism for studying the endocrine disruptors' action together with our findings on this line using the Nile tilapia, *Oreochromis niloticus*, : 1) the identification of sex-determining gene, 2) the cloning of genes involved in gonadal sex differentiation, and 3) an attempt to develop DNA microarrays for the identification of differentially regulated genes during gonadal sex differentiation and for routine use in environmental monitoring.

2. The identification and cloning of sex-determining gene

Since sex determination precedes gonadal sex differentiation, it is important to understand the cascade of genetic sex determination. This information will be useful to generate molecular probes for basic understanding of sex determination process and also for studying endocrine disruption. In mammals, the Y chromosome is male determining, and encodes a gene referred to as *TDF* (testis determining factor) which induces the indifferent embryonic gonad to develop as a testis [2]. In 1990, the *SRY* (sex determining region of Y) gene was isolated from the human Y chromosome by positional cloning [3].

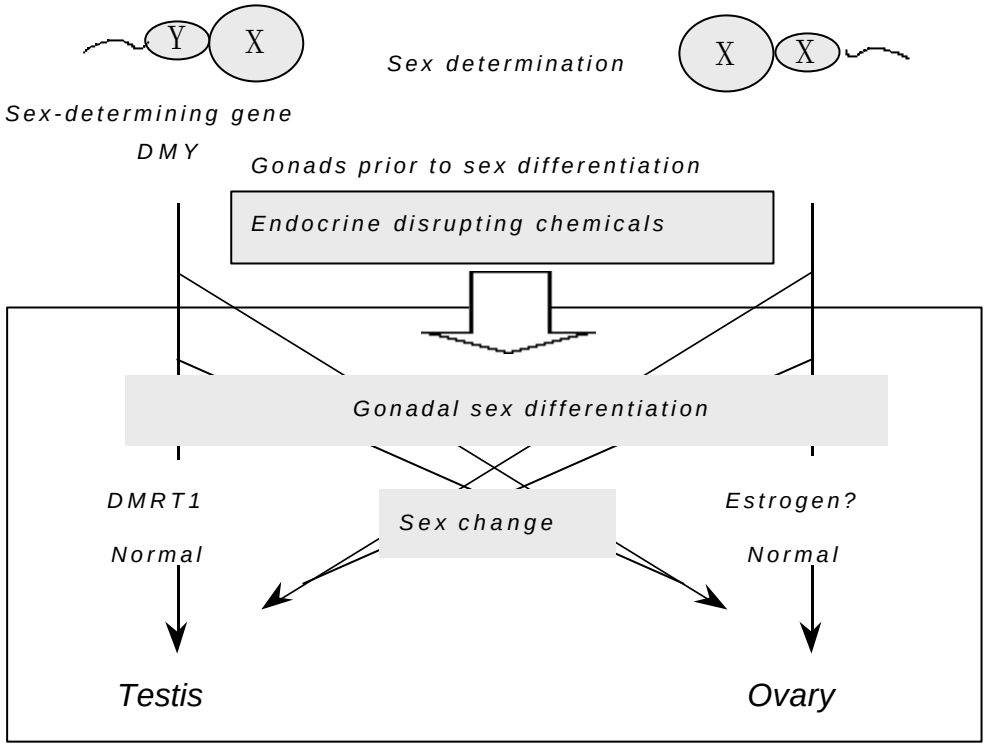

Fig. 2. Schematic diagram of the normal cascade of sex determination and possible mechanisms of endocrine disrupting chemical action. Endocrine disrupting chemicals can interchange the normal cascade of gonadal sex differentiation at different levels.

Not only were mutations detected in the *SRY* gene of XY females, indicating that *SRY* is required for normal testis development, but mice transgenic for *Sry* developed into sex reversed males, despite an XX karyotype [4]. Thus, among Y derived sequences *SRY* is both required and sufficient for male sex determination. In contrast, no genes comparable to *SRY/Sry* have been found in non-mammalian vertebrates.

Genetic determination of sex in non-mammalian vertebrates including fish can involve monogenic or polygenic systems, with factors located on the autosomes or on sex chromosomes. In the latter case, both male (XY) and female (ZW) heterogametic systems have been described, as well as many subtle variations on these themes [1]. To identify such a sex-determining gene, a positional cloning approach is suitable. The medaka fish is known to have a stable genetic XX/XY sex determining system, the male being heterogametic and dependent on the presence of the Y chromosome, although the chromosome is not cytogenetically distinct. Another major advantage to working with medaka is a large genetic diversity within the species [5] and the existence of several inbred strains [6]. Alteration of phenotypic sex with no reproductive

consequences, and recombination over the entire sex chromosome pair, suggest that there are no major differences, other than a sex-determining gene, between the X and Y chromosomes.

To clone positionally the sex-determining region, we generated a Y congenic strain to highlight the genetic differences between the X and Y chromosomes from inbred strains of medaka [7]. The Y congenic strain has a sex-determining region derived from the HNI-strain Y chromosome on the genetic background of an Hd-rR strain. Using this strain, we had previously constructed a genetic map of the medaka sex chromosome and constructed a BAC (bacterial artificial chromosome) genomic library [8]. Fluorescence *in situ* hybridization using one of the BAC clones as a probe was used to determine the location of the sex-determining region on the Y chromosome. As shown in Fig. 3, the sex-determining region is located on the centromere side of the long arms of the sex chromosomes.

