**Homozygous mutation of *foxh1* arrests oogenesis causing infertility in female Nile tilapia**

Wenjing Tao[1]*, Hongjuan Shi[1, 2]*, Jing Yang[1], Hamidou Diakite[1], Thomas D. Kocher[3], Deshou Wang[1]

[1]Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Sciences, Southwest University, Chongqing, 400715, China.

[2]Guangdong Research Center on Reproductive Control and Breeding Technology of Indigenous Valuable Fish Species, Key Laboratory of Marine Ecology and Aquaculture Environment of Zhanjiang, Key Laboratory of Aquaculture in South China Sea for Aquatic Economic Animal of Guangdong Higher Education Institutes, Fisheries College, Guangdong Ocean University, Zhanjiang 524088, China.

[3]Department of Biology, University of Maryland, College Park, Maryland, United States of America.

*Contributed equally

Corresponding author: Deshou Wang,

Tel: +86-68253702; Fax: 86-23-68253005; e-mail address: wdeshou@swu.edu.cn

**Competing interest**

The authors declare that they have no competing interests.

**Grant support:**

This work was supported by grant 2018YFD0900202 from the National key research and development program of China; grants 31630082, 31972778, 31502147, 31861123001, 31572609, 31972776 and 31872556 from the National Natural Science Foundation of China; grants cstc2018jscx-mszd0380 and cstc2018jcyjAX0283 from the Chongqing Science and Technology Commission; grant 2018IB019 from Yunnan Science and Technology project; grant XDJK2018B025 from the Fundamental Research Funds for the Central Universities.

**Abstract**

Foxh1, a member of *fox* gene family, was first characterized as a transcriptional partner in the formation of the Smad protein complex. Recent studies have shown *foxh1* is highly expressed in the cytoplasm of oocytes in both tilapia and mouse. However, its function in oogenesis remains unexplored. In the present study, *foxh1*$^{-/-}$ tilapia was created by CRISPR/Cas9. At 180 dah (days after hatching), the *foxh1*$^{-/-}$ XX fish showed oogenesis arrest and a significantly lower GSI. The transition of oocytes from phase II to phase III and follicle cells from one to two layers was blocked, resulting in infertility of the mutant. Transcriptomic analysis revealed that expression of genes involved in estrogen synthesis and oocyte growth were altered in the *foxh1*$^{-/-}$ ovaries. Loss of *foxh1* resulted in significantly decreased Cyp19a1a and increased Cyp11b2 expression, consistent with significantly lower concentrations of serum estradiol-17β (E2) and higher concentrations of 11-Ketotestosterone (11-KT). Moreover, administration of E2 rescued the phenotypes of *foxh1*$^{-/-}$ XX fish, as indicated by the appearance of phase III and IV oocytes and absence of Cyp11b2 expression. Taken together, these results suggest that *foxh1* functions in the oocytes to regulate oogenesis by promoting *cyp19a1a* expression, and therefore estrogen production. Disruption of *foxh1* may block the estrogen synthesis and oocyte growth, leading to the arrest of oogenesis and thus infertility in tilapia.

**Key words:** *foxh1*; CRISPR/Cas9; oogenesis arrest; tilapia

## Introduction

In teleosts, oogenesis is consisted of a prolonged growth phase with the remarkable enlargement of oocyte through primary oocyte growth, the cortical alveolar stage, as well as subsequent vitellogenesis, and a maturation phase characterized by the resumption of meiosis and ultimately egg formations prior to ovulation [1, 2]. Such an important differentiation process requires numerous circulating endocrine, locally-acting paracrine and autocrine factors, with interactions between the oocyte and the surrounding granulosa and theca cells [3]. These major regulators are the pituitary gonadotropins (luteinizing hormone LH and follicle-stimulating hormone FSH) and ovarian steroid hormones (estradiol-17β, and 17 α, 20β-DP), and the complex hormonal cross-talk at all stages between the developing oocyte and its surrounding follicle layers [1]. Gene expression profiling analysis has revealed that thousands of genes are involved in oogenesis. For example, transcripts of the FSH receptor are significantly higher in ovarian follicles displaying high maturational competence [4]. Another study has identified temporal expression patterns of *cyp19a1a* and *star* that suggest they have critical roles in oocyte growth [5, 6]. Many TGF-β family members specially produced by oocytes, *inhbb*, *bmp15* and *gdf9*, have fundamental roles in oogenesis by regulating the paracrine signaling between oocytes and the granulosa cells that control follicular development [6, 7]. A number of transcriptional regulators active in the germ line and somatic cells, including *figla*, *lhx8*, *nobox*, *sohlh1*, *pou5f1* and *sohlh2*, were also found to be critical in ovarian development and folliculogenesis [8, 9].

