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The germline-specific expression of *Foxl3a* and its paralogous *Foxl3b* are associated with male gonadal differentiation in the Japanese eel, *Anguilla japonica*

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ABSTRACT

Unlike its paralog *Foxl2*, which is well known for its role in ovarian development in vertebrates, the function of *Foxl3* is still unclear. *Foxl3* is an ancient duplicated copy of *Foxl2*. It is present as a single copy in ray-finned fish. But, due to repeated losses, it is absent in most tetrapods. Our transcriptomic data, however, show that two *Foxl3*s (*Foxl3a* and its paralog *Foxl3b*) are present in Japanese eel. *Foxl3a* is predominantly expressed in the pituitary, and *Foxl3b* is predominantly expressed in the gills. Both *Foxl3*s show a sex-dimorphic expression, being higher expression in testes than in ovaries. Moreover, *Foxl3a* and *Foxl3b* were exclusively expressed during gonadal differentiation in control eels (100% male). Conversely, *Foxl3a* and *Foxl3b* significantly decreased after gonadal differentiation in E2-treated eels (100% female). Furthermore, in accordance the difference in adhesive ability between somatic cells and germline cells in testes, *Foxl3*s showed a high expression in suspension cells (putative germline cells) and low expression in adhesive cells (putative somatic cells). *In situ* hybridization further showed that *Foxl3a* and *Foxl3b* were expressed in the testicular germline cells. In addition, *Foxl3*s expression was not changed by sex steroids in *in vitro* testes culture. Taken together, our results suggest that the teleost-specific *Foxl3* paralog was repeatedly lost in most fish after the third round of whole genome duplication. The two germline-expressed *Foxl3*s had higher expression levels in males than in females during gonadal differentiation in Japanese eel. These results demonstrated that *Foxl3*s might play an important role in germline sexual fate determination from ancient fish to modern fish.

1. Introduction

Sex determination is a rapidly evolving trait in many lineages of animals, and closely related species sometimes have different modes of sex determination (Bachtrog et al., 2014). In teleost fish, sex can be determined by genetic factors (genetic sex determination), environmental factors (environmental sex determination), or both (Devlin and Nagahama, 2002). Unlike the sex-determining gene *Sry* in mammals and *Dmrt1* in birds, which are conserved, there are a variety of sex-determining genes in fish (Kikuchi and Hamaguchi, 2013). For example, several *Dm*-domain genes are involved in sex determination, including a Y-linked duplicate of *Dmrt1* (*Dmy*) in Japanese medaka (*Oryzias latipes*) (Hornung et al., 2007; Matsuda et al., 2002) and a dosage-dependent *Dmrt1* in Chinese tongue sole (*Cynoglossus semilaevis*) (Chen et al., 2014; Cui et al., 2017). Moreover, several genes in the Amh signaling pathway are involved in sex determination, including a Y-linked duplicate of *Amh* (*Amhy*) in Patagonian pejerry (*Odontesthes hatcheri*) (Hattori et al., 2012) and Nile tilapia (*Oreochromis niloticus*) (Li et al., 2015) and a mutation in *Amhr2* in pufferfish (*Takifugu rubripes*) (Kamiya et al., 2012). In addition, several Y-linked duplicated genes are observed in fish, including *Sdy* in rainbow trout (*Oncorhynchus mykiss*) (Yano et al., 2012), *Gsdfy* in Luzon ricefish (*Oryzias luzonensis*) (Myosho et al., 2012), and *Sox3y* in Indian ricefish (*Oryzias dancena*) (Takehana et al., 2014). All of these sex-determining genes are expressed in the soma of the gonads. Germ-cell-deficient fish typically develop as phenotypic males in medaka (Kurokawa et al., 2007) and zebrafish (*Danio rerio*) (Siegfried and Nusslein-Volhard, 2008), while pond loach fish (*Misgurnus anguillicaudatus*) (Fujimoto et al., 2010), goldfish (*Carassius*

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Table 1
Primers used in this study.

	Gene Orientation (fragment size)	Sequence	Analysis
Gapdh	Sense (nt 861–880)	5′-CACCCACTCCTCCATCTTTG-3′	qPCR
Gapdh	Antisense (nt 940–959)	5′-TTGCTGTAGCCGAACTCATT-3′	
Vasa	Sense (nt 384–404)	5′-CGTGATTCAGGTGACCCAGTT-3′	qPCR
Vasa	Antisense (nt 429–447)	5′-GCCCGTGGTGTTCAGGAA-3′	
Foxl3a	Sense (nt 698–718)	5′-ACTGTTGCCATAGCCATCTAC-3′	qPCR
Foxl3a	Antisense (nt 764–785)	5′-GTATACGGGCTACTGTTGTGAG-3′	
Foxl3b	Sense (nt 704–723)	5′-CAAATCACCTCCACCATCCA-3′	qPCR
Foxl3b	Antisense (nt 779–800)	5′-GTTTGAGTTATCCCAGCGTAGT-3′	
Amh	Sense (nt 843–863)	5′-TCCTGGTCAGCACTGCGTATC −3′	qPCR
Amh	Antisense (nt 886–902)	5′-TCCCGCACCGACAGACA-3′	
Foxl3a	Sense (nt −235- −217)	5′-CTGACCGCCACGTGAACCT-3′	Full cDNA
Foxl3a	Antisense (nt 931–952)	5′-CGCACAGAACCGAATGCATATT-3′	
Foxl3b	Sense (nt −127- −106)	5′-GCAATGCCCTGAAGTGTCTCTT-3′	Full cDNA
Foxl3b	Antisense (nt 975–955)	5′-CGCTGCGTTGAAAGCTTTTCA-3′	

auratus) (Goto et al., 2012), and black porgy (*Acanthopagrus schlegelii*) (Wu et al., 2012), which lack germ cells can develop as either phenotypic sex. These findings suggest that the sexual fates of soma and germline cells are regulated by multiple factors.

Foxl3 is an ancient duplicated copy of *Foxl2* that has been repeatedly lost and is absent in most tetrapods (Crespo et al., 2013; Geraldo et al., 2013). Unlike its paralog *Foxl2*, which is well known for its role in ovarian development in vertebrates (Li et al., 2013, Schmidt et al., 2004; Uhlenhaut et al., 2009), the function of *Foxl3* is still unknown. *Foxl3* is expressed in gonads and shows a higher level of expression in testes than in ovaries in Atlantic salmon (*Salmo salar*) (von Schalburg et al., 2011), European seabass (*Dicentrarchus labrax*) (Crespo et al., 2013), and ricefield eel (*Monopterus albus*) (Gao et al., 2016). Conversely, *Foxl3* occurs in the differentiating ovary while its expression remaining undetectable in males in rainbow trout (Baron et al., 2004). Furthermore, early germline expression of *Foxl3*/Foxl3 has only been observed in Japanese medaka in XX female fish after gonadal differentiation; no such expression has been found in germ cells of XY fish after gonadal differentiation in Japanese medaka (Nishimura et al., 2015). In chimeric medaka (with *Foxl3*$^{-/-}$ germline donor cells are transplanted into recipient XX females), *Foxl3* mutant germ cells initiated spermatogenesis in normal female gonads (Nishimura et al., 2015). Thus, *Foxl3* is involved in sexual fate determination in Japanese medaka (Nishimura et al., 2015). However, the role of *Foxl3* is different in various fishes. An understanding of the original function of *Foxl3* in ancient teleosts might help to explain the diversity of *Foxl3* expression pathways in different fishes.