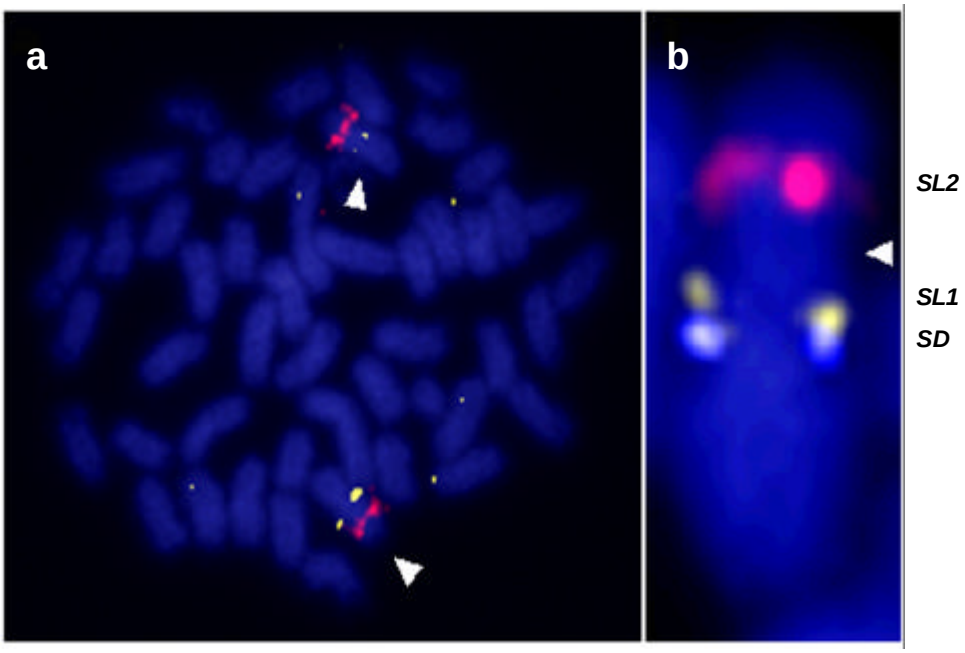

Fig. 3. Fluorescence *in situ* hybridization of medaka metaphase chromosome.
a, Sex-linked markers *SL1* (yellow) and *SL2* (red) hybridize to both X and Y chromosome.
b, A medaka sex chromosome with three different probes(*SL1*, *SL2*, sex-determining region, *SD*).

Shotgun sequencing was used to determine the sequence of the sex-determining region covered by the four BAC clones. The entire sequence of the two, centromere-side BAC clones was determined; however, the remaining

two, telomere-side BAC clones could not be completely sequenced due mainly to numerous repetitive sequences. Consequently, we sequenced a total of 422,202 nucleotides and estimated the four BAC clones covered about 530 kb. Genscan (a gene predicting program, Stanford Univ. ver. 1.0) predicted 52 genes in this region. Deletion analysis of the Y chromosome of a congenic XY female further shortened the region to 250 kb (Fig. 4). Shotgun sequencing of this region predicted 27 genes. Three of these genes were expressed during sexual differentiation. However, only the DM-related *PG17* was Y specific. We thus named it *DMY* [9]. The full-length cDNA sequence (1,320 bp) of *DMY* was obtained by 5' and 3' RACE. The longest open reading frame spans 6 exons and encodes a putative protein of 267 amino acids, including the highly conserved DM domain (Fig. 5). The DM domain was named after a related DNA binding motif found in two proteins, *doublesex* (*dsx*) and *mab-3*, involved in sexual development in the fruitfly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, respectively [10]. Since the initial characterizations of *dsx* and *mab-3*, DM-related genes have been identified from virtually all species examined to date including medaka. In vertebrate species, *DMRT1* (DM-related transcription factor 1), the DM-related gene most homologous to *DMY* (about 80%), correlates with male development [11-13]. Combined with its Y chromosome specificity, this finding suggests that *DMY* plays a pivotal role in testicular differentiation.

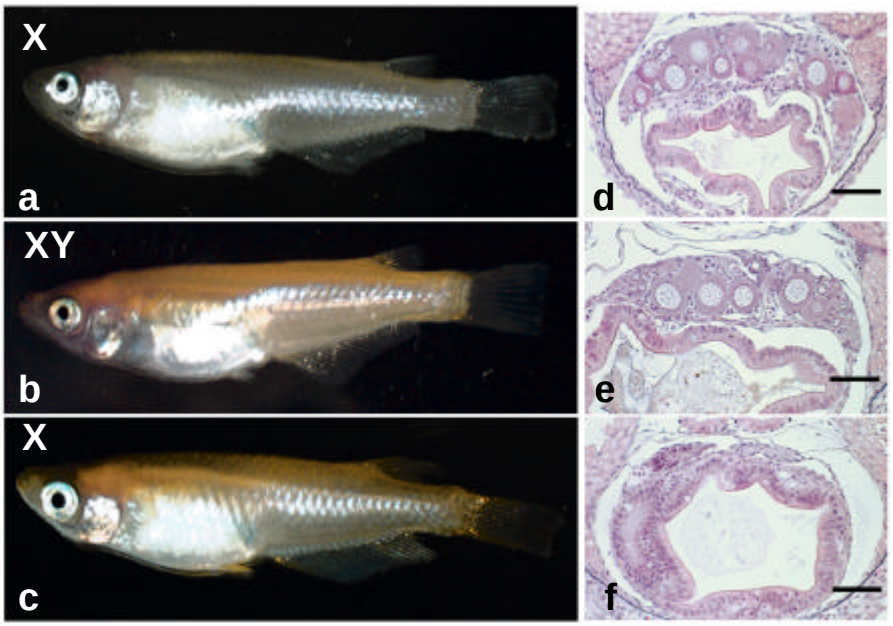

Fig. 4. Characteristics of medaka lacking a part of the Y chromosome. Phenotypes of the congenic strain (XX) (**a**), medaka lacking a part of the Y chromosome (XY-) (**b**) and the congenic strain (XY) (**c**). Histological cross-sections of medaka fry sampled at 30 dah. XX (**d**) and XY- (**e**) individuals have ovaries, whereas XY specimen has testes with spermatogonia (**f**). Scale bars, 50 μm.

