Sex Determination and Gonadal Sex Differentiation in Fish

Yoshitaka Nagahama
Okazaki National Research Institutes, Japan

This first slide shows the processes of gonadal sex differentiation and gametogenesis in fish. We know much about the hormonal regulation of gametogenesis in fish. In fact, we identified 3 steroidal mediators involved in spermatogenesis, oogenesis, and final oocyte maturation and sperm maturation.

These processes are possible targets of endocrine disrupters. Another possible target of endocrine disrupters is the period of sex determination and gonadal sex differentiation. But we do not know much about the mechanism of sex determination and gonadal sex differentiation in vertebrates including mammals and fishes. In my talk, I would like to discuss first gonadal sex differentiation followed by sex determination. I hope at the end of my talk, I will try to relate our findings to human or mammalian studies briefly.

This slide shows the regulation of sex determination and gonadal sex differentiation in vertebrates. As you heard in the previous talk, in mammals Sry, the sex-determining gene, was discovered in 1990. Since then, numerous genes listed in this slide have been reported to be involved in gonadal sex differentiation. However, Sry-induced cascade of gonadal sex differentiation is still unclear.

In other vertebrates such as birds and fishes, no Sry or equivalent gene has been identified. Instead environmental factors such as temperature or water temperature and particularly sex steroid hormones have been reported to be involved for gonadal sex differentiation in these vertebrates. We have been using several fishes to study sex determination and differentiation in fish, such as tilapia for gonadal sex differentiation and medaka for a sex-determining gene, which I will discuss today. We also use some sex changing fish, but I will not discuss this work today.

In tilapia we can produce all males and females by using androgen-treated XX male or estrogen-treated XY female as well as super male that is YY. In most fish we can change the sex by treating them with exogenous sex steroid hormones such as estrogens and androgens. But these sex changes can be induced only when treated with these sex hormones prior to sex differentiation. We call this, in the case of tilapia, 0 to 20 days after hatching. This is the critical window, but it is also a strong possible target of endocrine disrupters.

Morphological gonadal sex differentiation in tilapia becomes evident 20-25 days after hatching. Ovarian and testicular differentiation can be judged by the appearance of the ovarian cavity (ovary) and the efferent (sperm) duct (testis), respectively. Here is the ovarian cavity. This shows the steroidogenic pathway of fish gonads. The difference between fish and other vertebrates or mammals is that testosterone is the substrate for both potent androgen and potent estrogen, that is 11-ketotestosterone and estradiol-17 β. We have completed to clone most of these genes and to raise antibodies against some of these enzymes.

First, I will show our RT-PCR analysis for aromatase in both XX females and XY males. As shown in the slide, aromatase is expressed in XX gonads even 10 days to 2 weeks before ovarian differentiation. There is an aromatase-positive steroid-producing cell in the XX gonad 7 days after hatching. *In situ* hybridisation and RT-PCR analysis reveal that gonads of XX females collected between 5-10 days contain all these steroidogenic enzymes necessary for estrogen synthesis, or at least expressed, but not for enzymes for androgen synthesis.

Estrogen receptors α and β are also expressed in XX gonads even before ovarian differentiation. As RT-PCR, our immunocytochemistry analyses show expression of P450scc, 3β-HSD, P450arom in XX gonads prior to sex differentiation. These findings clearly indicate that estrogens may be synthesized

(or even released) in XX gonads prior to ovarian differentiation. Therefore, estrogen may play an important role in ovarian differentiation.

To prove endogenous estrogen or estrogen receptors are involved in ovarian differentiation, we treated an aromatase inhibitor (fadrozole) and also an estrogen receptor antagonist (tamoxifen). Fadrozole treatment induced the female to male sex change. Although this fish is still genetically XX, the fish has testis. In most cases, tamoxifen also caused sex change, but in some cases both testis and ovary were seen in a gonad.

Taken together, these results indicate that estrogens or estrogen receptors are critical for ovarian differentiation. We are now investigating the mechanism of steroidogenic enzyme gene expression that is sex (female)-, stage (5-7 days after hatching)- and cell (steroid-producing cell)-specific. We are also looking at the mechanism of estrogen action – search for target genes for estrogens.

Here I briefly discuss the regulation of aromatase gene expression. There are 2 forms of aromatase genes in fish, they are, the ovarian and brain forms, but I will concentrate here only on the ovarian form.

The promoter region of the tilapia aromatase gene identified sequences that are identical to nanomeric Ad4 motifs found in the promoter region of mammalian aromatase. Using various 5' end deletions of the tilapia aromatase gene, we showed that the Ad4 motifs may be required for the aromatase gene expression. Gel shift assays revealed that *in vitro* translated tilapia Ad4BP/SF-1 and nuclear extracts from tilapia ovarian follicles formed complexes with oligonucleotide probes containing Ad4 motifs.