In the present study, an ancient teleost, the Japanese eel (*Anguilla japonica*), is used to investigate *Foxl3* function during various stages of gonadal differentiation and development. This species is catadromous, with the adult eels living in freshwater and migrating into salt water to spawn. Before they mature, the immature eels are called yellow eels because of their golden pigmentation. During their migration into the ocean to spawn, the mature eels are called silver eels after the gold pigment disappears. Japanese eels spawn in the Philippine Sea. In this species, the sex ratio becomes more biased towards males under culture conditions than in the field conditions (Jeng et al., 2018; Satoh et al., 1992). To explain this, environmental factors, such as temperature and pH, as weel as eel density and social interactions, have been suggested to override genetic sex determination in eels (Davey and Jellyman, 2005). In addition, the period of gonad differentiation and development is related more to body size rather than to age, both in the field and in culture (Colombo and Grandi, 1996, Jellyman, 2001; Jeng et al., 2018; Oliveira and McCleave, 2000). In this study, we found two *Foxl3*s (*Foxl3a* and its paralog *Foxl3b*) in the Japanese eel. We investigated the development and expression pattern of both *Foxl3*s during gonadal differentiation in raised Japanese eels (all males). To better understand the ovarian development, an estradiol-17b (E2) treatment was conducted to feminizes, and the effect of E2 on the expression of both *Foxl3*s was also evaluated. In addition, the types of *Foxl3*-expressing cell were determined by *in vitro* primary cell culture of testes (adhesion cells is correlated to somatic cells and suspension cells correlated to germ cells) and *in situ* hybridization. The effects of androgen (11-ketotestosterone, 11-KT) and E2 on the expression of both *Foxl3*s were also evaluated by *in vitro* culture of testicular tissues. Our results demonstrated that germline-expressed *Foxl3*s are involved in gonadal differentiation in Japanese eel.

2. Material and methods

2.1. Animals and experimental design

Elvers of Japanese eel (body length = 9.54 ± 0.96 cm; body weight = 0.88 ± 0.34 g) were purchased from a commercial eel dealer in Pingtung in southern Taiwan and transferred to the culture station of National Kaohsiung Marine University, Taiwan. Yellow eels (immature eels) were purchased from a commercial eel dealer in Changhua in central Taiwan and transferred to the culture station of National Taiwan Ocean University. The experimental fish were acclimated to a freshwater pond environment (a 2.5-ton FRP tank) with natural lighting. All procedures and investigations were approved by the National Taiwan Ocean University Institutional Animal Care and Use Committee and were performed in accordance with standard guidelines.

Fish were treated with E2. In our study, all elvers developed into males in a freshwater pond environment (all males) (Jeng et al., 2018). To understand ovarian development, E2 treatment was conducted to feminization (all female). Before the experiment began, the gonads of juvenile fish were collected to check the gonadal stage. Undifferentiated fish (body length smaller than 12 cm) were treated with E2 by oral feed administration. E2 treatments induced the fish to undergo female sexual development. In our previous studies (Jeng et al., 2018), according to a diet that contained E2 (n = 300; 10 mg/kg of diet) or control (n = 300) was applied for 1 year. Fish were fed the diet twice daily and the amount of feed was more than 1% of their body weight (to make sure all fish received enough feed). Fish were randomly collected from both groups at one month intervals until most fish reached 40 cm. Due to the small quantity of gonadal tissue in these small fish (body length < 30 cm), gonads from individual fish were collected either for histology or for molecular biology only.

2.2. Gonadal histology

To detect any correlation between body size and gonadal stage, fish of different sizes were collected. The gonads were fixed with 4% paraformaldehyde in PBS at 4 °C for 16 h. The fixed gonads were dehydrated in ethanol, and then embedded in paraffin. Sections (6 μm in

Fig. 1. Alignment of deduced amino acid sequences of fish Foxl2 and Foxl3 protein. Sequences were aligned using ClustalW. The forkhead domain is labeled in bold and underlined in black. The nuclear localization signal (NLS) has a red underline. The conserved amino acids of NLS for Foxl2 (Methionine) and Foxl3 (Valine) are highlighted in gray and labeled green with a square. The name and accession numbers of the sequences obtained in the analysis are listed in Supplemental Table 1.

thickness) were rehydrated and stained with hematoxylin and eosin (H &E). The gonads of all of the fish were examined histologically.

2.3. Cloning of Japanese eel Foxl3a and Foxl3b

Gonads were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted following the manufacturer's protocol. Total RNA was reversely transcribed to the first-strand cDNA using Superscript III (Invitrogen) with the oligo (dT)15 primer (Promega, Madison, WI, USA). To amplify the full cDNA fragment of Japanese eel, *Foxl3a* (GenBank accession no. MF966996) and *Foxl3b* (GenBank accession no. MF966997) PCR primers were designed based on nucleotide sequences of the genome and the transcriptome database (Supplemental Table 1). The confirmed cDNA sequence of *Foxl3a* and *Foxl3b* was used to design a specific qPCR primer for the RNA analysis.

2.4. Sequence alignment and phylogenetic analysis

For phylogenic analysis, a subset of FOXL (FOXL1s, FOXL2s, FOXL3s) sequences from various taxa was retrieved from GenBank. The deduced amino acid sequences of Japanese eel were aligned by a multiple sequence alignment using MUSCLE. The phylogenetic tree was constructed using the neighbor-joining method on MEGA5. The number at each node represents the bootstrap probability (% from 1000 replicates). The accession numbers of the sequences obtained in the analysis are listed in Supplemental Table 1.