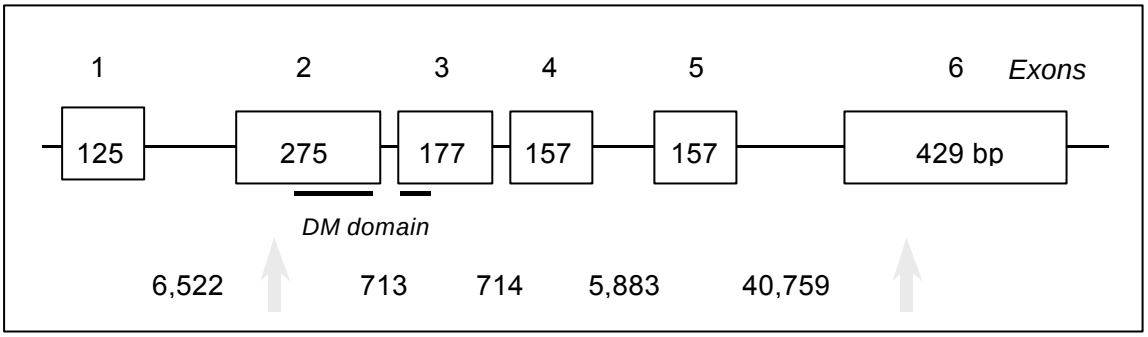

Fig. 5. Exon and intron structures of *DMY*.

Two lines of evidence established the critical role of *DMY* in testicular differentiation. Firstly, the analysis of two naturally occurring XY females from two separate populations (Awara and Shirone). One of these mutants contained a single nucleotide insertion in exon 3 of *DMY* that causes premature termination of the DMY protein. When mated, all XY offspring with the mutant Y were female. The absence of about two-thirds of the protein presumably renders *DMY* nonfunctional, thus resulting in XY sex reversal (female phenotype). The other mutant had a severe depression in *DMY* expression in the embryo and 60% of its XY offspring with the mutant Y developed as females. Secondly, specific RT-PCR and *in situ* hybridization were used to determine the temporal expression of *DMY* during sexual differentiation. At hatching and 5 days after hatching, *DMY* mRNA was present in XY embryos. *DMY* signals were detected only in the somatic cells (probably pre-Sertoli cells) surrounding germ cells in XY gonads at the time when sex determination occurs (Fig. 6).

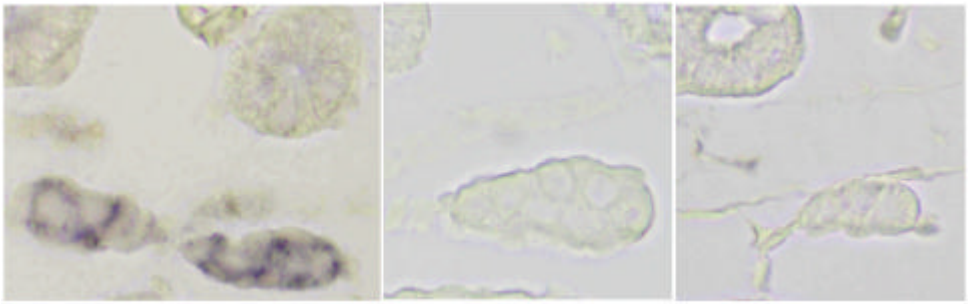

Fig. 6. *DMY* mRNA expression in fry gonads shown by *in situ* hybridization of larval sections. **a**, XY gonads on hatching day; antisense probe. Strong signals for *DMY* are seen in cells surrounding the germ cells. **b**, XX gonad on hatching day; antisense probe. *DMY* signal is undetectable in XX individuals. **c**, XY gonad on hatching day; sense probe. Control hybridization shows no signal. Germ cells (arrows).

Oryzias curvinotus has the same sex-determining mechanism (XX-XY) as *O. latipes* and has *DMY* on the Y chromosome, which suggests that *DMY* also has a role in sex determination of *O. curvinotus*. A phylogenetic tree based on the amino acid sequence including the DM-domain shows that *DMY* was derived from *DMRT1* immediately before speciation of *O. latipes* and *O. curvinotus* (Fig. 7). The branch length of *DMY* is longer than that of *DMRT1* in the phylogenetic tree. This means that *DMY* has more mutations than *DMRT1* and suggest that *DMY* accumulated these mutations after it acquired its sex-determining function. It is of interest that the rate of evolution of *Sry*, which is the sex-determining gene on the Y chromosome of mammals, is also higher than that of *Sox* family genes. These results suggest that a new sex-determining gene generated by a gene duplication event in the sex chromosome tends to evolve fast.

