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Molecular cloning and analysis of gonadal expression of *Foxl2* in the medaka, *Oryzias latipes*

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Abstract

Foxl2 is a member of the winged helix/forkhead family of transcription factors and is known to be involved in ovarian development in some vertebrates. To address the role of *Foxl2* in ovarian differentiation in medaka, we isolated *Foxl2* cDNA and analyzed its expression patterns during sex differentiation. Expression of *Foxl2* started in somatic cells surrounding germ cells in XX gonads, just after initiation of ovarian differentiation, and was maintained in granulosa cells throughout ovarian development. In the adult ovary, *Foxl2* was expressed in previtellogenic and vitellogenic follicles, but expression ceased in postvitellogenic follicles. In contrast, *Foxl2* mRNA could not be detected in testes. In addition, *Foxl2* and *aromatase* mRNAs were co-localized in some somatic cells located on the ventral side of developing XX gonads. Our results suggested that *Foxl2* was not involved in ovarian determination, but was involved in differentiation of granulosa cells in medaka.

Keywords: Foxl2; Aromatase; Sex differentiation; Ovarian development; Granulosa cell; Folliculogenesis; Teleost; Medaka

The teleost fish medaka, *Oryzias latipes*, contains the genetic XX–XY sex-determining system like mammals [1]. In medaka, *DMY* was found to be a prime candidate of Y-linked testis determining genes [2,3]. Although, many vertebrates also have XX–XY sex-determining systems, medaka is the only non-mammalian vertebrate with an identified testis determining gene. *DMY* is specifically expressed in males, in somatic cells of primordial gonads surrounding primordial germ cells (PGCs) which are probably precursors of Sertoli cells, during testicular differentiation [4]. Expression of *DMY* initiates at stage 36, corresponding to complete formation of primordial gonads. At stage 38, shortly after onset of *DMY* expression, the first morphological difference between male and female gonads is observed [4]. On the other hand, genes involved in female sex differentiation remain completely unknown. In general, it has been considered that estrogen plays critical roles in sex differentiation and subsequent ovarian development in teleost fish. However, in medaka, *aromatase*, the key enzyme for estrogen synthesis, is expressed in female gonads after oogenesis [5]. Moreover, in *Scl* mutants, in which sex steroids are absent, early oogenesis proceeds normally [6]. Therefore, estrogen is not involved in early female sex differentiation in medaka, and there must be other factor(s) involved in the process.

Foxl2 is a putative winged helix/forkhead transcription factor involved in ovarian development. In humans, mutations in *Foxl2* cause blepharophimosis/ptosis/

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epicanthus inversus syndrome (BPES) type I [7]. BPES is characterized by malformations of the eyelid and premature ovarian failure (POF). It is known that in some vertebrates, expression of *Foxl2* is specifically initiated before morphological sex differentiation in female gonads and is maintained throughout ovarian development [8–13]. Thus, to date, *Foxl2* is the earliest known gene showing sex dimorphic expression patterns in ovarian somatic cells, suggesting that *Foxl2* may be involved in basic processes of female sex differentiation that are conserved through evolution among vertebrates.

However, functions of *Foxl2* in early ovarian differentiation have yet to be clarified. In goats, Polled Intersex Syndrome (PIS) mutation is associated with absence of horns and female to male sex-reversal in XX animals. The PIS mutation is characterized by a deletion of an 11.7-kb *cis*-regulatory element and affects transcription of at least three genes; i.e., *Foxl2*, *PISRT1*, and *PFOXic* [9,14]. In XX PIS mutants, gonadal sex reversal occurs at a very early stage of gonadal development, and expression of *Foxl2* and *aromatase* is reduced at the stage when the first morphological difference between male and female gonads appears [9,15]. In chick, estrogen is critical for female gonadal sex differentiation [16]. The expression profile of *Foxl2* highly correlates with that of *aromatase* in chick gonads during sex differentiation. Both *Foxl2* and *aromatase* co-localize in the medullar part of the ovary [11]. These results suggest that *Foxl2* is involved in regulation of estrogen synthesis via transcriptional regulation of *aromatase* during ovarian development.

On the other hand, estrogen is not involved in early ovarian differentiation in mice. In knockout mice of aromatase, estrogen receptor-α, estrogen receptor-β, or both receptors, folliculogenesis proceeds normally up to the large antral stage [17–21]. Knockout mice of *Foxl2* are not affected in early sex differentiation, and female to male sex reversal does not occur, but differentiation of granulosa cells is blocked at the squamous to cuboidal transition, followed by follicle degeneration [22,23]. Moreover, genes involved in testis differentiation such as *Sox9* are upregulated after birth, in *Foxl2* null mice [24]. These results suggest that *Foxl2* has a critical role in granulosa cell differentiation, and its function does not depend on estrogen.