The Fox (forkhead box) genes encode an evolutionarily conserved family of transcription factors that play important roles in a diverse range of biological processes, including the establishment of the body axis, metabolic and immune regulation, control of cell cycle and cellular survival, as well as sex determination and differentiation [10, 11]. Previous studies have also shown that *fox* genes are essential for ovarian differentiation, primordial follicle activation, female germ cell migration and folliculogenesis [12-16]. For example, *Foxo3* is a PI3K-dependent molecular switch controlling the initiation of oocyte growth in mammals [17]. Foxl2 is essential for ovarian granulosa cell differentiation and proliferation. Disruption of Foxl2 prevents development of follicle cells [18]. Although the potential target genes of

Foxc1 remains unknown, it is required for follicle maturation beyond the early antral stage [14]. Due to their important roles in oogenesis, the *fox* genes have attracted considerable interest.

Foxh1 was first characterized as a transcriptional partner for Smad proteins, and has been demonstrated to play an important role in the embryonic development of *Xenopus* embryos [19, 20]. Foxh1 has a conserved N-terminal Forkhead domain and a distinct C-terminal domain essential for DNA binding and regulation of transcription, which interacts with various Smad proteins including Smad2, Smad3 and Smad4, affecting the expression of target genes via an activin-response element [21]. Overexpression of *foxh1* induces the expression of a broad range of downstream activins and leads to abnormal axis formation in *Xenopus* [22, 23]. Recent studies indicate that Foxh1 is also an essential regulator of nodal signaling during the key developmental processes of node formation and gastrulation in mouse [24, 25], as well as the progression of colorectal cancer [26]. In zebrafish, *foxh1* is involved in blood vessel formation by negatively regulating *flk1* gene expression, subsequently affecting gastrulation, development of dorsal axial structures and left-right asymmetry [27-29]. *foxh1* was identified to be overexpressed in zebrafish ovary, indicating its possible involvement in suppression of the biological activity of androgens [30]. Our recent study reported the sexually dimorphic expression of *foxh1* in tilapia gonad, with extremely high expression in the cytoplasm of phase I and II oocytes in tilapia ovary. Remarkably, expression in XX gonads increased continuously from 20 to 240 dah (days after hatching), while expression in XY gonads remained at a low level [31]. A recent study in the mouse also demonstrated that *foxh1* is expressed in oocytes during early embryonic development, and subsequently in the thecal cells during ovarian follicle development, ovulation, and luteinization, indicating an important role for *foxh1* in these physiological processes [32]. However, there have been no functional studies of *foxh1* during oogenesis.

In the present study, we knocked out *foxh1* in tilapia to study its roles in oogenesis. We analyzed the ovarian phenotype, gene expression, serum estradiol-17β (E2) and 11-ketotesterone (11-KT) levels and fertility of the mutant fish. We found that *foxh1* is critical

for oogenesis, through its possible effects on the estrogen synthesis and oocyte growth in Nile tilapia.

## Materials and Methods

### Animals

Nile tilapia (*Oreochromis niloticus*) were reared in aerated recirculating freshwater tanks at 26ºC under a natural photoperiod. All-XX progenies were obtained by crossing an XX pseudomale with a normal XX female. All-XY progenies were obtained by crossing an YY supermale with a normal XX female. Animal experiments were conducted in accordance with the regulations of the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University.

### *In situ* hybridization (ISH)

To identify the cell type expressing *foxh1* in the developing ovaries, ISH was performed using ovaries from fish at 30, 90, 120 and 180 dah. Sense and antisense probes of *foxh1*were previously described [31]. ISH was performed as described in previous study [33].