2.5. RNA analysis

The correlation between body length and gonadal status was determined by histology. RNA extraction and cDNA synthesis were performed as described above. This first-strand cDNA was used for quantitative real-time PCR analyses (qPCR). The number of PCR cycles was preliminarily tested and was in the range of the linear curve for the relationship between the number of cycles and the amount of PCR product. As an internal control, *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*; GenBank accession no. AB075021) was used to normalize the gene expression level. Specific qPCR primers for *Foxl3a*, *Foxl3b*, *Vasa* (germline marker; GenBank accession no. KX987991), *Amh* (*anti-Müllerian hormone*, a spermatogenesis-preventing substance; GenBank accession no. AB074569), and *Gapdh* are listed in Table 1. The qPCR analysis was performed as in our previous study (Wu et al., 2016). Gene quantification of standards, samples, and controls was conducted simultaneously by qPCR (GeneAmp 7500 Sequence Detection System; Applied Biosystems, Foster City, CA, USA) with the SYBR green Master Mix (Applied Biosystems, Vilnius, Lithuania). PCR specificity was confirmed by the single melting curve (at the same temperature) observed for unknown samples and standards. In addition, PCR specificity was confirmed by gel electrophoresis in both testicular tissue and plasmids (Supplemental Fig. 1A). The respective standard curve of log (transcript concentrations) vs CT (the calculated fractional cycle number at which the PCR-fluorescence product is detectable above a threshold) was obtained. The values detected from different amounts of plasmid DNA contained a fragment of the target gene (10 times serial dilution) of the representative samples in parallel with the respective standard curve. The correlations of the standard curve for the gene analyses were at least −0.999. The qPCR assay was conducted with duplicate repeats in each sample. All samples were normalized to *Gapdh*, and the highest value (control value) of each gene was defined as 100%. According to both qPCR and gel electrophoresis (Supplemental Fig. 1B) in testicular samples (n = 5) and DNase I-treated testicular samples (n = 5), PCR amplification of *Foxl3a* (1 ± 0.126 and 0.806 ± 0.069, respectively) and *Foxl3b* (1 ± 0.111 and 0.946 ± 0.175, respectively) was not due to contaminated genomic DNA.

2.6. Preparation of testicular cells and cell selection

The method for investigating cell dispersion is modified from that used to study gonadal cell preparations in rainbow trout (Shikina et al., 2008) and black porgy (Wu et al., 2016). Testes collected from five yellow eels (with immature testes) contained type A and type B spermatogonia. These testes were dissociated using 0.36% trypsin (pH 8.2, 5% FBS) with gentle pipetting. Dispersed testicular cells were filtered through a 70-μm and then a 40-μm pore-size nylon screen to eliminate large cell clumps. Filtered testicular cells were washed with serum-free Leibovitz's L-15 medium and then cells were seeded in 6-well plates containing 2 ml of culture medium (L-15 with 5% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin). Based on the difference of adhesive

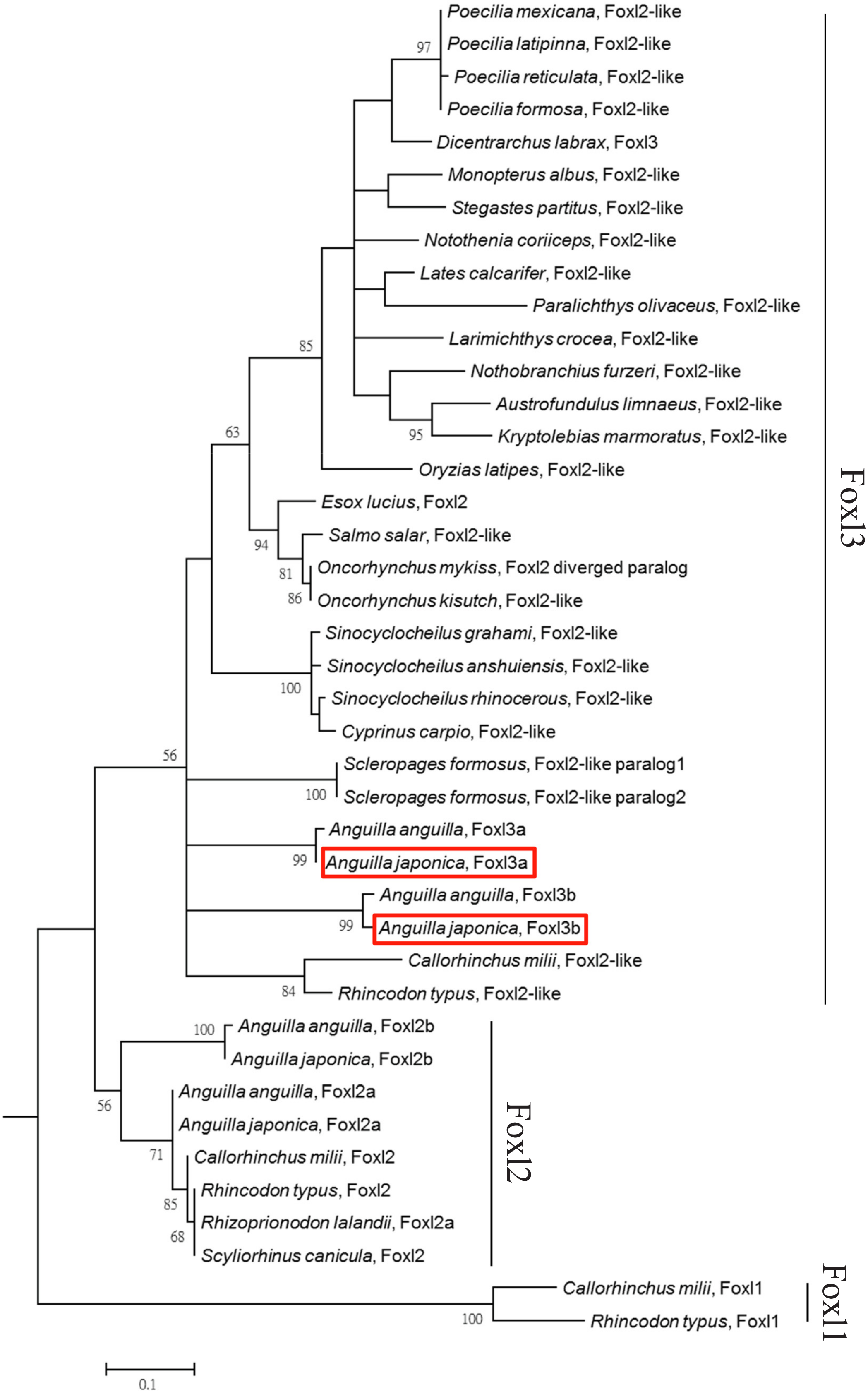

Fig. 2. Phylogenetic tree comparing the amino acid sequences of Foxl3s and other Foxls from various taxa. The open reading frame of Foxls was used for analysis. The phylogenetic tree was constructed using the neighbor-joining method. The number at each node represents the bootstrap probability (% from 1000 replicates). Branches correspond to values of 50% and higher. The name and accession numbers of the sequences obtained in the analysis are listed in Supplemental Table 1.