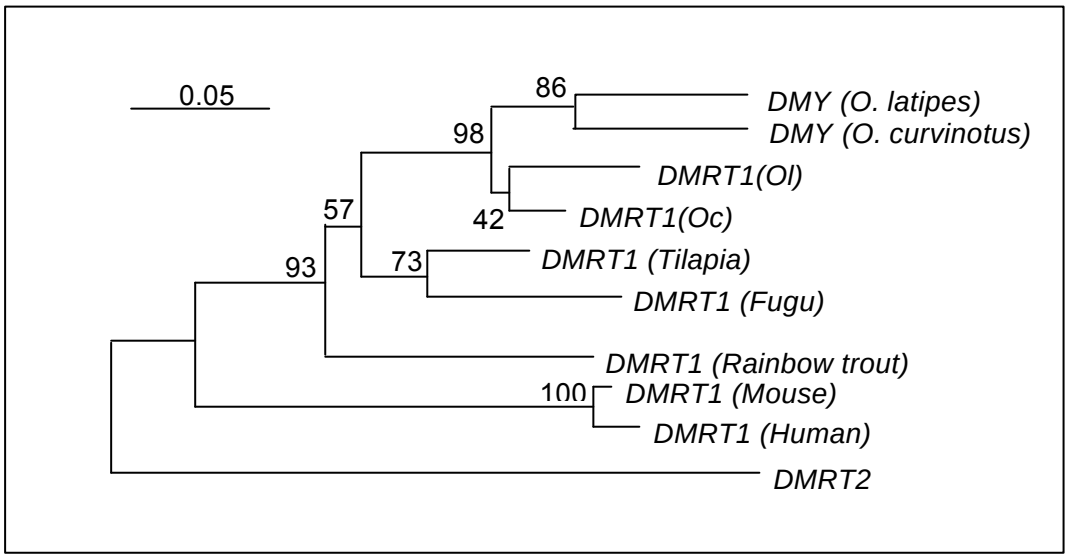

Fig. 7. Phylogenetic tree of DMY and its associated DM-domain genes from various

The precise role of *DMY* remains to be elucidated. It would seem likely that the protein product of *DMY* is a regulatory factor, probably DNA binding element that regulates expression of a secondary or downstream testis determinant. The downstream locus could be either Y or X-linked or autosomal. *DMY* is the first identified sex-determining gene in any non-mammalian vertebrates (Fig. 8) and it seems to be a useful molecular probe to understand the effects and molecular mechanisms of endocrine disruptors in wild life.

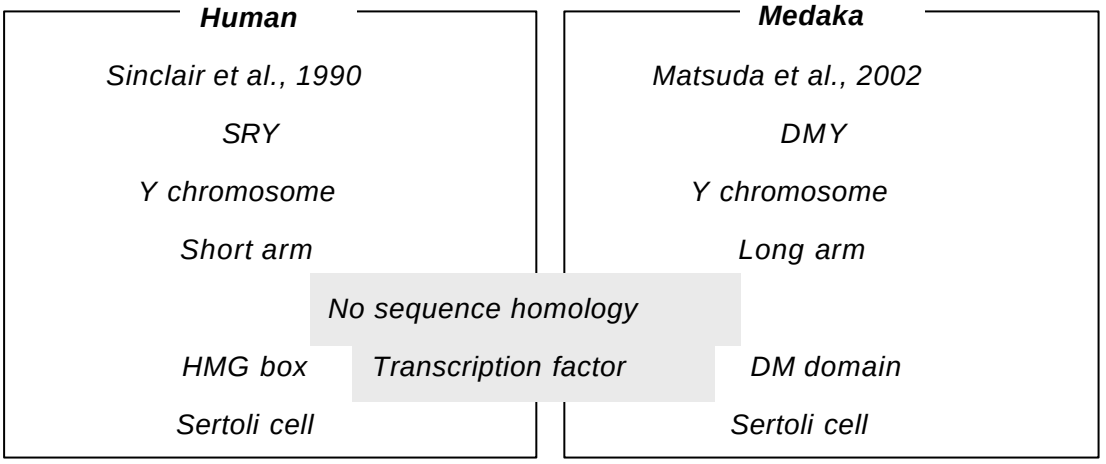

Fig. 8. Comparison of two identified sex-determining genes, *SRY* and *DMY*, in vertebrates.

3. Gonadal sex differentiation

3-1. Germ cells and *vasa* gene

The morphological criteria for judging the initial differentiation of ovaries and testes have been studied in detail in medaka by Yamamoto [14]. Germ cells in putative ovaries outnumber those in putative testes just after hatching, and mitosis and meiosis of germ cells take place earlier in ovaries than in testes [15] (Fig. 9). No sex-specific differences have been identified in the gonadal architecture, including the supporting lineage cells. The sex-specific structure among supporting cells first appears in the acinous structure of males, 10 days after hatching [16]. Thus, unlike mammals, the sex differentiation in medaka is first observed in germ cells and then in supporting cells.

The gene *vasa* encodes a DEAD family of putative RNA helicase and is present in both the polar granules at the posterior end of the oocyte and the nuage structure in the germ cells in *Drosophila*. In several vertebrate species, the *vasa* homologs are found to be expressed specifically in the germ line [17]. We cloned a medaka *vasa* homolog (*olvas*). *In situ* hybridization studies revealed that the transcripts for *olvas* are exclusively detected in the cytoplasm of germ cells in the testis and ovary [18]. We then generated transgenic medaka lines that express green fluorescent protein (GFP) exclusively in germ cells that can be visualized in a living vertebrate. Two medaka strains, himedaka (orange-red variety) and inbred QurtE (this strain lacks most pigments except for leucophores, a Y-linked trait). Around the day

of hatching, the QurtE strain clearly exhibited sexual dimorphisms in the number of GFP fluorescent germ cells [19]. The apparent sexual dimorphism of the gonads indicated by GFP fluorescence can also provide a useful indicator of the effects of environmental substances on gonadal development (Fig. 10).