In this study, to investigate whether *Foxl2* was involved in ovarian differentiation and development in medaka as in other vertebrates, we isolated medaka *Foxl2* cDNA and analyzed its expression patterns in gonads during sex differentiation by RT-PCR and in situ hybridization. In addition, to investigate whether *Foxl2* was regulated by estrogen, we analyzed expression of *Foxl2* during estrogen-mediated male to female sex reversal. Our results suggested that *Foxl2* was not involved in the first morphological difference between males and females, but might be involved in granulosa cell differentiation and ovarian development in medaka.

Materials and methods

Fish. The Hd-rR/SIN-A inbred strain of medaka, *Oryzias latipes*, was used for this study. Fish were maintained in aquaria under an artificial photoperiod of 16L:8D at 27 ± 2 °C. Embryos were staged using morphological criteria by Iwamatsu [25]. Genetic sex was identified with a PCR-based strategy according to a previous report [26]. Estradiol-17β (E_2) treatments were carried out as previously described [27]. Eggs were incubated in estrogen-containing aged tap water at a concentration of 0.2 μg/ml from 1 day after fertilization to hatching (F7d). Hatched larvae were transferred to normal tap water and were fed with commercial pet food.

cDNA cloning of medaka Foxl2. To clone the medaka *Foxl2* ortholog, we searched available medaka genome databases (http://dolphin.lab.nig.ac.jp/medaka/) using the BLAST program for primer design. The designed primers mFoxl2-F (GCG ATG ATG GCC ACT TAC CAA AG) and mFoxl2-R (TTA AAT ATC AAT CCT CGT GTG CAA AGC) were used to amplify a 921-bp *Foxl2* cDNA fragment from ovaries. To obtain full-length cDNA sequence of *Foxl2*, we have made cDNA library from adult ovary using the Creator™ SMART™ cDNA library construction kit (BD biosciences). This system is useful for obtaining enriched full-length cDNAs by Long-Distance PCR and the cDNAs have asymmetrical *Sfi*I restriction enzyme sites at the 5′ and 3′ ends. LD-PCR products were digested with *Sfi*I and purified using MicroSpin S-400 HR columns (GE Healthcare) and ligated with SfA vectorette adapter (BPBI, 5′ phosphorylated CAA GGA GAG GAC GCT GTC TGT CGA AGG TAA GGA ACG GAC GAG AGA AGG GAG AG, and BPHIISfA, 5′ CTC TCC CTT CTC GAA TCG TAA CCG TTC GTA CGA GAA TCG CTG TCC TCT CCT TGT TA) for 5′ end template, and with SfB vectorette adapter (BPBI and BPHIISfB, CTC TCC CTT CTC GAA TCG TAA CCG TTC GTA CGA GAA TCG CTG TCC TCT CCT TGC CT) for 3′ end template, respectively. These linkers were designated based on the vectorette PCR method, which are used for the isolating of end-probes from BAC and cosmid clones [28]. Then vectorette PCR, using 5′ template, was performed with 224 (CGA ATC GTA ACC GTT CGT ACG AGA ATC GCT) and mFoxl2-R primers to obtain 5′ end of cDNA sequence, whereas vectorette PCR, using 3′ template, was performed using 224 and mFoxl2-F primers to obtain 3′ end of cDNA sequence. The amplified fragments were subcloned using a TOPO TA Cloning kit for Sequencing (Invitrogen) in pCR4-TOPO vectors and were sequenced on both strands.

RT-PCR. Total RNA was extracted from adult gonads using an RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized from 50 ng of total RNA in a 20-μl reaction using Sensiscript (Qiagen) with oligo-dT primers. PCR was carried out in 25-μl reaction mixtures containing 2 μl of first-strand cDNA. PCR conditions were 5 min at 95 °C; 30–35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 60 s at 72 °C; and 7 min at 72 °C. A pair of Foxl2 gene-specific primers (mFoxl2-F and mFoxl2-R) was used to amplify 921-bp cDNA fragments by PCR. As an internal control, the *ef-1*α (GenBank Accession No. AB013606) primers, i.e., ef1-F (TCC ACC TCC ACC GGT CAC CT) and ef1-R (CAC CAA CGC CAG CAG CGA CGA T) were used to amplify a 304-bp cDNA fragment.

Histological observations. For histological observations, specimens were fixed in Bouin's solution, embedded in paraffin, sectioned serially at 5 μm, and stained with hematoxylin and eosin.