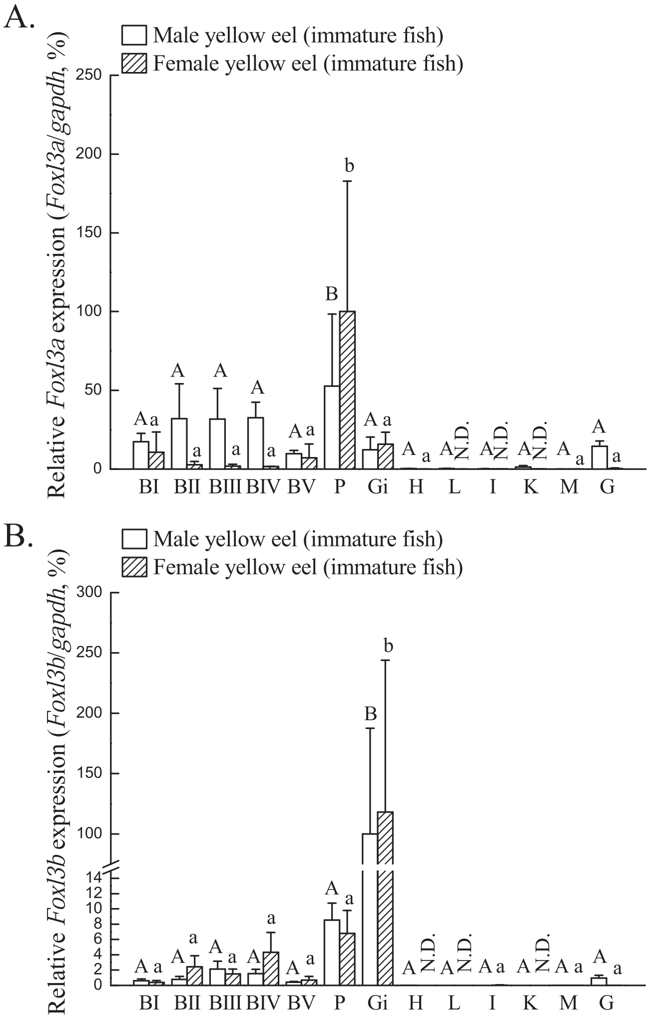

Fig. 3. Expression of *Foxl3a* and *Foxl3b* in various tissues of Japanese eel. Distinguished by morphological criteria, male yellow eels (immature males, n = 3) and female yellow eels (immature females, n = 3) were used to check for the gene expression in different tissues of Japanese eel. *Foxl3a* (A) and *Foxl3b* (B) were used to analyze the gene expression. Differences between the different groups were normalized with *Gapdh*, and the value of each gene in the highest group was defined as 100%. The qPCR assay was conducted with duplicate repeats in each sample. Small and capital letters indicate a one-way ANOVA and Tukey's multiple test ($P < 0.05$). N.D., nondetectable; BI, olfactory bulb; BII, telencephalon including rostral preoptic area; BIII, optic tectum including dorsal tegmentum and diencephalic and mesencephalic area; BIV, corpus cerebellum; BV, medulla oblongata; G, gonad (testis in male and ovary in female); Gi, gill; H, heart; L, liver; I, intestine; K, kidney; M, muscle; P, pituitary.

ability in the somatic cells and germline cells, dispersed cells were incubated in wells overnight and then divided into 2 groups, adhesive cells (putative somatic cells) and suspension cells (putative germline cells). The cell types were confirmed by gene expression for germ line markers (*Vasa*).

2.7. *In situ hybridization*

In situ hybridization (ISH) staining was performed as described previously (Wu et al., 2017). Eel gonads were fixed with 4% paraformaldehyde in PBS. cDNA fragments of *Foxl3a* (nucleotide 430-952) and *Foxl3b* (nucleotide 413-975) were used to synthesize the RNA probe for ISH. For ISH, digoxigenin-11-UTP (DIG) was used to label RNA with digoxigenine (Roche, Penzberg, Germany). Anti-sense DIG-labeled antisense and sense probes were used to detect the localization of VTG1 and VTG2. The rehydrated ovarian sections (6-μm-thickness) were treated with proteinase K (5 μg/ml) at 37 °C for 30 min, post-fixed with 4% paraformaldehyde, and incubated with the DIG-labeled RNA probes (150 ng/ml) at 58 °C overnight. After washing with saline-sodium citrate (SSC) at 58 °C, the sections were blocked with a 0.5% blocking reagent (Roche). Immunostaining was performed with pre-absorbed alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche) at room temperature. Finally, the NBT/BCIP Detection System (Sigma-Aldrich) was used to detect RNA expression.

2.8. *Tissue culture*

The effects of sex steroids (E2 and 11-KT) on the expression of *Foxl3a* and *Foxl3b* were analyzed using a previously described organ culture system (Wu et al., 2012). Testes were collected from male yellow eels (immature testes) and cut into 0.5 μm thickness pieces in 24-well plastic culture dishes. Tissues were incubated in the absence or presence of E2 (2 ng/ml, 20 ng/ml, and 200 ng/ml) and 11-KT (2 ng/ml, 20 ng/ml, and 200 ng/ml) for 24 h. To examine the long-term effects of androgen, tissues were incubated in the absence or presence of 11-KT (10 ng/ml) for 5 days. The medium was changed every day. The basal culture medium using a previously described formulation (Miura et al., 1991) consisted of a Leibovitz L-15 medium supplemented with 1.7 mM proline, 0.1 mM aspartic acid, 0.1 mM glutamic acid, 0.5% bovine serum albumin fraction V, retinol at 50 mg/L, and 10 mM Hepes, adjusted to pH 7.4 with 1 M NaOH.

2.9. *Data analysis*

The data are presented as mean ± standard deviation (SD). The values were subjected to analysis by one-way ANOVA, followed by a Turkey test, with $P < 0.05$ indicating a significant difference. Student's *t*-test was also conducted to determine significant differences ($P < 0.05$) between treatments.

3. Results

3.1. *Molecular identification and phylogenetic analysis of Japanese eel Foxl3s*

According to the deduced amino sequences in the transcriptome database of immature gonads of males and females, four *Foxl2*-like genes were found in Japanese eel through a local blast on CLC Genome Workbench 8.0 and the sequences of these four genes were determined by cDNA cloning. According to the difference in nuclear localization signal (NLS) sequences in Foxl2 (RRRRR$\underline{\text{M}}$KRPFRP) and Foxl3 (RRRR$\underline{\text{V}}$R/KRX) found in teleosts (Crespo et al., 2013), two Foxl2s and two Foxl3s were separated (Fig. 1). To further clarify the relationship between these genes, the deduced sequences of the L group of FOX family (Foxl) genes were retrieved and the phylogenetic relationship was analyzed. The accession numbers of the sequences obtained in the analysis are listed in Supplemental Table 1. The phylogenetic analysis showed that two *Foxl2*-like genes (named *Foxl2a* and *Foxl2b*) were clustered with Foxl2 in teleosts (Fig. 2). In addition, two *Foxl2*-like genes (named *Foxl3a* and *Foxl3b*) were clustered with Foxl3 in teleosts (Fig. 2).