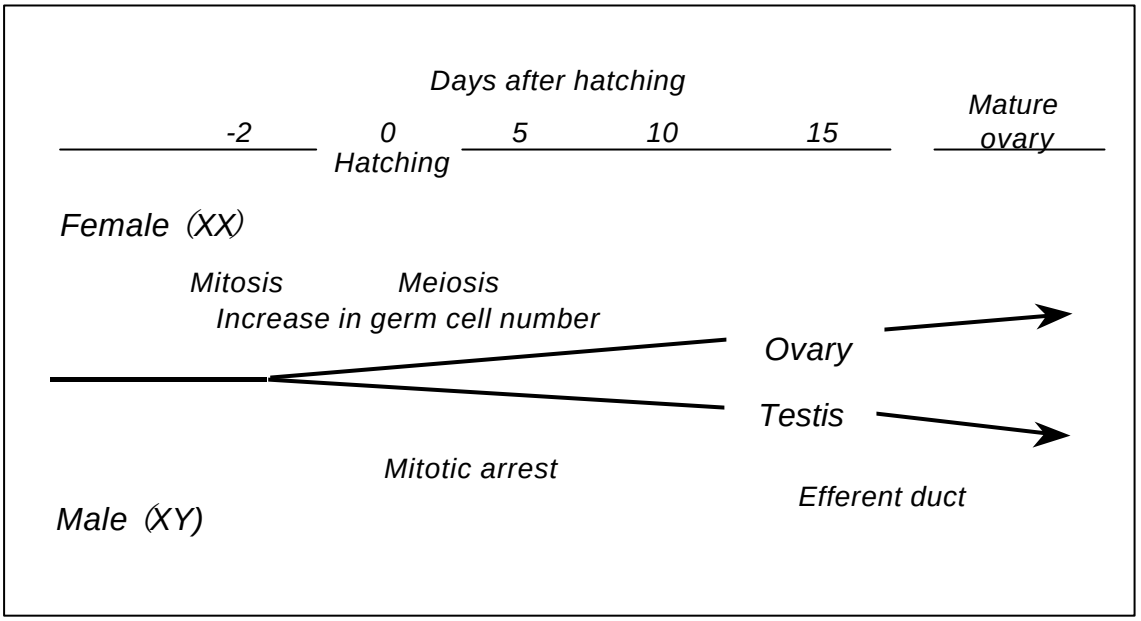

Fig. 9. Gonadal sex differentiation in medaka.

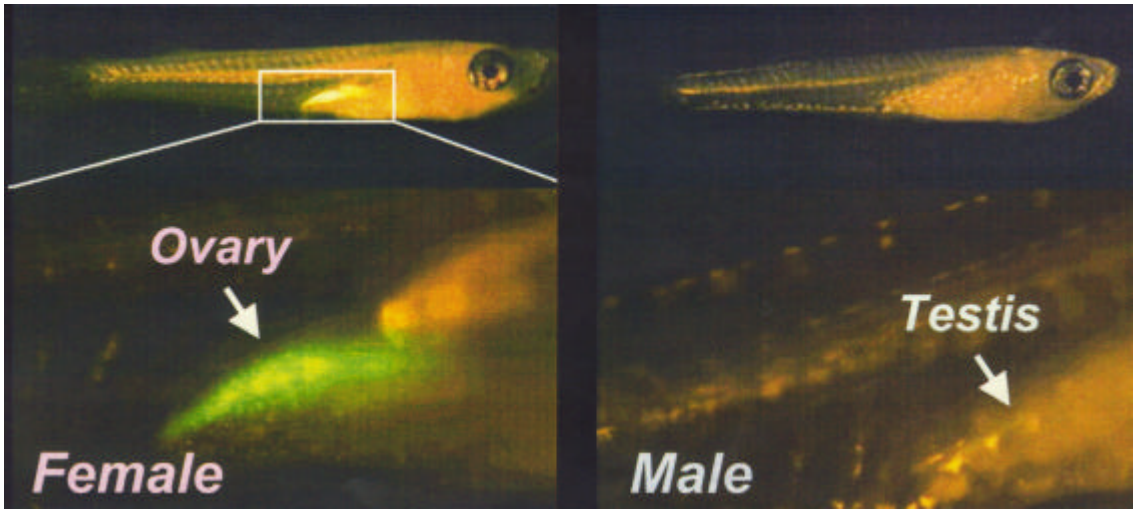

Fig. 10. GFP fluorescent germ cells in the ovary and testis of medaka. The ovary contains numerous oocytes which can be seen as a green fluorescence.

3-2. Steroidogenic enzymes

As in other vertebrates, a complex series of enzymes are responsible for the biosynthesis of sex steroids in fish [20] (Fig. 11). Several biologically important steroidal mediators have been identified in fish gonads. In females, estradiol-17β and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) are two major steroids which are responsible for oocyte growth and maturation, respectively [20], while in males 11-ketotestosterone has been identified as a spermatogenesis-inducing hormone in the Japanese eel, *Anguilla japonica* [21].