### Establishment of *foxh1* mutants

The tilapia *foxh1* F0 XY mutants were generated by CRISPR/Cas9-induced gene knockout, as described previously [34]. The gRNA target site was selected from *foxh1* sequences corresponding to GGN18NGG on the sense or antisense strand of DNA (http://zift.partners.org/ZiFiT/). Candidate target sequences were compared with the entire tilapia genome using the Basic Local Alignment Search Tool (BLAST) to avoid cleavage of off-target sites [35]. One-cell stage embryos were divided into two batches, one for microinjection and the other for control. Briefly, gRNA and Cas9 mRNA were co-injected into one-cell stage embryos at an optimal concentration of 250 ng/μl and 500 ng/μl, respectively. To assay the rate and pattern of mutation we collected and separately pooled 20 control and 20 injected embryos 72 hours after injection. Genomic DNA fragments spanning the target site were amplified using the primers listed in Table S1. The mutated sequences were analyzed by digestion with *Hae*III and by Sanger sequencing of cloned PCR fragments.

$F_1$ offspring were produced by crossing $F_0$ XY males with wild-type XX females. Male

and female siblings of the heterozygous $F_1$ mutants (*foxh1*$^{+/-}$) carrying the same mutation (11 bp deletion and 15 bp insertion) were mated to generate homozygous $F_2$ mutants (*foxh1*$^{-/-}$). Heteroduplex mobility assays were performed using polyacrylamide gels electrophoresis (PAGE) to detect the mutations as described previously [36]. In-frame and frame-shift deletions were again confirmed by Sanger sequencing.

**Oocyte counting**

The ovaries of six *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ XX fish were dissected at both 90 and 180 dah. Ovarian tissues were fixed in Bouin's solution for 24 hours at room temperature, dehydrated, and embedded in paraffin. Tissue blocks were sectioned at 5 μm for histological analysis. The histological classification of the oocytes has been described previously [37]. The phase I oocyte is morphologically similar to but larger than oogonia (size range: 7-11 μm). The phase II oocyte contains several nucleoli and chromatin strands (size range: 36-240 μm). The phase III oocyte is with numerous vesicles close to oocyte periphery and with the appearance of small yolk granules (size range: 224-658 μm). The phase IV oocyte is with larger yolk granules and empty large vacuoles (size range: 428-1416 μm). The number of oocytes were counted in at least 10 sections of each ovary from the *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ XX fish.

**Measurement of steroid hormones**

Serum estradiol-17β (E2) and 11-ketotestosterone (11-KT) levels were measured using the Enzyme Immunoassay Kit (Cayman Chemical Co., Ann Arbor, MI). Sample purification and assays were performed according to the manufacturer's instructions. Data were expressed as the mean ± SD. Significant differences between groups were tested with a Student's t-test. P values $< 0.05$ were considered to be statistically significant.

**Immunohistochemistry, fluorescence Immunohistochemistry (FIHC) and western blot**

Ovaries of the *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ XX fish were dissected at 90 and 180 dah. The gonads were fixed in Bouin's solution for 24 hours at room temperature, dehydrated, and embedded in paraffin. Tissue blocks were sectioned at 5 μm thickness. Immunohistochemical analysis was performed as described previously [38]. Cyp19a1a (RRID: AB_2629226) antibody was donated by Prof. Nagahama, the National Institute for Basic Biology, Okazaki, Japan [39].

Steroid 11beta-hydroxylase (Cyp11b2) antibody (RRID: AB_2650466) was prepared by our laboratory [40]. Antibodies against Cyp11b2 and Cyp19a1a were diluted 1:500 and 1:3000 for use, respectively. Caspase 3 antibody from cell signaling technology (5A1E-9664S) was used at 1:200 to label cell apoptosis. 4', 6-diamidino-2-phenylindole (DAPI) was used for DNA visualization. The specificity of these antibodies have been checked previously [16]. The IHC positive signals were quantified using Image J software (Bethesda, USA) according to the instructions of the manufacturer. For FIHC, the secondary antibody Alexa Fluor 586-conjugated goat anti-rabbit (Invitrogen) was used to detect primary antibodies by immunofluorescence. For the negative control, the primary antibody was replaced with normal rabbit serum. A BX51 light microscope (Olympus) was used to image the stained sections.