3.2. *Tissue distribution of Foxl3a and Foxl3b in immature eels (yellow eels)*

Male yellow eels (n = 3) and female yellow eels (n = 3) were used to analyze the gene expression in various tissues. According to the qPCR results, *Foxl3a* was predominantly expressed in the pituitary (Fig. 3A) but was also detected in brain, heart, liver, intestine, kidney, muscle, testis, and ovary (Fig. 3A). However, only slight or no expression of *Foxl3a* was found in the liver, intestine, and kidneys of female eels (Fig. 3A). *Foxl3b* was predominantly expressed in the gills and also

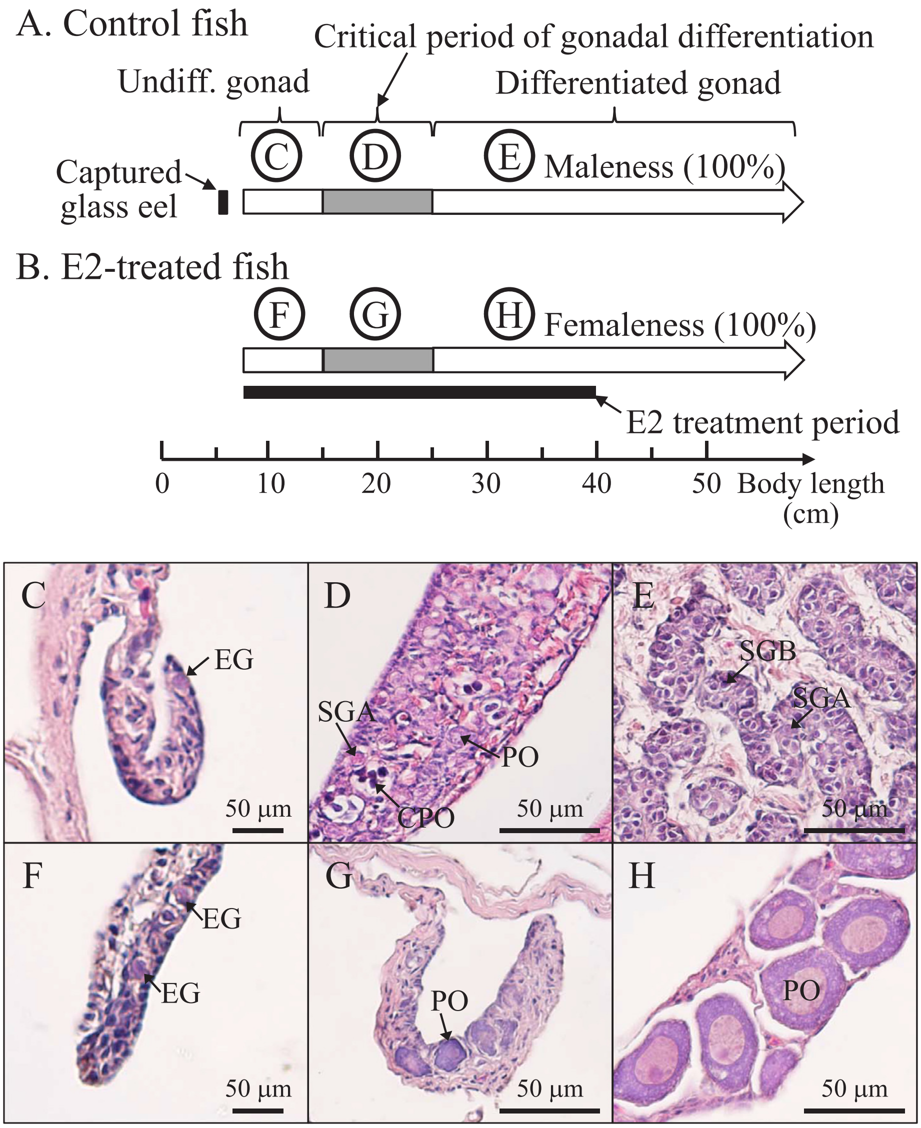

Fig. 4. Culture-induced masculinization and E2-induced feminization in Japanese eel. Relationship between body size and gonadal stage in control (A) and E2-treated fish (B). Gonadal stages were shown as follows: C) undifferentiated gonad in a control fish; D) differentiating testis in a control fish; E) developed testis in a control fish; F) undifferentiated gonad in an E2-treated fish; G) differentiating ovary in an E2-treated fish; H) developed ovary in an E2-treated fish. EG, early germ cell; CPO, cystic primary oocyte; PO, primary oocyte; SGA, spermatogonia type A; SGB, spermatogonia type B.

detected in the brain, pituitary, heart, liver, intestine, kidney, muscles, testis, and ovary (Fig. 3B), with only slight or no expression in the heart, liver, and kidney (Fig. 3B).

3.3. *The expression profiles of Foxl3a and Foxl3b during gonadal differentiation*

Different gonad stages were used to analyze the gene transcript levels in both sexes. Under normal culture conditions, an all-male population was found among the control fish (Fig. 4A), but an all-female population was induced by long-term administration of E2 (Fig. 4B). Based on histological characteristics, three different stages were found in the control group (100% male), including fish with undifferentiated gonads with early germ cells (Fig. 4C), fish with differentiating testes with spermatogonia (Fig. 4D), and fish with immature testes with germinal cysts (Fig. 4E). The characteristics of the different male germ cells are shown in Supplemental Fig. 2. Three different stages were also found in the E2-treated group (100% female), including undifferentiated gonads (Fig. 4F), differentiating ovaries with early primary oocytes (Fig. 4G), and immature ovaries (Fig. 4H). Combining the data of size and age, the critical period of gonadal differentiation was associated with body length, but not with age, in Japanese eel (Table 2). In both the control fish and E2-treated fish, undifferentiated gonads were found in fish with a body length smaller than 15 cm (Table 2). Gonadal differentiation started when the fish reached to 15 cm (Table 2). All fish became either immature males (with differentiated testes) or immature females (with differentiated ovaries) after reaching a size of 25 cm (Table 2). Because histology and qPCR analysis could not be performed on the same individual fish the gonadal condition of fish selected for qPCR analysis was inferred based on their body size. Analysis by qPCR showed that *Foxl3a* had a wide range of expression in the control fish (Fig. 5A); for the individual fish, see Supplemental Fig. 3A. However, *Foxl3a* expression levels significantly decreased after female gonadal differentiation and were maintained at a low level in ovaries compared with testes (Fig. 5A). Similarly, *Foxl3b* also showed a steady level of expression during male gonadal differentiation (Fig. 5B, Supplemental Fig. 3B) whereas its expression level significantly decreased after female gonadal differentiation and was maintained at a low level in ovaries compared with testes (Fig. 5B). To confirm the sex-dimorphic expression of *Foxl3a* and *Foxl3b* in the

Table 2
Gonadal stage and corresponding fish size.