In situ hybridization. Sense and antisense digoxigenin-labeled RNA probes were generated using in vitro transcription with a DIG RNA labeling kit (Roche). *Foxl2* RT-PCR products were obtained using the primers Foxl2-RT-f (AGA GGG GGG CTG GAC TTC GTC TG) and Foxl2-RT-r (GGG TTT TTA CGC AGA CGG AAA ACT T), and were cloned into pCR4-TOPO vectors as probe templates. A probe for *aromase* (D82969) was made as described previously [5]. Specimens were fixed in 4% paraformaldehyde in 0.85× phosphate-buffered saline (PBS) at 4 °C overnight. After fixation, tissues were embedded in paraffin, and 5-μm cross-sections were cut. At least eight specimens were prepared at and earlier than hatching day, and five specimens were prepared at later stages. For in situ hybridization,

sections were deparaffinized, hydrated, and treated with 4 mg/ml proteinase K (Roche) at 37 °C for 5 min, and then hybridized with sense or antisense DIG-labeled RNA probes at 60 °C for 24 h. Hybridization signals were then detected using alkaline phosphatase-conjugated anti-DIG antibody (Roche) and NBT/BCIP (Roche) as chromogen. For two-color in situ hybridization, fluorescein isothiocyanate (FITC)-labeled and DIG-labeled probes were used. Alkaline phosphatase-conjugated anti-DIG antibody (Roche) was used for the first detection with NBT/BCIP. Following staining with the first dye, alkaline phosphatase was inactivated by incubating samples in 0.1 M glycine–HCl, pH 2.2, at room temperature for 10 min. Then, specimens were subjected to alkaline phosphatase-conjugated anti-FITC antibody (Roche) for the second detection with INT/BCIP (Roche).

Results

Molecular cloning of medaka Foxl2

To isolate medaka *Foxl2* ortholog, we performed a BLAST search of medaka genome databases and obtained a partial sequence of medaka *Foxl2*. Then, we screened cDNA library from adult ovary to obtain full-length cDNA of medaka *Foxl2*. The isolated medaka *Foxl2* was 1166-bp long, with a predicted ORF encoding a protein of 306 amino acids (GenBank Accession No. AB252055).