Total proteins extracted from ovaries of *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ XX tilapia (n=5/genotype) at 180 dah were separated using 12% SDS-PAGE under reducing condition. Western blot was carried out as reported previously [38] using the purified Cyp19a1a and Cyp11b2 antibodies at dilution of 1:500. The abundance of α-tubulin was examined as a loading control using rabbit anti-α-tubulin at 1:1000 (AB_2619646). Immunofluorescence signal was detected with BeyoECLPlus Kit (Beyotime, Shanghai, China) and was visualized on a Fusion FX7 (Vilber Lourmat, East Sussex, France).

**Transcriptome analysis**

Six ovaries from *foxh1*$^{-/-}$ XX fish at 180 dah were used for transcriptome analyses. Total RNA was extracted from ovaries using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions，and subsequently treated with ribonuclease-free DNaseI to eliminate genomic DNA contamination. The oligo (dT) bead-enriched mRNA was disrupted into short fragments (200-700 nt). These short fragments were used as templates for first- and second-strand cDNA synthesis using a DNA synthesis kit (Invitrogen). A QiaQuick PCR purification kit (Qiagen) was used to purify these cDNA fragments, and elution buffer was used for end repair and addition of the poly (A) tail. These short fragments were ligated to sequencing adapters and sequenced on an HiSeq2000 instrument (Illumina). The reads have

been deposited in the NCBI Sequence Read Archive (SRA) database with accession number SUB4835705. Gonadal transcriptome data from ovaries of XX fish at the same development stage [41] were used as a control. Clean reads from each library were aligned to the reference genome (ftp://ftp.ensembl.org/pub/release-94/fasta/oreochromis_niloticus/dna/) using HISAT with default parameters [42]. The Fragments Per Kilobase of transcript per Million mapped reads (FPKM) method was used to estimate gene expression levels. The abundance of transcripts and differentially expressed genes in *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ XX were analyzed as using Ballgown [43]. Comparison of expression levels between the gonads of control and *foxh1*$^{-/-}$ fish identified non-differentially expressed genes (NDGs), *foxh1*$^{-/-}$ XX up-regulated genes (URGs), and *foxh1*$^{-/-}$ XX down-regulated genes (DRGs). Enrichment for gene ontology (GO) terms was assessed with the DAVID Bioinformatic Database [44] using *Danio rerio* as background ($P < 0.05$).

**Real-time PCR**

Gonads were dissected from the *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ XX fish at 180 dah. Total RNA extraction and reverse transcription were performed as described previously [45]. Real-time PCR was carried out on an ABI-7500 real-time PCR machine (Applied Biosystems) according to the protocol provided with the SYBR® Premix Ex TaqTM II (Takara, Japan). The relative abundance of the differentially expressed genes (*cyp19a1a*, *foxl2*, *star2*, *42sp50*, *dmrt1* and *cyp11b2*) was evaluated using the formula: $R = 2^{-\triangle\triangle Ct}$ [46]. The geometric mean of the copy number of the three reference genes (β-*actin*, *gapdh* and *eef1a1a*) was used to normalize the expression of the other genes. Data were expressed as the mean ± SD. Statistical analysis was performed using GraphPad Prism4 software (GraphPad Software, USA). Significant differences between groups were tested by one-way ANOVA with a post-hoc significance threshold of $p < 0.05$.

**Rescue experiment**

The *foxh1*$^{-/-}$ XX fish were divided into an E2-treatment group and a control group. Following our previous work [38], the treatment fish were fed a diet sprayed with 95% ethanol containing E2 at 50 ng/g feed, whereas control fish were fed a diet sprayed with 95%

ethanol only. The treatment was applied from 120 dah to 180 dah, at which point both groups were fed a normal diet until 240 dah. The gonads of six fish from each group were sampled at 240 dah for FIHC analyses.

**Results**

**Expression profile of foxh1 in developing ovaries**

Our previous study demonstrated the abundant expression of *foxh1* in the ovary from 20 to 240 dah by qPCR [31]. By *in situ* hybridization, robust expression of *foxh1* was found in the cytoplasm of phase I and II oocytes at 30, 90, 120 and 180 dah (Fig. S1).