Group	Body length (cm)	No. of fish	Gonadal stages		
			Undiff. Gonad Number (%)	Testis Number (%)	Ovary Number (%)
Control fish	< 15	6	6/6 (100)	0/6 (0)	0/6 (0)
	15–19.9	11	6/11 (55)	5/11 (45)	0/11 (0)
	20–24.9	9	3/9 (33)	6/9 (67)	0/9 (0)
	25–29.9	6	0/6 (0)	6/6 (100)	0/6 (0)
	30–34.9	14	0/14 (0)	14/14 (100)	0/14 (0)
	35–39.9	6	0/6 (0)	6/6 (100)	0/6 (0)
	40–44.9	7	0/7 (0)	7/7 (100)	0/7 (0)
	> 45	3	0/3 (0)	3/3 (100)	0/3 (0)
E2-treated fish	< 15	5	5/5 (100)	0/5 (0)	0/5 (0)
	15–19.9	20	8/20 (40)	0/20 (0)	12/20 (60)
	20–24.9	20	1/20 (5)	0/20 (0)	19/20 (95)
	25–29.9	10	0/10 (0)	0/10 (0)	10/10 (100)
	30–34.9	12	0/12 (0)	0/12 (0)	12/12 (100)
	35–39.9	1	0/1 (0)	0/1 (0)	1/1 (100)

gonads of both sexes, the gonads of immature males and females were collected for qPCR analysis. Both *Foxl3a* and *Foxl3b* were exclusively expressed in the testes but not in the ovaries in immature fish as well (Fig. 6A, Supplemental Fig. 4), but with a wide range of expression of both genes in the testes (Supplemental Fig. 4). To summarize, *Foxl3a* and *Foxl3b* were exclusively expressed in undifferentiated gonads and in differentiated testes, respectively.

3.4. *Germline-specific expression of Foxl3a and Foxl3b in testis*

To examine the *Foxl3a* and *Foxl3b* expression in the cell type (germline cells or somatic cells) in the testes, we developed an *in vitro* culture system to separate the germline cells and somatic cells. Different cell types were isolated based on a previous report on the difference in adhesive ability between somatic cells and germline cells (Wu et al., 2016, 2017). In our *in vitro* cell culture system, qPCR data showed much higher *Vasa* (germline cell marker) expression in suspended cells than in adhesive cells (Fig. 6B). This shows that suspended cells are associated with germline cells. Furthermore, qPCR data showed that both *Foxl3a* and *Foxl3b* were more highly expressed in the suspended cells than in the adhesive cells (Fig. 6B). To further understand the *Foxl3a* and *Foxl3b* expression in various cell types in the testes, *in situ* hybridization (ISH) was used to analyze the gene transcripts in immature male testes (Fig. 7A). In our effort to analysis gene transcripts by ISH, no signal was observed from sense probes for *Foxl3a* (Fig. 7B) but antisense probes of *Foxl3a* demonstrated *Foxl3a* expression in male germ cells (Fig. 7C and D). No signal was observed from sense probes for *Foxl3b* (Fig. 7E), but again, *Foxl3b* expression was observed in male germ cells (Fig. 7F and G). Neither *Foxl3a* nor *Foxl3b* expression could be detected (slight or no expression) in ovarian tissue of immature female (data not shown). In sum, *Foxl3a* and *Foxl3b* were expressed in germline cells in testes but hardly at all in germline cells in ovaries.

3.5. *Sex steroids did not influence Foxl3a and Foxl3b expression*

To further understand the correlation between the *Foxl3a* and *Foxl3b* expression in germline cells and the testicular environment, we used E2 and 11-KT to study the effects of sex steroids on gene expression. For *in vitro* tissue culture study, testes were collected from immature male with type A and type B spermatogonia. Dissected gonads were incubated with various doses of E2 (2 ng/ml, 20 ng/ml, and 200 ng/ml) or 11-KT (2 ng/ml, 20 ng/ml, and 200 ng/ml) for 24 hrs. Results from qPCR showed that E2 and 11-KT treatments had no effect on *Foxl3a* and *Foxl3b* expression in eel testicular tissue (Fig. 8); for individual fish treated with E2 and 11-KT, see Supplemental Fig. 5A and B. Furthermore, to examine the long-term effect of androgen, dissected gonads were incubated with 11-KT (10 ng/ml) for 5 days. Results of qPCR on dissected gonads showed that long-term 11-KT treatment had no effect on *Foxl3a* and *Foxl3b* expression in the testicular tissue (Supplemental Fig. 6). To determine the gonadal status, the spermatogonia self-renewing factor (spermatogenesis-preventing substance) *anti-Müllerian hormone* (*Amh*) was used to determine the status of incubated testicular tissue (Miura et al., 2002). *Amh* expression was significantly lower in the 11-KT-treated group compared to the control group

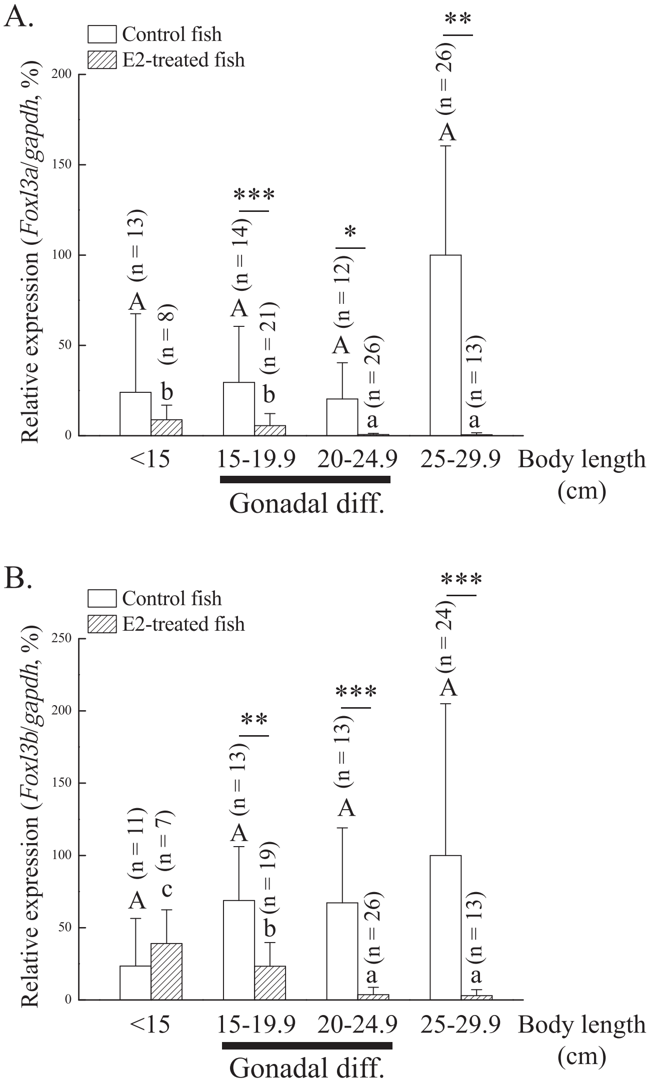

Fig. 5. Gonadal *Foxl3a* and *Foxl3b* gene expression profiles during gonadal differentiation. Distinguished by histological criteria (Fig. 4), three different stages of gonads were used to ascertain gene expression pattern, including an undifferentiated stage (body length smaller than 15 cm), a differentiating stage (body length between 15 and 24.9 cm), and a developed stage (body length greater than 25 cm). Sample size of different-size fish were 6, 15, 13, and 25 for the control group (100% male) and 10, 21, 29, and 29 for the E2-treated group (100% female). Expression of *Foxl3a* (A) and *Foxl3b* (B) was analyzed by qPCR. Differences between the two groups were normalized with *Gapdh*, and the value of each gene in the highest group was defined as 100%. The qPCR assay was conducted with duplicate runs for each sample. Lower and upper-case letters indicate one-way ANOVA and Tukey's multiple test ($P < 0.05$), respectively. The number of asterisks indicates the results of Student's *t*-test as follow: *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. The data of gene expression for individual fish are shown in Supplemental Fig. 3.