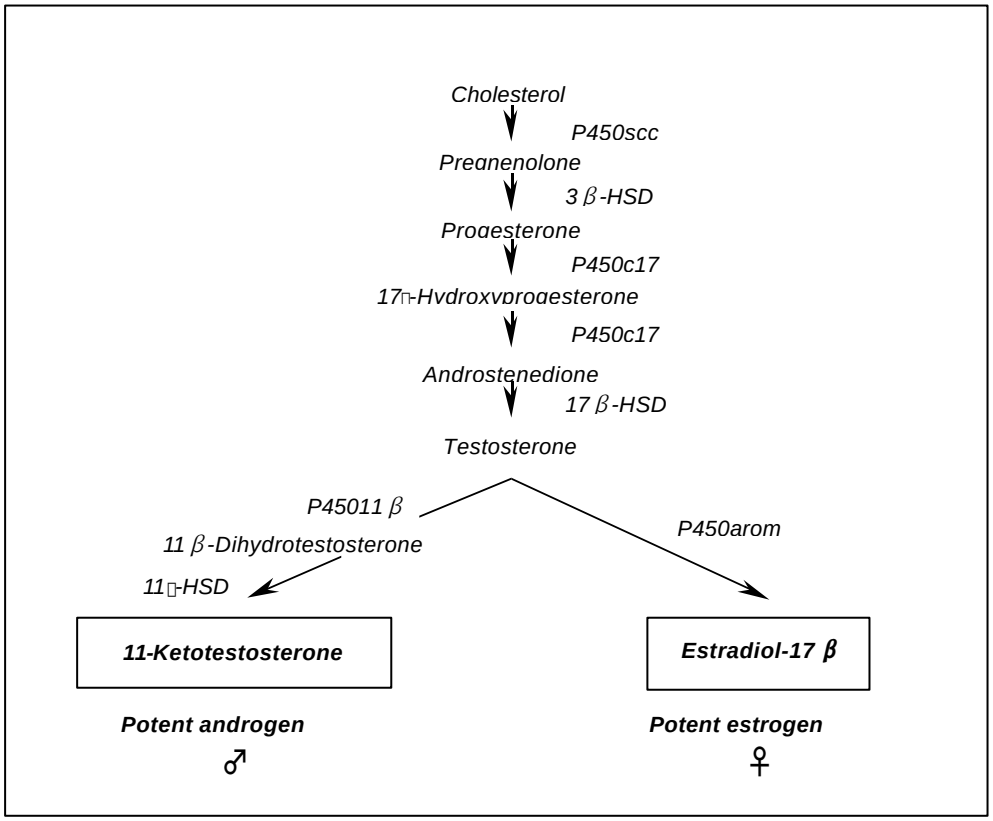

Fig. 11. Schematic diagram of steroidogenic pathway in fish

Exogenous sex steroids administered at the time of sex determination can strongly influence the course of sex differentiation in fish, suggesting that they play a critical role in assignment of gonad determination as well as subsequent differentiation [1]. Similarly, if steroid-synthesizing capability is interfered with, sex determination can be disrupted; for example, inhibition of estrogen synthesis in early development using inhibitors of the enzyme aromatase can cause masculinization of several teleost fishes [1]. If steroids are critical for

directing initial sex differentiation rather than being consequence of it, then the appearance of steroid-producing cells and differences in steroid production between the sexes should be apparent prior to morphological differentiation of the gonad. Indeed, immunohistochemistry has shown that P450scc, P450c17, 3β-HSD and P450arom are found at high levels in female gonadal anlagen of tilapia at the undifferentiated and differentiating stages, but is only seen weakly in genetic males and not until 30 days after hatching. These results strongly suggest that endogenous estrogens act as the natural inducers of ovarian differentiation in tilapia [22]. This hypothesis is further supported by evidence of masculinization of genetic female tilapia by inhibition of estrogen synthesis using an inhibitor of P450arom.

On the basis of extensive experiments using various steroid hormones, Yamamoto [23] hypothesized that sex hormones act as endogenous sex inducers during sex differentiation of medaka, androgens acting as testicular inducers and estrogens acting as ovarian inducers. However, some of the morphological studies do not seem to support the critical role of endogenous steroid hormones in gonadal sex differentiation in medaka. For example, it was shown that steroid-producing cells appeared initially long after the completion of sex differentiation [24, 16]. Similarly, 3β-HSD, an important enzyme for steroid hormone biosynthesis, also appeared after sex differentiation. The use of aromatase inhibitors and antiestrogens has also shown that ovarian development may occur independent of the action of estrogens [25]. More recently, using transgenic medaka with over-expressed medaka estrogen receptors, Kawahara *et al*. [26] examined the effects of exogenous estrogen and androgen on sexual differentiation. The results showed that over-expression of estrogen receptors did not affect the sexual development in either male or female after estrogen or androgen treatment, suggesting an estrogen/estrogen receptor-independent female sex determination pathway. Certainly, further studies are required to determine whether endogenous steroids are critical for directing initial sex differentiation in fish including medaka.

Specific genes involved in steroid biosynthesis are differentially expressed in the somatic cells of testis and ovary, which results in the production of an array of sex steroids. To provide a basis for the possible involvement of endogenous sex steroids in gonadal sex differentiation in medaka, it is necessary to examine the expression pattern of various steroidogenic enzymes in gonads before, during and after sex differentiation. We previously isolated two enzymes, P450c17 (D. Kobayashi *et al*., unpublished) and P450arom [27, 28] from medaka ovaries. These cDNA inserts were confirmed to encode each

steroidogenic enzyme by introducing it into non-steroidogenic COS-1 monkey kidney tumor cells. The coding region of the medaka *P450arom* gene is composed of nine exons; human P450arom gene also contains nice exons. Although the intron – exon boundary sequences of medaka P450arom gene are exactly the same as those of mammals, introns are much shorter in medaka than mammals. The promoter region of medaka *P450arom* gene also contains potential Ad4BP/SF-1 sites and estrogen responsive element (ERE) half-sites. These results suggest that the basic structural organization of *P450arom* genes and the regulatory mechanisms of expression are well conserved throughout the vertebrates [27]. Multiple *P450arom* genes have been identified in several fish species, and sequence comparisons have revealed that the forms of *P450arom* expressed in brain and ovary are distinct *P450arom*. Recently, the brain form of *P450arom* has been shown to be expressed at two distinct levels among individual zebrafish embryos around the time of sex differentiation, suggesting a possible role of this gene in controlling sex differentiation [29]. However, no multiple forms of *P450arom* genes have been reported in medaka.

More recently, we have cloned a cDNA encoding the steroidogenic acute regulatory (StAR) protein from medaka ovaries. StAR protein mediates the rate-limiting and acutely-regulated step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane where P450scc initiates the synthesis of all steroid hormones (M. Tokumoto *et al.*, unpublished).

Using our acquired knowledge of nucleotide sequences, we synthesized peptides and used them to raise polyclonal antibodies against four steroidogenic enzymes essential for the biosynthesis of all major steroid sex steroid hormones including androgens and estrogens [30]. Some of these antibodies can also be used to stain immunohistochemically gonads during the process of sex differentiation in medaka. Our current efforts, in collaboration with Dr. N. Shibata, Shinshu University, center on the cloning and sequencing of the genes encoding several other steroidogenic enzymes such as P450scc, 3β-HSD, and 11β-hydroxylase.