**Establishment of a *foxh1* mutant line**

The gRNA target sequence containing a *Hae*III restriction site adjacent to the protospacer adjacent motif (PAM) sequence was located in the second exon of *foxh1* (Fig. 1A). The genomic DNA from a pool of 20 embryos was used as the template for amplification and the mutation assay. Complete digestion with *Hae*III produced two fragments (118 and 453 bp) in the control group, while an intact DNA fragment (571 bp) was observed in embryos co-injected with Cas9 mRNA and gRNA (Fig. 1B). The mutation frequency of *foxh1* in the pool of 20 embryos was 49%. $F_1$ mutant fish were obtained by crossing a wild-type XX female with a $F_0$ mosaic XY male. Heterozygous *foxh1* $F_1$ offspring with a 4 bp insertion (created by an 11 bp deletion followed by a 15 bp insertion) in exon 2 were selected to breed the $F_2$ generation. The survival rate of the F2 offspring was ~87%, similar to that of the same $foxh1^{+/-}$ XX fish mating with normal XY fish. And the $foxh1^{-/-}$ XX and XY fish amounted to 1/4 of the F2 progeny (Fig. 1C). Unlike $foxh1^{-/-}$ zebrafish [28, 29], no defects in the embryonic epiboly movements during gastrulation and axial mesendoderm structures during gastrulation were observed in the homozygous $foxh1^{-/-}$ tilapia. Heterozygous $foxh1^{+/-}$ individuals were identified by the presence of both heteroduplex and homoduplex amplicons in a heteroduplex mobility assay, while the $foxh1^{+/+}$ and $foxh1^{-/-}$ individuals were found to present only homoduplex amplicons (Fig. 1D). The 11 bp deletion followed by a 15 bp insertion of F2 progeny was confirmed by Sanger sequencing (Fig. 1E). This frame-shift

mutation might result in the premature termination of *foxh1* translation before the Forkhead domain based on *in silico* predication (Fig. 1F).

**Phenotype and gonadal gene expression of *foxh1* homozygous mutants**

At 90 dah, no obvious difference was observed in the ovarian morphology between *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ fish (Fig. 2a A-B). Histological analysis showed that both *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ XX fish displayed phase I and II oocytes and no significant difference were identified in GSI or oocyte number at 90 dah (Fig. 2a C-D, Fig. 2b-c). Nevertheless, remarkable gonadal dysgenesis was observed in *foxh1*$^{-/-}$ XX fish compared with the *foxh1*$^{+/+}$ control at 180 dah (Fig. 3a A-B). By DAPI staining, the *foxh1*$^{+/+}$ XX fish at 180 dah contained phase III and IV oocytes besides phase I and II oocytes, and had two layers of follicular cells including one layer of granulosa cells and one layer of theca cells. The gonads of *foxh1*$^{-/-}$ XX fish contained phase I and II oocytes with a single layer of pre-granulosa cells at this stage (Fig. 3a E-F). In addition, the number of phase I and II oocytes was significantly higher in the ovaries of *foxh1*$^{-/-}$ fish compared with *foxh1*$^{+/+}$ fish (Fig. 3b), indicating failed transitions of oocytes from phase II to phase III and of follicle cells from one layer to two layers. oocytes The GSI of the *foxh1*$^{-/-}$ XX fish was significantly lower than that of the control fish (Fig. 3c).

To understand the mechanisms by which *foxh1* works in driving female gonadal development, we performed a transcriptome analysis at 180 dah. Transcriptome sequencing of the ovaries from the *foxh1*$^{-/-}$ XX fish at 180 dah yielded a total of 49,155,870 reads. The total gene counts of the *foxh1*$^{-/-}$ XX fish (17,813) were slightly less than those of the *foxh1*$^{+/+}$ XX fish (18,336). In total, 15,503 genes were not differentially expressed and 9,882 genes were at background expression level (FPKM<1) (Fig. 5a). Compared with the control ovaries, 472 genes were upregulated and 1,231 genes were downregulated in the *foxh1*$^{-/-}$ XX fish (Table S2 and S3). Further analysis revealed that the differentially expressed genes were involved in the regulation of transcription, protein phosphorylation, wnt signaling, notch signaling and cellular responses to estrogen stimulus (Fig. 5b). Interestingly, genes promoting estrogen synthesis (e.g. *foxl2*, *star2*, *cyp19a1a*) were significantly downregulated, while genes promoting androgen synthesis (e.g. *dmrt1* and *cyp11b2*) were upregulated. The Differentially expressed genes also included genes associated with growth factor activity (e.g. *inhbb* and

*igf3*) and regulation of transcription (e.g. *tbp2* and *nobox2*). Though expression level of *gdf9* and *bmp15* were relatively high, these two genes were not among the differentially expressed genes. Real-time PCR analyses confirmed that mRNA levels of *cyp19a1a*, *foxl2* and *star2* were significantly downregulated, while *dmrt1*, *42sp50* and *cyp11b2* were significantly upregulated in *foxh1*$^{-/-}$ ovaries, validating the gonadal transcriptomic data (Fig. 5c).