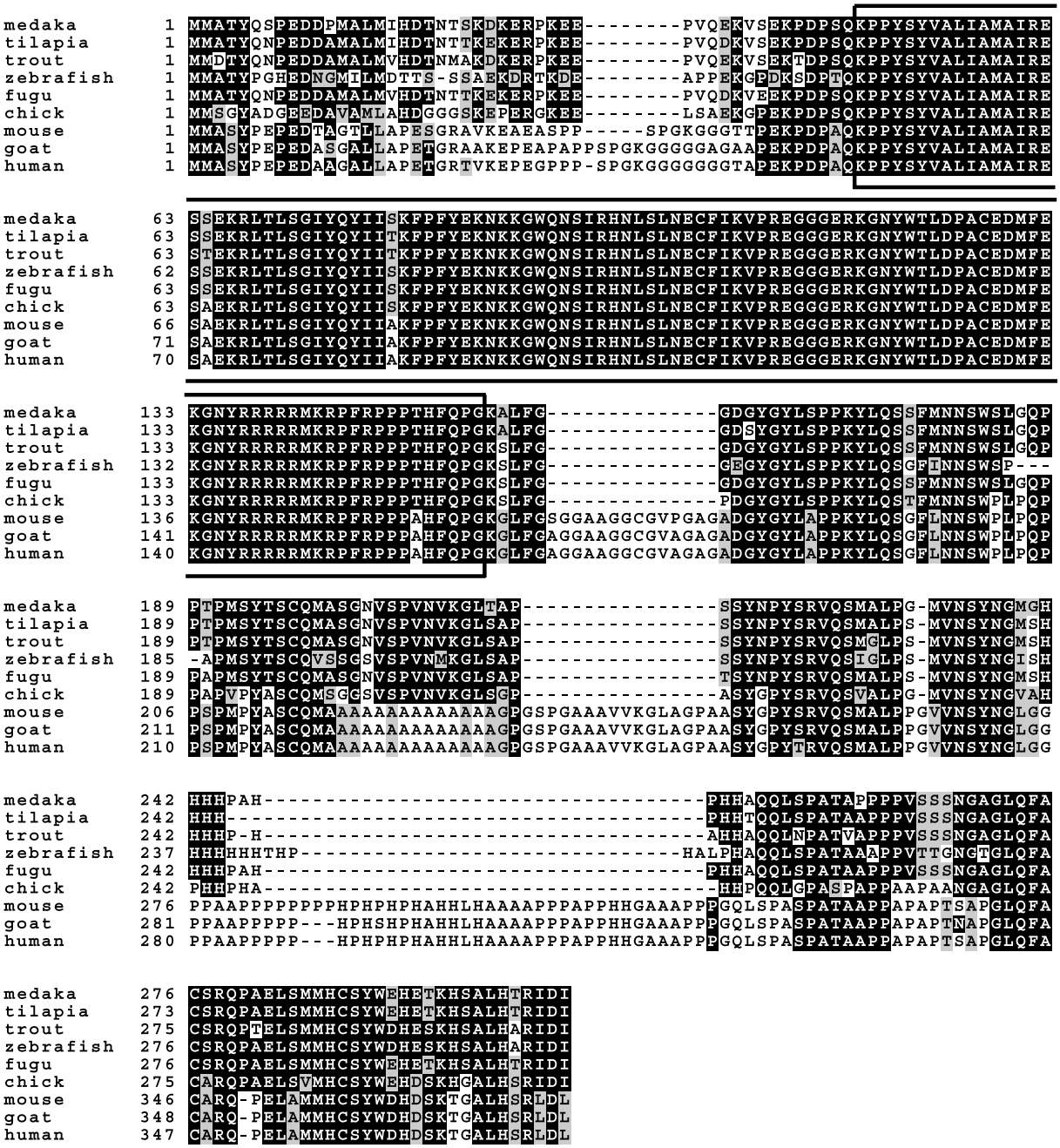

Fig. 1. Alignment of the predicted amino acid sequence of medaka Foxl2 with that of other vertebrate Foxl2. Sequences were aligned using ClustalW. The Forkhead domain is boxed. For GenBank accession numbers, please refer to legends in Fig. 2. BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) was used to make this figure.

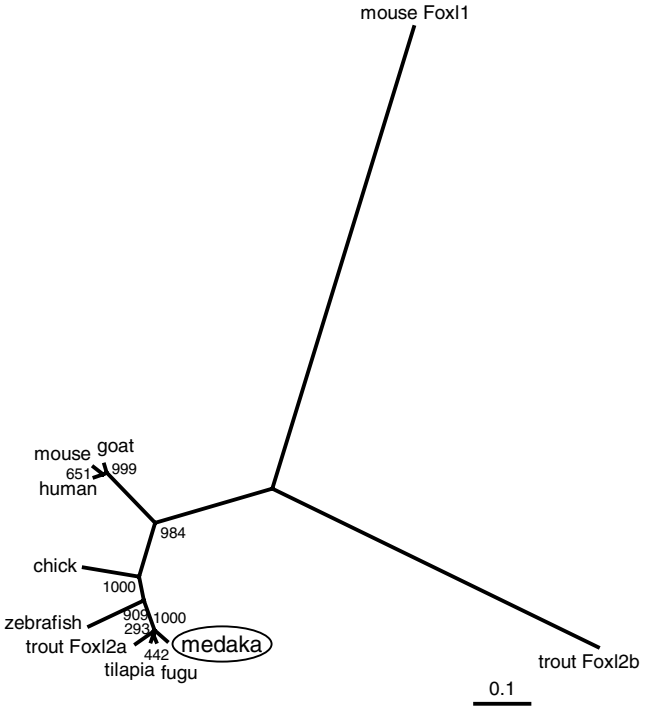

Fig. 2. Unrooted phylogenetic tree calculated by the neighbor-joining method using amino acid sequences of vertebrate Foxl2 proteins. Bootstrap values (1000 resamplings) are indicated by numbers. Accession numbers for the sequences are as follows: mouse Foxl2 (AF522275); human FOXL2 (AF301906); goat Foxl2 (AY112725); chick Foxl2 (AY487165); zebrafish Foxl2 (XM_693823); rainbow trout Foxl2a (AY507927); rainbow trout Foxl2b (AY507926); tilapia Foxl2 (AY554172); fugu Foxl2 (CAAB01001061); and mouse Foxl1 (NM_008024).

Similar to other vertebrates' *Foxl2*, medaka *Foxl2* was a single-exon gene. Alignment of the predicted Foxl2 amino acid sequence with those of other teleost fish, chick, and mammals indicated that the Forkhead domain and the C-terminal region were highly conserved (Fig. 1). A phylogenetic tree was constructed using a neighbor-joining method, and revealed that medaka *Foxl2* fitted within the same branch of other species' *Foxl2* (Fig. 2).

Medaka Foxl2 is expressed in granulosa cells throughout ovarian development

To assess expression of *Foxl2* in adult gonads, we examined expression of *Foxl2* by RT-PCR and in situ hybridization. Using RT-PCR, a strong expression of *Foxl2* mRNA was detected in ovaries, but not in testes (Fig. 3A). To determine cell types expressing *Foxl2*, we analyzed expression of *Foxl2* using in situ hybridization. In ovaries, *Foxl2* mRNA was detected in follicular cells of previtellogenic and vitellogenic follicles, but was not detected in mature follicles (Fig. 3E). In testes, *Foxl2* mRNA was not detected as expected (Fig. 3D).