Then we examined changes in Ad4BP/SF-1 and aromatase gene mRNA levels during ovarian or testicular differentiation. Again, aromatase transcripts were expressed in XX gonads before ovarian differentiation but not in XY gonads. It is of interest to note that Ad4BP/SF-1 is expressed also in XX gonads prior to ovarian differentiation but not in XY gonads, strongly suggesting that Ad4BP/SF-1 acts as a transcriptional modulator for the tilapia aromatase gene expression.

This slide shows expression of vasa, Ad4BP and also aromatase genes. One day after hatching, primordial germ cells appear in the coelomic epithelium. By 3 days, cells, probably steroid-producing cells, appear near the blood vessel, which have both Ad4BP/SF-1 and aromatase positive. By 10 days these cells increase in number. These events are probably important for ovarian differentiation. Now I will move on to the mechanism of testicular differentiation.

In tilapia, as ovarian differentiation, testicular differentiation occurs in XY gonads at 20-25 days after hatching. But unlike ovarian differentiation, prior to testicular differentiation neither steroidogenic enzymes nor androgen receptors α and β were expressed in XY males.

We also examined expression of other genes such as Sox9, DAX1, etc, but there are no sex differences in gonads prior to or during or immediately after sex differentiation.

So far, I have discussed the important roles of estrogens and estrogen receptors in ovarian differentiation in XX genetic females. However, in genetic XY males neither androgen synthesizing enzymes nor androgen receptors are expressed prior to testicular differentiation. So what is important for testicular differentiation?

Then we came across a gene called DM domain gene. Probably you are familiar with this gene that has a DNA binding motif shared between Drosophila Doublesex and C. elelgans nematode mab-3. It is known that both Doublesex and Mab-3 play a key role in sex determination of fly and nematode.

These DM domain genes are known to be present in various vertebrate species including humans. We examined the expression of the DM domain genes, that is, DMRT1. In adult tilapia, DMRT1 is testis specific and also by *in situ* hybridization DMRT1 is expressed in testicular Sertoli cells. Then we examined DMRT1 expression by *in situ* hybridization in XX and XY gonads 15 days after hatching. DMRT1 expression was seen in somatic (Sertoli) cells of XY males, but not in XX females. Then we examined changes during sex differentiation, at 7, 20, 25 days. Even 7 days after hatching, 10 days to 2

weeks prior to gonadal sex differentiation, DMRT1 is expressed in XY genetic males and this expression was male-specific. Thus, DMRT1 expression is sex (male)-, stage (prior to testicular differentiation)-, and cell (Sertoli cell)-specific. We are now studying the mechanisms of DMRT1 gene expression and action, searching for target genes of DMRT1.

I will summarize what I have discussed so far. Specific DMRT1 expression in Sertoli cells, and estrogens (estrogen-synthesizing enzymes in steroid-producing cells) and estrogen receptors appear to be critical for testicular (XY males) and ovarian (XX females) differentiation, respectively. Also I indicated that this critical period prior to sex differentiation is very sensitive to various sex steroid hormones and environmental factors including endocrine disrupters. But to determine the effects of endocrine disrupters for example, we need to know the genetic sex of each individual (fish). It is important to determine whether the effects we see are genetic or epigenetic.

To do so, we need to know the sex-determining gene of the species concerned. As I already discussed, a sex-determining gene, SRY/Sry, which was first discovered in 1990 in humans, has only been reported in mammals. This SRY/Sry gene is located on the short arm of the Y chromosome, and its transient expression occurs in testis of 10.5-11.5 dpc. In nonmammalian vertebrates, however, no SRY/Sry or equivalent gene has been identified.

Recently we identified a strong candidate of sex-determining gene in medaka. As in mammals, sex determination in medaka is male heterogametic, although the Y chromosome is not cytogenetically distinct. To clone positionally the sex-determining gene, we generated a Y congenic strain to highlight the genetic differences between the X and Y chromosomes from inbred strains of medaka. 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 genomic library. Fluorescence *in situ* hybridisation using one of the BAC clones as a probe was used to determine the location of the sex-determining region on the Y chromosome.

Then using this congenic medaka we developed 2 DNA markers, DNA marker 1 and 2. The sex-determining gene is located between these DNA markers (the sex-determining region).

Shotgun sequencing was used to determine the sequence of this sex-determining region. We sequenced a total of 422,202 nucleotides and estimated the four BAC clones covered about 530 kb. Our computer analysis revealed that 52 predicted genes (PG) were present in these four BAC clones. We further narrowed to identify the sex-determining gene. Deletion analysis of the Y chromosome of a congenic XY females further shortened the region to 250 kb. Shotgun sequencing of this region predicted 27 genes. Three of these genes were expressed during sex differentiation. Among these 3 genes, only 1 gene, PG17, was Y specific. The remaining 2 genes were not Y specific and found in both XY males and XX females.