3-3. Sex steroid receptors

Steroid hormone action is mediated by binding to specific receptors. Like higher vertebrates, fish also possess two estrogen receptors typical of the α and β forms. In tilapia, while both genes are expressed in early male and female gonads at 10-15 days after hatching, they display different expression patterns suggesting important roles in regulating early sex differentiation. Genomic

and complementary DNA fragments encoding an estrogen receptor homolog have been cloned from medaka and used to examine its expression patterns during gonadal sex differentiation. Estrogen receptor mRNA was expressed in both sexes, but at very low levels, during the period of sex determination [25, 26]. The α and β forms of estrogen receptors are present in medaka ovaries (M. Nakai, see Chapter 2 in this issue; T. Todo, personal communication). Unlike other vertebrates, some of fish have two forms of androgen receptors. Two functional androgen receptors were cloned for the first time from an eel testis cDNA library [31]. A cDNA encoding medaka androgen receptors has also been cloned (T. Todo, unpublished).

3-4. Other genes involved in gonadal sex differentiation

Numerous genes have been shown to be involved in sex determination and differentiation [2]. These genes include *Sox9*, *Ad4BP/SF-1*, *DMRT1*, *Dax1*, *WT1*, *MIS* and others. Several of these genes have also been cloned from fish. The *SRY*-related gene *Sox9* is highly conserved in vertebrate evolution. At the onset of gonadal sex differentiation, *Sox9* is up-regulated in testes but down-regulated in ovaries. *Sox9* is an autosomal gene involved in the differentiation of Sertoli cells in mouse and chicken, two species with different genetic mechanisms of sex differentiation. We isolated several kinds of *Sox* sequences and then investigated the expressions of *Sox* transcripts in medaka oocytes and embryos [32]. Medaka *Sox* sequences are present in both males and females and can be classified into five subgroups based on the analysis of the amino acid homologue in the HMG box and further that one of the medaka *Sox* genes is expressed in unfertilized eggs. More recently, two more *Sox* transcripts (*Sox9* and *Sox9lf*) were also isolated from a testis cDNA library [33]. The *Sox9* encodes 487 amino acids and shows approximately 70% amino acid identity with known vertebrate SOX9 proteins. The *Sox9lf* is a longer from of the *Sox9*, which is transcribed from an additional exon in the 5' upstream region. Interestingly, the expression of medaka *Sox9* is predominantly observed in the adult ovary by northern blot and *in situ* hybridization, whereas in the testis, its expression is detectable only by RT-PCR. During medaka embryogenesis, its expression is observed in the cranial cartilage and pectoral fin endoskeleton. These observations suggest that the function of *Sox9* in the cartilage is conserved among vertebrates, while that in the gonad is quite different in medaka.

A cDNA encoding Ad4/BP/SF-1 (FTZ-F1) has also been cloned from ovarian follicles of medaka [34]. Distribution of *Ad4BP/SF-1* transcripts in various

tissues of medaka was very similar to that of mammalian *Ad4BP/SF-1*. The expression pattern of *Ad4BP/SF-1* transcripts during oogenesis coincides with that of *P450arom* transcripts. Transfection assays further suggest a potential transcriptional regulatory activity of Ad4BP/SF-1 in the transcriptional regulation of *P450arom* in the ovarian follicle of medaka. Sequence analyses revealed three putative Ad4BP/SF-1 binding motifs in the medaka *P450arom* promoter region. Since the role of Ad4BP/SF-1 in P450arom regulation in fish granulosa cells during vitellogenesis proved essential, the usage of this probe might be further helpful to test whether estrogens are necessary for ovarian differentiation.

Another gene family (the family of genes that contain DM-domain) involved in insects and nematodes has also been shown to be involved in vertebrate sex determination and gonadal sex differentiation. One member of this group, *DMRT1*, has been shown to be essential for postnatal testis differentiation [35]. In tilapia, *DMRT1* possesses the male-specific motif present in *doublesex*, and expression appears in testis (Sertoli cells) specific, whereas another DM homologue (*DMO*) lacked this motif and was found to be expressed only in ovary [13]. Masculinization of tilapia genetically female (XX) gonads with androgen induces *DMRT1* expression, whereas treatment of genetically male (XY) with estrogen resulted in reduced expression [13, T. Kobayashi, unpublished]. These results suggest that *DMRT1* genes are expressed in response to testis differentiation induced by other factors (i.e. steroids), and in these species, are located somewhat downstream in the determining pathway.

There are several forms of DM-domain genes in medaka. Brunner *et al*. [36] cloned the DM gene family from medaka such as *DMRT1*, *DMRT2*, and *DMRT3*, and determined their structural and functional characteristics. Gene structure and order are highly conserved within a larger region of synteny with human chromosome 9. While the chromosomal localization of the *DMRT* cluster argues against its role as a primary sex-determining locus, the comparative analysis uncovered putative regulatory elements that might be important during sex differentiation and sex reversal [36]. *DMRT1* mRNA has been found expressed in embryos of male medaka around 20 days after hatching [37, Kobayashi *et al*., unpublished].

4. EST and DNA microarray

Up to now, I discussed the process of sex determination and identification of the sex-determining gene, together with several other genes involved in

gonadal sex differentiation in medaka. Since this did not account for the entire gene cascade of sex determination/differentiation, we cloned a series of genes and generated EST clones during the early stages of development of medaka. Later we re-arrayed these EST clones in a DNA chip for DNA microarray evaluation of genes. By this we will be able to distinguish the molecular mechanisms of endocrine disruptors to know how it bring about its biological effects. Thus DNA microarray-based gene expression profiling might be highly useful gene technology to understand mRNA-level control of sex differentiation and also to examine endocrine disruptors in medaka embryos.