To investigate whether *Foxl2* was involved in ovarian determination and ovarian development in medaka, we next examined expression patterns of *Foxl2* during gonadal development using in situ hybridization. At and earlier than stage 38, expression of *Foxl2* was not detected in both XY and XX gonads while the first morphological difference between male and female gonads appeared at stage 38 in the medaka Hd-rR strain [4] (Figs. 4C and D). At hatching day (H0d) when germ cells just enter meiosis in XX gonads, *Foxl2* mRNA was detected in somatic cells surrounding germ cells in almost half of XX specimens, but not in any XY gonads (Figs. 4G and H). Five days after hatching (H5d) and 10 days after hatching (H10d), expression of *Foxl2* was detected in all female specimens, especially in somatic cells surrounding oocytes throughout the entire gonads (Figs. 4K and 5C). Subsequently, 30 days after hatching (H30d), *Foxl2* mRNA was detected in follicular cells of previtellogenic follicles in XX gonads (Fig. 5G). No signal was detected in XY gonads throughout this period.

Foxl2 and aromatase are co-localized in XX gonads

A previous study showed that expression of *aromatase* was initially detected on the ventral side of XX gonads from H4d to H10d [5]. As mentioned above, the first appearance of *Foxl2* mRNA was detected in somatic cells surrounding germ cells in XX gonads from H0d to H5d. To investigate whether *Foxl2* and *aromatase* were expressed in the same cell linages, we compared expression patterns of *Foxl2* and *aromatase* at H10d using double labeling in situ hybridization. Double staining of *Foxl2* and *aromatase* showed co-localization of signals in somatic cells located on the ventral side of XX gonads (Fig. 5I). However, in other regions, somatic cells only expressed *Foxl2*. In contrast, no cells were found that expressed *aromatase* only.

Expression of Foxl2 is upregulated during estrogen-mediated sex reversal

To investigate expression of *Foxl2* during exogenous-estrogen-mediated male to female sex reversal, we examined expression of *Foxl2* using in situ hybridization in medaka larvae that had been incubated in E_2-containing water at a concentration of 0.2 μg/ml from 1 day after fertilization to hatching (7 days after fertilization). At stage 38, expression of *Foxl2* was not detected in both E_2-treated XY and XX gonads (Figs. 6A and B; control untreated gonads are shown in Fig. 4C). Later, at H5d, expression of *Foxl2* was detected in E_2-treated XY gonads in somatic cells surrounding germ cells as in E_2-treated XX gonads (Figs. 6C and D; control untreated gonads are shown in Fig. 4K).

Discussion

Foxl2 is involved in granulosa cell differentiation

In medaka, at stage 36, PGCs reach the future gonadal region, and formation of primordial gonads is complete. At this stage, expression of *DMY* initiates in somatic cells

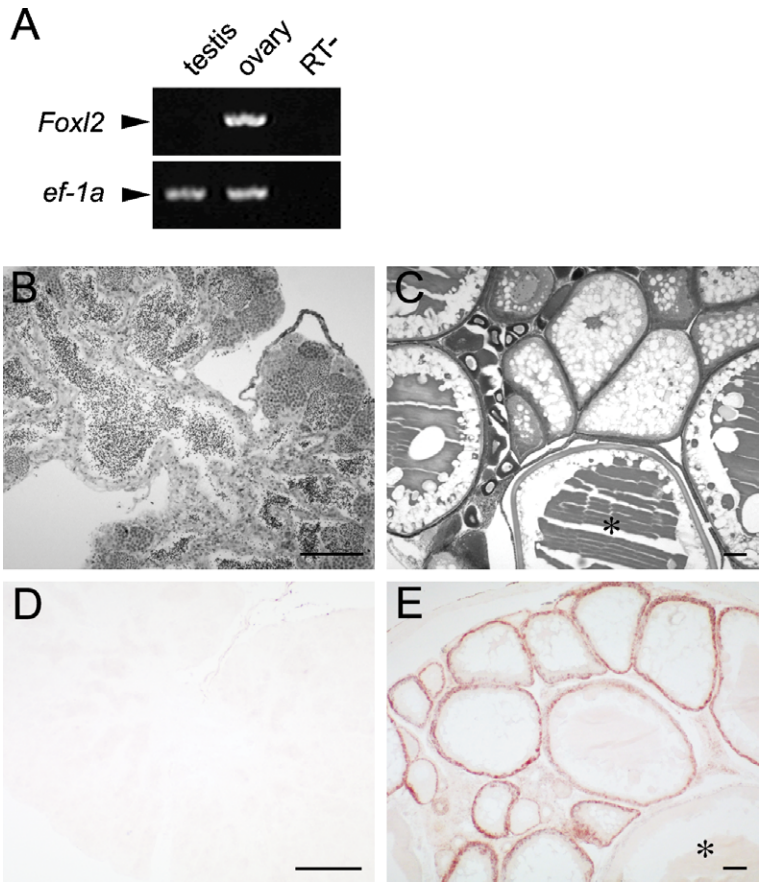

Fig. 3. Expression of *Foxl2* in medaka adult testes and ovaries. (A) RT-PCR analysis of *Foxl2* mRNA in medaka adult testes and ovaries. *ef-1α* was used as an internal control. (B,C) Hematoxylin/eosin-stained sections of medaka adult testes (B) and ovaries (C). (D,E) In situ hybridization for *Foxl2* in medaka adult testes and ovaries. No signal was detected in adult testes (D). *Foxl2* mRNA was detected in follicular cells of previtellogenic and vitellogenic follicles, but not in mature follicles in adult ovaries (E). Asterisk: mature follicles. Scale bars: 100 μm.

surrounding PGCs in XY primordial gonads [4]. At stage 38, the first morphological difference between males and females is the number of germ cells. The number of germ cells in female gonads is larger than that in male gonads [4]. Germ cells in females continue to proliferate and then enter into meiosis just after hatching [4,29].