PG 17 spans 6 exons and encodes putative proteins of 267 amino acids, including the highly conserved DM domain. Again, the DM domain is Doublesex and Mab-3. We named this gene (PG17) DMY, DM domain gene on the Y chromosome. Then we examined detailed expression profiles of DMY as well as DMRT1 during gonadal sex differentiation of medaka. RT-PCR analysis revealed that DMY is expressed prior to and during testicular differentiation, followed by DMRT1 expression after testicular differentiation. *In situ* hybridization studies showed specific and strong expression of DMY in somatic cells (Sertoli cells) of XY gonads. Of course, DMY expression was never found in XX gonads.

As shown, DMY is located within the sex-determining region of Y chromosome, Y chromosome specific, and expressed in somatic Sertoli cells of XY gonads prior to and during sex differentiation, is a transcription factor with a DM domain. Therefore, DMY is a very strong candidate for the sex-determining gene of medaka. However, to conclude that DMY is the sex-determining gene of medaka,

we have to provide evidence produced by both gain-of-function and loss-of-function studies. We are now doing gain-of-function that DMY can induce male development in XX medaka.

The analysis of two naturally occurring XY females from two separate populations (Awara and Shirone) established the critical role of DMY in testicular differentiation. One of these mutants contained a single 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 non-functional, 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.

These are normal XX female and XY male. The female is white, while the male is yellow. The Awara mutant (XY) has male body color, but the anal fin is that of normal genetic XX females. Of course XX has ovary, XY testis. How about Awara mutant? The Awara mutant has ovary. So it is a complete sex change.

We also looked at whether other medaka have DMY. We found Hainan medaka have DMY. Then we looked at the phylogenetic tree of DMY and DMRT1 from Japanese and Hainan medaka. We found that DMY is derived from DMRT1 just prior to the speciation of these 2 medaka species.

Finally, human sex chromosomes and medaka sex chromosomes are compared. Mammal X and Y chromosomes differ in size and gene content. The human 165-Mb human X contains about 1500 genes. The Y chromosome is smaller and almost devoid of genes. The 60-Mb human Y contains only about 50 functional genes. At least a half of these Y-borne genes are specialized for sex and spermatogenesis. In contrast, as I have drawn here, medaka X and Y chromosomes are almost identical.

Both Sry and DMY are located on the Y chromosome, but importantly, no sequence homology at all. They are transcriptional factors, as Sry has the HMG box and DMY has the DM domain, and both are expressed in a certain stage of a critical period at the Sertoli cell.

Many interesting questions still remain to be answered in future studies. DMY's generality among vertebrate species? Is DMY the sex-determining gene only in medaka? If so, how about other vertebrates? DMY-induced cascade of sex differentiation? Evolution of Y chromosomes in various species of vertebrates? All of these studies are very important and will certainly provide basic information to understand the molecular mechanisms underlying sex determination and differentiation in vertebrates in general, as well as to determine the mechanisms of action of endocrine active chemicals on gonadal sex differentiation that is characterized by a well defined estrogen sensitive period of development.

I would like to thank my colleagues for sex determination. My postdoc Matsuda with his family; and particularly some of the work I described today for sex determination is a collaboration with 2 professors in Niigata University, that is Drs. Sakaizumi and Hamaguchi. And for gonadal sex differentiation I thank Assistant Prof. Toru Kobayashi and others, and also I thank Prof. M. Nakamura who is here from Ryukyu University for tilapia sex differentiation.

Finally, I would like to acknowledge support from the JSPS, Ministry of the Environment, and CREST. Thank you very much.

Q&A

Sakuma: Thank you very much Prof. Nagahama. Now Professor Nagahama's presentation is open for question and comments. We have time for just a few questions. Please come forward.

Q: You said in medakas there is not much difference between the X chromosome and the Y chromosome. Actually, in the first place, how can you distinguish between them at the beginning of your work? Morphologically they are very similar, right? So how can you say medaka?

Nagahama: We are still in the process of detailed analysis of gene structures of X and Y chromosomes. In our studies to identify the sex-determining gene of medaka, we generated a Y congenic strain to highlight the genetic differences between X and Y chromosomes from inbred strains of medaka. In this congenic strain, a sex-linked pigment gene is located only on the Y chromosome.

Q: I see, thank you. Another question: in mammals, generally speaking, estrogen receptor α is basically a receptor for the general sex hormone action for females. But when I look at the literature in fish, on some occasions, the expressions of estrogen receptor α and β are very different. For example, estrogen receptor β in some cases is very high in almost all tissues or something like that. Do you know the physiological differences between the estrogens α and β in fish?

Nagahama: I really don't know. We have been examining the expression of estrogen receptors only in gonads. As I mentioned in my talk, the expression profiles of estrogen α and β appear to be different in XX gonads during sex differentiation, suggesting their respective functions. It is important to examine the detailed expression pattern of these two estrogen receptors in ovarian follicles during various stages of development and maturation.

Sakuma: I am sorry to interrupt your discussion, but time is up and every officer here is very nervous about finishing this session. I thank you very much for the elegant presentation, Prof. Nagahama and everyone else, particular thanks to the people on the floor staying so late at night and committing their time for this valuable meeting. Thank you very much for your cooperation.