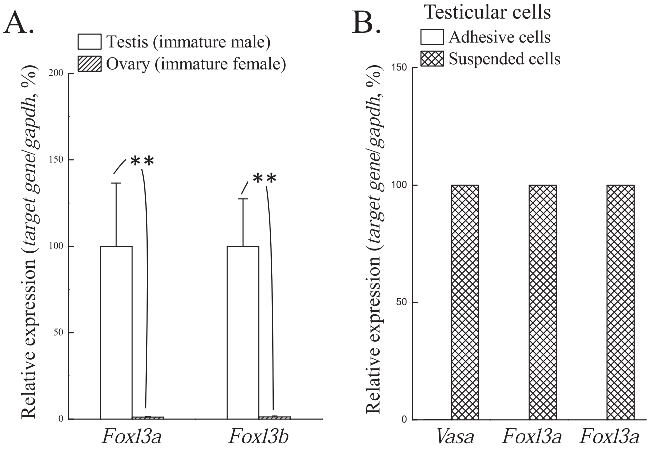

Fig. 6. Sex-specific and lineage-specific expression patterns in *Foxl3a* and *Foxl3b*. A) Relative expression of *Foxl3a* and *Foxl3b* in both sexes (ovaries and testes) of yellow eels (immature eel). Six males and five females were used for RNA analysis. B) Distinguished by adhesive ability, the proportions of somatic cells (adhesive cells) and germline cells (suspended cells) among dispersed cells (as a pool) obtained from testes of 5 immature male eels. The relative expressions of germ line marker (*Vasa*) and *Foxl3s* (*Foxl3a* and *Foxl3b*) were analyzed for the adhesive cells and suspension cells by qPCR with duplicate measurement for each sample. Differences were normalized with *Gapdh* and the value of each gene in the highest sample/group was defined as 100%. Two asterisks indicate the results of Student's *t*-test ($P < 0.01$). The gene expression data for individual fish are shown in Supplemental Fig. 4.

(Supplemental Fig. 6). Taken together, all these results show that *Foxl3a* and *Foxl3b* expression in the testes was not directly influenced by sex steroids.

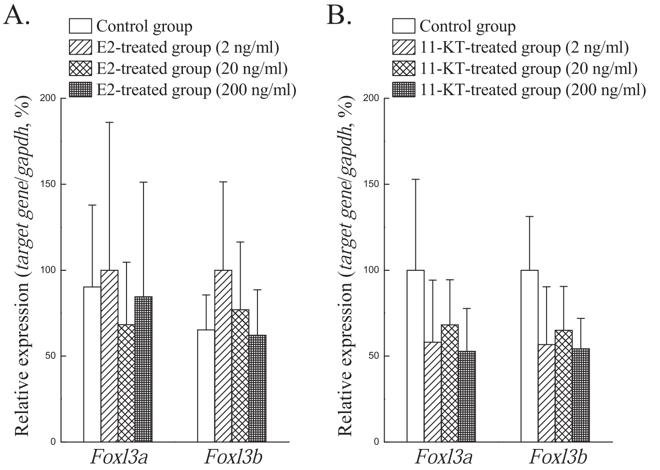

Fig. 8. Effects of E2 and 11-KT on *Foxl3a* and *Foxl3b* expression in testicular tissue. Different dosages of E2 (estradiol-17ß; 2 ng/ml, 20 ng/ml, and 200 ng/ml) and 11-KT (11-ketotestosterone; 2 ng/ml, 20 ng/ml, and 200 ng/ml) were administered for a 24 h period and then testicular tissues (n = 4–6 fish in each group) was collected for RNA analysis. A qPCR assay was conducted with duplicate replication for each sample. Differences were normalized with *Gapdh*, and the value of each gene in the highest group was defined as 100%. The gene expression data for individual fish are shown in Supplemental Fig. 5.

4. Discussion

4.1. Duplication of Foxl3 in ancient fish

In a recent study of whole genome sequences in vertebrates (Crespo et al., 2013; Geraldo et al., 2013), the *Foxl2* paralog (*Foxl2b*) was renamed as *Foxl3*. In the present study, two *Folx3*s were cloned and showed a teleost-conserved NLS of Foxl3 in Japanese eel (Fig. 1).

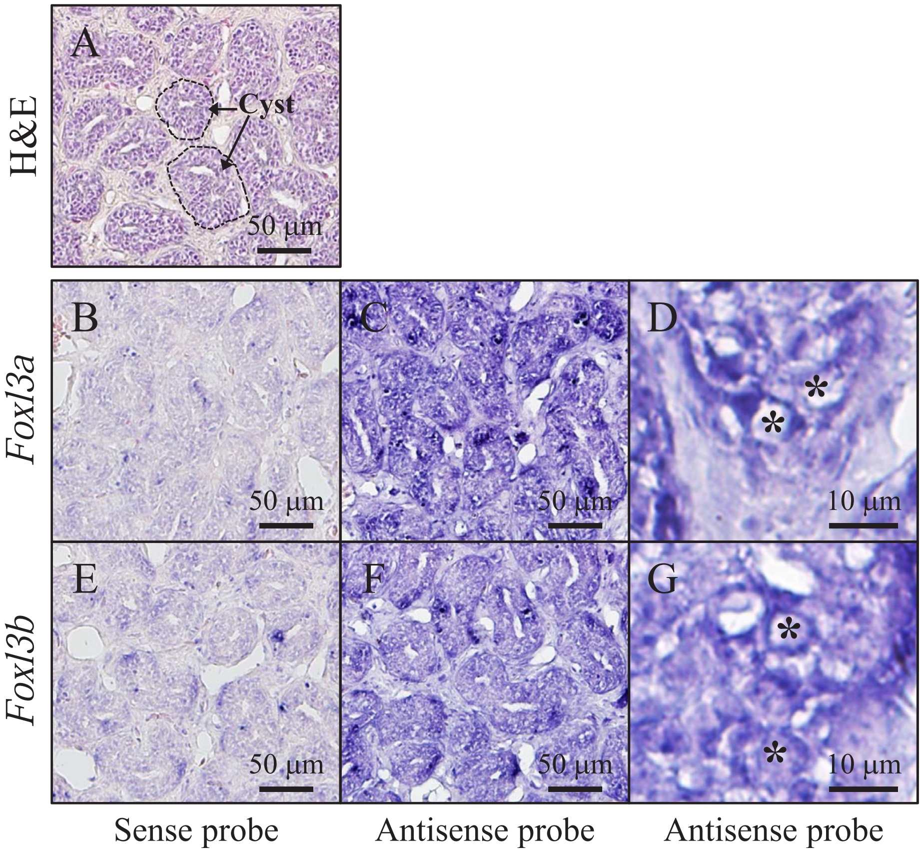

Fig. 7. Location of *Foxl3a* and *Foxl3b* transcripts in the testis. *Foxl3a* and *Foxl3b* mRNA expression was detected by *in situ* hybridization (ISH) using immature male eels (A). The reference of *Foxl3a* expression was detected by the sense probe of *Foxl3a* (B). Expression of *Foxl3a* (C and D) was detected in male germ cells. The reference of *Foxl3b* expression was detected by the sense probe of *Foxl3b* (E). Expression of *Foxl3b* (F and G) was detected in male germ cells. Asterisks indicate germline cells in testes.