Foxl2 is expressed in somatic cells in XX gonads from hatching day in medaka, and these *Foxl2* positive cells are assumed to differentiate into granulosa cell lineages. At hatching day, we found that only half of XX specimens expressed *Foxl2*, suggesting that expression of *Foxl2* was very weak at this stage. Thus, expression of *Foxl2* initiated around hatching day. These results indicate that initiation of *Foxl2* expression followed the first morphological difference between male and female gonads. Therefore, *Foxl2* is not an ovarian determining gene in medaka. On the other hand, these results show the possibility that onset of meiosis in germ cells induced expression of *Foxl2* in somatic cells or vice versa. Further studies are necessary to reveal relationships between meiotic germ cells and expression of *Foxl2* in surrounding somatic cells.

In mouse, chick, and turtle, expression of *Foxl2* specifically starts in female gonads around the time of sex determination [10,11]. It is well conserved among vertebrates that expression of *Foxl2* starts before folliculogenesis. Although function of *Foxl2* at that stage remains unknown, conservation of expression patterns of *Foxl2* suggests that *Foxl2* has a basic function in granulosa cell differentiation.

As mentioned above, in medaka, *Foxl2* was continuously expressed in the granulosa cell linage throughout ovarian development. Thereafter, in postvitellogenic mature follicles, expression of *Foxl2* disappears. In mouse, *Foxl2* mRNA is expressed in small and medium follicles, but not in preovulatory antral follicles and the corpus luteum [30]. Moreover, *Foxl2* represses transcription of *StAR*, a marker of granulosa cell differentiation in preovulatory follicles [30]. Thus, the limited expression profile of *Foxl2* mRNA during the vitellogenic phase to the premature phase in medaka well corresponds to that in mouse. These results suggest that *Foxl2* possibly acts as a suppressor of progression of follicles from the growing phase to the mature phase.

Foxl2 is involved in the regulation of aromatase expression

In chick, it has been suggested that *Foxl2* is involved in regulation of *aromatase* transcription during early sex