FIHC and IHC revealed that Cyp19a1a was expressed in the interstitial, theca and granulosa cells of the ovary of the *foxh1*$^{+/+}$ fish at 180 dah, but only in the interstitial cells of the *foxh1*$^{-/-}$ fish (Fig. 4a A and B, Fig. S2 A and B). Expression of Cyp11b2, an enzyme responsible for the synthesis of 11-KT (a potent androgen in fish), was not found in normal XX fish (Fig. 4aC and D, Fig. S2 C and D). Unexpectedly, Cyp11b2 was found to be expressed in the interstitial cells of the ovary in the *foxh1*$^{-/-}$ fish at this stage. Western blot analysis showed that the protein level of Cyp19a1a was significantly downregulated, while the Cyp11b2 was significantly upregulated in the *foxh1*$^{-/-}$ fish (Fig. 4b and c). Consistently, the serum E2 concentrations were significantly lower, and the 11-KT concentrations were significantly higher, in the *foxh1*$^{-/-}$ fish compared to the *foxh1*$^{+/+}$ fish (Fig. 4d and e).

**Phenotype of *foxh1*$^{-/-}$XX fish could be rescued by E2 treatment**

All E2-treatment (*foxh1*$^{-/-}$ + E2) fish developed as normal females with gonad size similar to *foxh1*$^{+/+}$ fish at 240 dah, whereas all untreated *foxh1*$^{-/-}$ fish displayed gonadal dysgenesis. Histological analyses showed besides phase I and II oocytes, there were phase III and IV oocytes with two layers of follicle cells, including one layer of theca cells and one layer of granulosa cells in *foxh1*$^{+/+}$ fish at 240 dah. However, *foxh1*$^{-/-}$ fish still displayed only phase I and II oocytes at this stage, similar to that observed at 180 dah. Massive ovarian follicle enlargement and higher expression of Cyp19a1a were observed in the follicle cells and interstitial cells of the *foxh1*$^{-/-}$ + E2 fish, compared with the unrescued *foxh1*$^{-/-}$ fish (Fig. 6 A-C, Fig. S3 A-C). In addition, Cyp11b2 was not detected in *foxh1*$^{+/+}$ fish and *foxh1*$^{-/-}$ + E2 fish, while its expression was still apparent in *foxh1*$^{-/-}$ fish, indicating masculinization of the female tilapia after disruption of *foxh1* (Fig. 6 D-E, Fig. S3 D-F). To further investigate the cellular changes in ovaries of *foxh1*$^{-/-}$ XX fish at 240 dah, we used Caspase3 staining to examine cell

apoptosis. Homozygous mutation of *foxh1* resulted in apoptosis of pre-follicle cells, while no apoptosis was observed in *foxh1*$^{+/+}$ fish and *foxh1*$^{-/-}$ + E2 fish (Fig. 6 G-I).

**Discussion**

The evolutionarily conserved family of Fox genes is involved in many developmental processes, including sex determination and differentiation, as well as oogenesis. It is becoming evident that loss of *Foxl2*, *Foxo3* and *Foxc1* is detrimental to folliculogenesis and female fertility, indicating that Forkhead transcription factors are essential for follicle assembly, growth, and ovulation [14, 15]. Previous studies of *foxh1* focused primarily on its involvement in early embryogenesis, as well as the coordination of the TGF-beta/Activin/Nodal signaling pathways [20-22, 24, 25]. In a previous study, we reported the sexually dimorphic expression of *foxh1* in gonad, with extremely high expression in the cytoplasm of tilapia phase I and II oocytes, indicating a possible role of *foxh1* during early oogenesis [31]. A recent study in mice also indicated that *foxh1* is abundantly expressed in mouse oocytes, thecal cells and newly formed corpora lutea [32]. As a germ cell-specific transcription factor with higher expression in females, it is conceivable that *foxh1* plays an important role in driving ovarian differentiation, and it is likely that this function is highly conserved. Thus, functional studies are required to understand the molecular mechanisms by which *foxh1* regulates oogenesis and ovarian differentiation.