4-1. EST clones

The first step towards microarraying is to isolate gene fragments. Gene targets for arraying may be randomly picked from a cDNA library, characterized by sequencing to produce ESTs (Exposed Sequence Tag) and used for arraying. Embryos are from the Hd-rR strain. For library construction, embryos two days before hatching (7 days after fertilization), 0 day after hatching (dah), 5 dah, 10 dah, 15 dah, and ovary (adult) were used (Table 1). Total RNA was extracted from whole body of embryos and an ovary. Bacterial clones were plated, colonies picked robotically and glycerol stocks constructed in 384-well format. Random clones (3,072 - 6,528) were 5'-end sequenced. Within 24,960 sequence reads, 19,290 sequences were evaluated as high-quality sequences (Table 1). Batch sequence alignments were performed on a computer workstation using a program (programmed by Computer Laboratory of National Institute for Basic Biology). As a result, 7,513 sequences were nonoverlapping sequences. Glycerol stocks correspond to these 7,513 sequences were re-arrayed to 20 plats of 384-well microtiter plates using a GENESIS workstation (Tecan).

Table 1. EST clones obtained from medaka embryos during sex determination and gonadal sex differentiation adult ovary

	2dbh*	0dah*	5dah	10dah	15dah	Ovary	All
Reads	6,528	3,840	3,840	3,840	3,840	3,072	24,960
Available	4,935	3,419	3,148	2,577	2,462	2,749	19,290
Clusters	3,548	1,282	2,259	1,847	1,784	1,479	7,51

* days before hatching; ** days after hatching

4-2. DNA arrays

Amplification of target cDNA clones was carried out. PCR amplification was performed using 384-well formatted glycerol stocks. Amplified PCR products were purified using the 384-well glass filter membrane (Corning). The quality and quantity of purified PCR products were analyzed by 1% agarose gel electrophoresis, and the PCR was repeated if required. PCR products were dried under a 37 ℃ oven and dissolved in Microarray Crosslinking Reagent D (Amersham). DNA solutions were spotted on metal coated Microarray slides Type 7 Star (Amersham) using a Molecular Dynamics GenIII Arrayer (Amersham). Each slide had two identical sets of up to 4,608 DNA spots (Fig 12). Spotted slides were UV cross-linked at 100 mJ. Target DNA spotted on slides was stored dry until used.

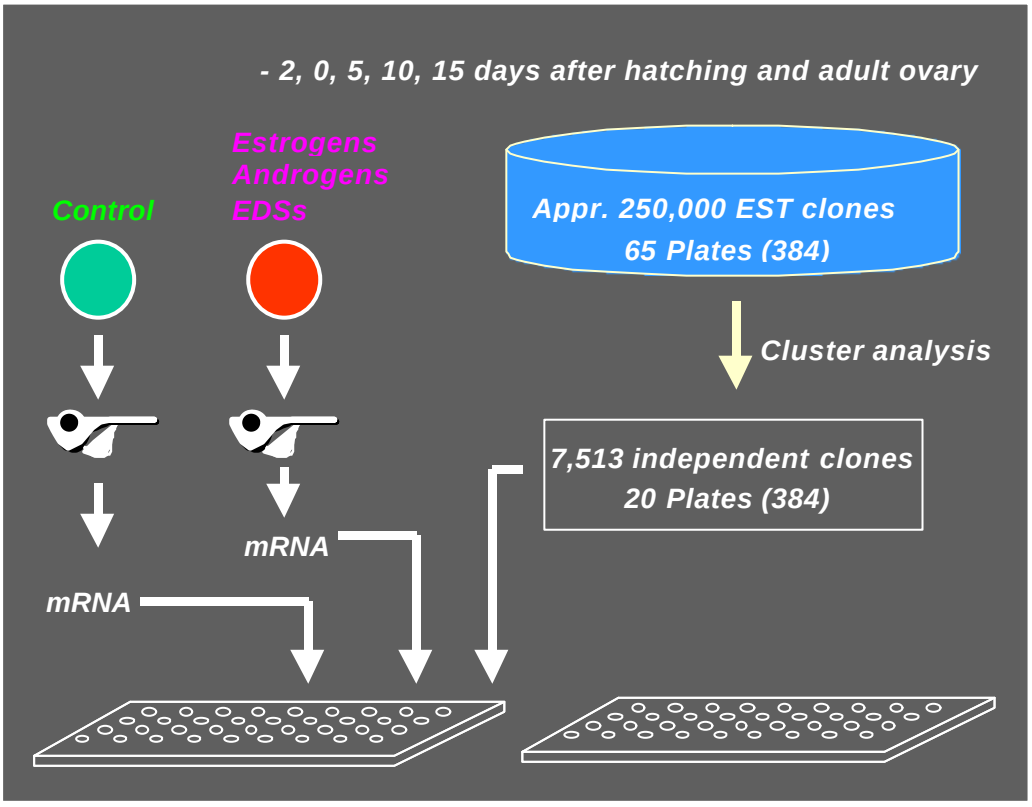

Fig. 12. Procedures and methodology to assess normal and endocrine disrupting chemicals' mechanisms of sex determination and differentiation.

5. Future directions

As described above, 7,513 individual EST clones that are thought to be essential for gonadal sex differentiation in medaka were selected and re-arrayed. In the next step, we are planning to use this microarray DNA chip to detect mRNA changes after the treatment for several days either with

estrogenic or androgenic compounds or endocrine disruptor chemicals. In brief, we prepare RNA from both from the control and treated groups and then label them with two different dyes (fluorophores Cy3 and Cy5) followed by hybridization on the DNA chip. The difference in the dye reveals those genes that had either undergone up or down regulation due to the treatments. The localization of these genes will be further analyzed by whole mount *in situ* hybridization. Later we plan to prepare the DNA chip containing only these important genes. This will later serve as more comprehensive DNA chip for our future studies related to sex differentiation as well as screening of endocrine disrupting chemical in wild life.

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