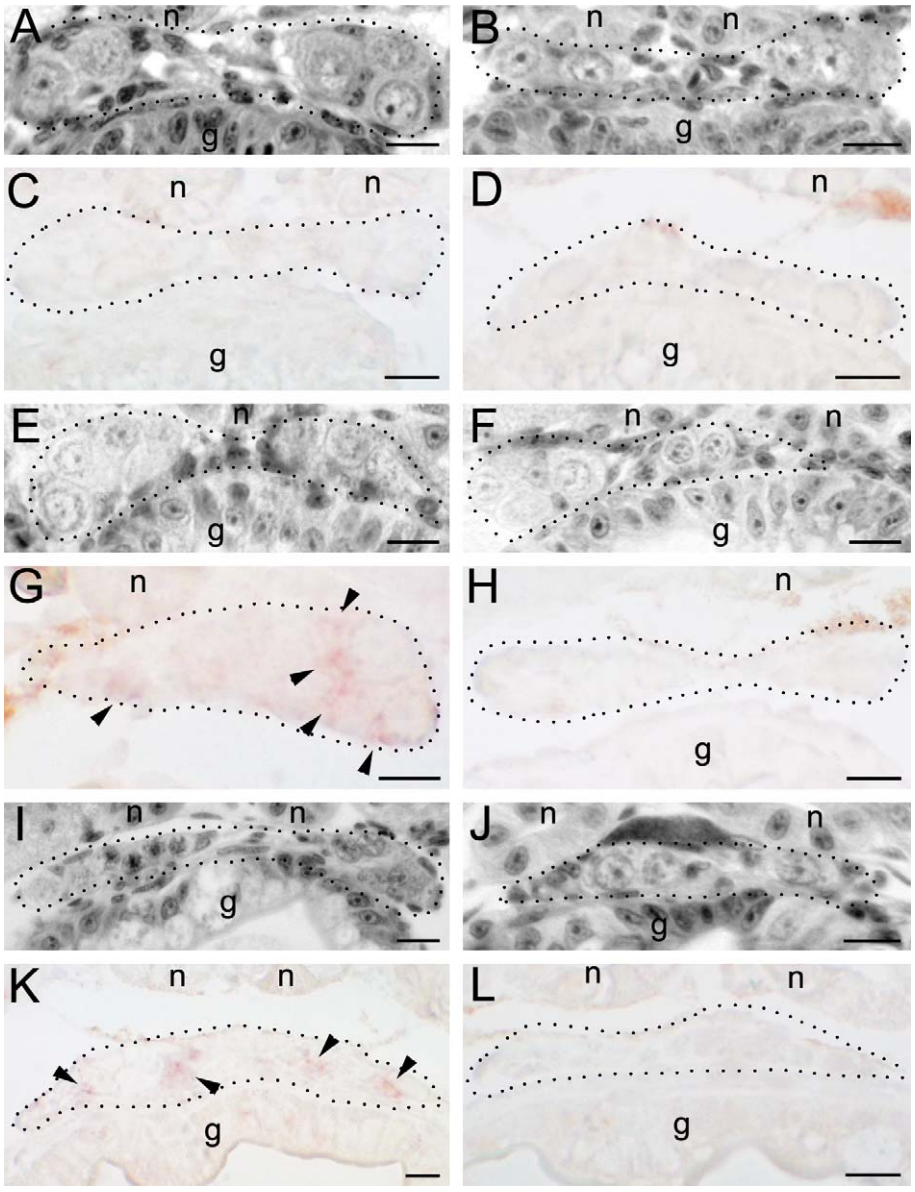

Fig. 4. Expression patterns of medaka *Foxl2* during early gonadal differentiation. Stage 38 XX (A,C), stage 38 XY (B,D), H0d XX (E,G), H0d XY (F,H), H5d XX (I,K), and H5d XY (J,L) are shown. H/E-stained sections (A,B,E,F,I, and J); in situ hybridization of *Foxl2* mRNA (C,D,G,H,K, and L). *Foxl2* is present in somatic cells surrounding germ cells in XX gonads from H0d (G). The arrowhead indicates positive signals of *Foxl2*. Dotted lines indicate the outline of the gonad. n, pronephric duct; g, gut. Scale bars: 10 μm.

differentiation [11,31]. Our results showed that onset of *Foxl2* expression was earlier than that of *aromatase*. Double staining of *Foxl2* and *aromatase* showed that *Foxl2* and *aromatase* were co-expressed in some somatic cells located on the ventral side of XX gonads. These results suggested that *Foxl2* was involved in initiation of *aromatase* transcription during early ovarian differentiation in medaka as in chick. However, not all *Foxl2*-positive cells eventually expressed *aromatase*, and there was a significant delay from onset of expression of *Foxl2* to that of *aromatase*. These expression profiles indicate that expression of *Foxl2* alone was insufficient for *aromatase* expression, and other specific factor(s) might be required.

Exogenous estrogen does not directly induce expression of Foxl2

In medaka, sex reversal of XY to functional females can be induced by estrogen treatment during the embryonic period [32]. Under estrogen-induced male to female sex reversal conditions, effects on proliferation of germ cells are observed from H3d, and effects in oocytes are also observed from H3d in XY gonads, suggesting that the testis differentiation pathway is initiated as usual, then secondary sex reversal occurs after hatching in E_2-treated XY embryos [27]. Under the same sex reversal conditions, we observed that expression of *Foxl2* was not detected at stage 38. Therefore, exogenous E_2 does not directly induce