According to our phylogenetic analysis, these two *Foxl3*s (*Foxl3a* and it paralog *Foxl3b*) occur only in Japanese eel and European eel (*Anguilla anguilla*) but also in Asian arowana (*Scleropages formosus*) (Fig. 2). Contrary to recent reports, the *Foxl3* gene only has a single copy in rayfinned fish (actinopterygians) (Crespo et al., 2013). Furthermore, this gene was lost in lobefinned fish (sarcopterygians), and is not found in amphibians, most birds, and eutherian mammals (Bertho et al., 2016; Crespo et al., 2013; Geraldo et al., 2013). Taken together, these results suggested that the teleost-specific *Foxl3* paralog was repeatedly lost in most fish after the third round of whole genome duplication.

4.2. The role of Cyp19a1 and estrogen in Japanese eel

Cyp19a1 transcripts were significantly increased in differentiating and differentiated gonads of Japanese eel, but did not show any differential expression between control males and E2-treated females (Jeng et al., 2018). Aromatase activity was very low in ovaries compared to brains in female eels (Jeng et al., 2005, 2012) and female eels treated with E2 showed no change in either the expression of *Cyp19a1* or aromatase enzymatic activity in their ovaries (Jeng et al., 2012). These results suggest that *Cyp19a1* is not a direct target for E2 action during gonad differentiation in Japanese eels. Nonetheless, E2 somehow induces all-female gonad development in them (Table 2). Unlike in eels, estrogens have long been considered important for female sexual differentiation in non-eutherian vertebrates (Guiguen et al., 2010). In addition, high estrogen levels result in the appearance of ectopic female germline cells in male testes (Hecker et al., 2006) and low estrogen levels conversely result in ectopic male germline cells in female ovaries (Sun et al., 2014) in gonochoristic fish. In hermaphroditic fish, high plasma estrogen levels are also considered important for female fate maintenance (protogyny) and male-to-female sex change (protandry) (Devlin and Nagahama, 2002; Wu and Chang, 2013b; Wu et al., 2015). Taken together, low *Cyp19a1* expression and low aromatase activity in ovaries of female eels reveal that both an E2-independent pathway and an E2-dependent pathway are associated with female differentiation in Japanese eel. The gene *Foxl2* has been reported to be related to *Cyp19a1a* and ovarian development (Yao, 2005). *Foxl2* is predominantly expressed in the ovaries during and after female differentiation in vertebrates, including mice (Schmidt et al., 2004), chickens (Govoroun et al., 2004), Japanese medaka (Nakamoto et al., 2006), and black porgy (Wu et al., 2008). Moreover, Foxl2 upregulates *Cyp19a1a* expression *in vitro* in tilapia (Wang et al., 2007). Thus, Foxl2 plays an important role in female differentiation through the E2-dependent pathway in fish. However, *Cyp19a1* is involved in gonadal differentiation and development in both sexes in eels. Taken together, we suggest that the E2-independent pathway might play an important role in gonadal differentiation in eels.

4.3. Germline-specific Foxl3a and Foxl3b in Japanese eel

Foxl3a and *Foxl3b* both showed a stable level of expression during male gonadal differentiation in eels, but their expression levels significantly decreased during female gonadal differentiation and maintained at a low level in ovaries compared to testes (Fig. 5). Moreover, *Foxl3a* and *Foxl3b* were predominantly expressed in germline cells in testes (Figs. 6 and 7). The wide range of *Foxl3a* and *Foxl3b* expression found in the testes of male eels maybe due to different developmental stages (related to difference in the ratio of germinal cells to connective tissues in the gonads) of immature eels.

Similarly to the present study, *Foxl3* shows a higher expression in testes than ovaries in Atlantic salmon (*Salmo salar*) (von Schalburg et al., 2011), European seabass (*Dicentrarchus labrax*) (Crespo et al., 2013), and ricefield eel (*Monopterus albus*) (Gao et al., 2016). Conversely, *Foxl3* is expressed in the differentiating ovaries while its expression remains undetectable in males in rainbow trout (Baron et al., 2004). Furthermore, the expression of *Foxl3*/Foxl3 in the early germline has only been observed in XX female Japanese medaka after gonadal differentiation, while such expression has not been found in germ cells of XY fish (Nishimura et al., 2015). Thus, the expression pattern of gonadal *Foxl3* is highly various in fish. Conversely, the expression of *Foxl3* in gills is conserved in fish, including Japanese eel (the present study), European seabass (Crespo et al., 2013), and ricefield eel (Gao et al., 2016).

In the present study, both the E2 and 11-KT treatments had no effect on either germline-expressed *Foxl3a* or *Foxl3b* expression in testicular tissue (Fig. 8, Supplemental Fig. 6). In black porgy, ectopic oocytes can survive in the testicular environment and alter the sexual fate of surrounding cells from male to female under low plasma E2 levels even after E2 has been withdrawn from E2-treated fish (Wu and Chang, 2013a; Wu et al., 2017). In chimeric medaka (with $Foxl3^{-/-}$ germline donor cells transplanted into recipient XX females), *Foxl3* mutant germ cells initiate spermatogenesis in normal female gonads (Nishimura et al., 2015). Taken together, these results reveal that sexual fate of germline cells may be determined through a pathway independent of sex steroids. Furthermore, sex-steroid-insensitive *Foxl3a* and *Foxl3b* might play an important role in male sexual fate maintenance while germ cells of both sexes experience similar environment conditions (E2 levels).

4.4. Conclusion

We demonstrated that two *Foxl3*s (*Foxl3a* and its paralog *Foxl3b*) showed higher levels of expression in males than in females during gonadal differentiation in the Japanese eel. The data indicated that the two *Foxl3*s are expressed in male germline cells and demonstrated that *Foxl3s* might play an important role in germline sexual fate determination in both ancient fish (Japanese eel) and modern fish. Our data thus shed light on the *Foxl3* genes evolutionary profile in teleosts. Further studies including *Foxl3s*/Foxl3s regulation in eel are required for a better understanding of the conserved mechanisms and pathways in fish.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.03.008.

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