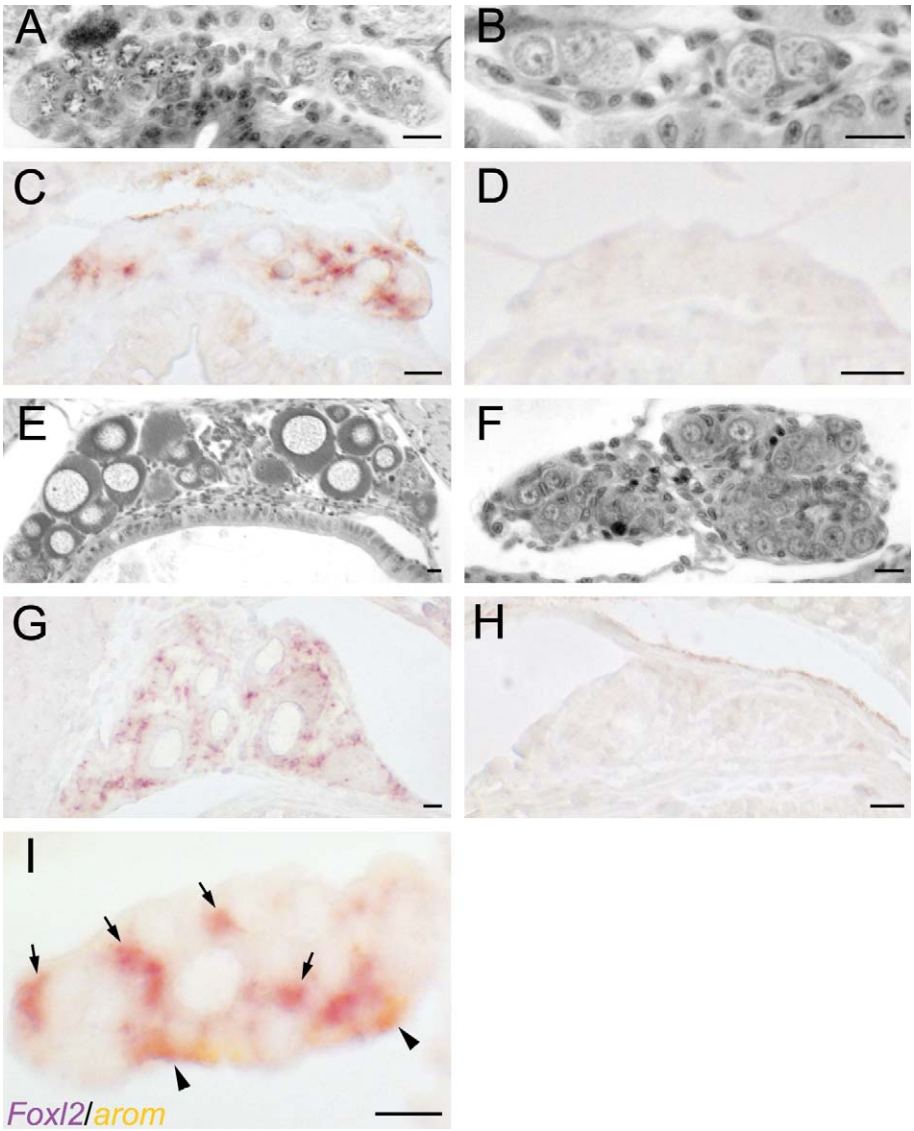

Fig. 5. (A–H) Expression patterns of *Foxl2* during gonadal differentiation. H10d XX (A,C), H10d XY (B,D), H30d XX (E,G), and H30d XY (F,H) are shown. H/E-stained sections (A,B,E, and F); in situ hybridization of *Foxl2* mRNA (C,D,G, and H). Expression of *Foxl2* was detected in somatic cells surrounding oocytes at H10d (C) and in follicular cells of previtellogenic follicles at H30d (G). No signal was detected in XY gonads throughout this period (D,H). (I) Double labeling in situ hybridization of *Foxl2* and *aromatase* in H10d XX gonads. *Foxl2* and *aromatase* were co-expressed in somatic cells located on the ventral side of XX gonads. Arrows indicate cells expressing *Foxl2* only. The arrowhead indicates position of both *Foxl2* and *aromatase*-positive cells. Scale bars: 10 μm.

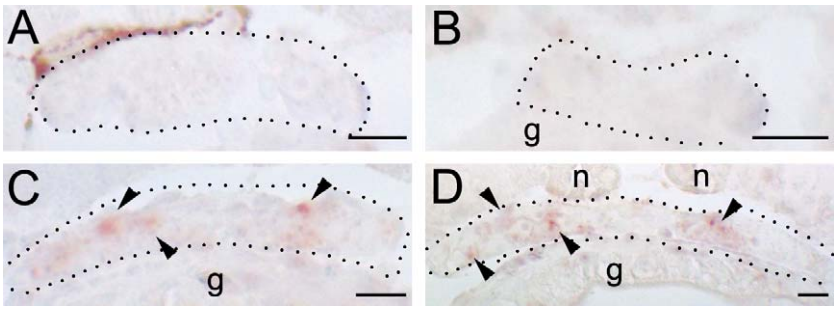

Fig. 6. Effects of E_2 on *Foxl2* expression. Expression of *Foxl2* in E_2-treated XY at stage 38 (A), in E_2-treated XX at stage 38 (B), in E_2-treated XY at H5d (C), and in E_2-treated XX at H5d (D). Expression of *Foxl2* was not detected at stage 38 in both E_2-treated XY and XX gonads (A,B); while at H5d, *Foxl2* mRNA was detected in sex reversed XY gonads as in XX gonads (C,D). The arrowhead indicates positive signals of *Foxl2*. Dotted lines indicate outlines of the gonad. n, pronephric duct; g, gut. Scale bars: 10 μm.

expression of *Foxl2*. Rather, our results suggest that after the ovarian differentiation pathway was somehow activated by exogenous E_2 following hatching, expression of *Foxl2* was induced by unknown factors involved in the pathway. This was also supported by the fact that *Foxl2* was expressed in E_2-treated XY gonads at H5d, shortly after the earliest phase of sex reversal.

In conclusion, our results indicated that *Foxl2* was not an ovarian determinant, but the gene was expressed just after initiation of ovarian differentiation, suggesting that *Foxl2* might play a critical role in differentiation of granulosa cell linages, and its function might be conserved throughout evolution among vertebrates.

Acknowledgments

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