In the present study, a *foxh1* mutant line was established in tilapia using CRISPR/Cas9. Embryos of mouse, frog and zebrafish Foxh1 mutants displayed a loss of organizer and disruption of embryonic epiboly movements, indicating requirement of maternal foxh1 for development of dorsal axial structures and left-right asymmetry [25, 28, 29, 47]. However, normal development of dorsal axial structures and establishment of left-right asymmetry, as well as viable homozygous *foxh1*$^{-/-}$ tilapia, were observed in present study. One explanation could be that the maternal *foxh1* from *foxh1*$^{+/-}$ fish can partially compensate for the loss of zygotic *foxh1* function during gastrulation, as up to 50% of the *foxh1*$^{-/-}$ homozygotes survived to adulthood in zebrafish [48]. Consistent with this, more severe defects were also observed in the maternal-zygotic *foxh1* disrupted zebrafish embryos [29, 30]. Another possible explanation is the differences in target sites, as well as different knockdown and knockout

methods [28]. Of course, the possibility of some gene products compensates for the loss of function of *foxh1* could not be completely excluded in fish, as *foxh1*$^{-/-}$ homozygotes were lethal in mouse [25].

The ovarian morphology, histology and the GSI were indistinguishable from *foxh1*$^{+/+}$ gonads at 90 dah, as both were filled with a similar number of phase I and II oocytes. These results indicated that disruption of *foxh1* does not affect the initiation of meiosis and primary oocyte growth. Oocytes receive signals and nutrients from follicle cells throughout their development. Thus, it has long been recognized that somatic cells support oocyte development [2, 49, 50]. In contrast, compelling evidence of an active role for oocytes in regulating the progression of follicular development, by exchanging germ and somatic cell components of follicles at different stages of development, during oogenesis in mammals, has emerged only in the last few decades [51, 52]. In human, paracrine and autocrine signals derived from oocytes seem to be extremely important in influencing the microenvironment of the developing follicle, by regulating its own maturation as well as somatic cell proliferation, differentiation and ovulation rate [53]. Less is known about the role of oocytes in teleost folliculogenesis, as the focus has mostly been on the role of follicular cells in the steroidogenic control of oogenesis. Existing evidence in the zebrafish indicates that the oocyte is a paracrine important signaling center in driving follicle development [54]. Recent study has found that oocyte-specific *bmp15* is also required to maintain the differentiated female state throughout the adult life of zebrafish [6]. In the present study, significantly lower GSI and extremely small ovaries with no phase III and IV oocytes were observed in *foxh1*$^{-/-}$ fish at 180 dah, suggesting the failure of transition from primary growth to the cortical alveolar stage and subsequent vitellogenesis. Therefore, the signals of *foxh1* in oocytes regulate the function of surrounding follicle cells. Growing evidence suggested that the TGF-beta superfamily of receptors, ligands and binding proteins are critical for follicle recruitment, granulosa and theca cell proliferation, and oocyte maturation and ovulation [55-60]. Conditional granulosa cell deletion of *Smad2* (R-Smad) and *Smad4* causes female infertility, with defects in follicle development and ovulation [61, 62]. Previous studies demonstrated that Foxh1, as a transcriptional partner for Smad proteins [8, 63]. Therefore, Foxh1 possibly activates transcription of downstream genes involved in estrogen synthesis by binding to heteromeric

complexes of R-Smad/Smad4. Foxh1, through its interaction with the R-Smad/Smad4, also participates in mediating TGF-β/activin signaling [64]. It has been reported that recombinant goldfish activin B significantly increases the maturational rate of the full-grown post-vitellogenic follicles in the zebrafish [65]. In the present study, one of the important activin member *inhbb* was among one of the most downregulated genes in *foxh1*$^{-/-}$ ovary based on gonadal transcriptome analysis. Thus, we speculated that *foxh1* affects the transition of oocytes from phase II to phase III and follicle cells from one to two layers through interaction with *Smad2* and *Smad4* to regulate transcription levels of growth factors, such as *inhbb* and *igf3*, involved in oogenesis. Moreover, our results also supported that oocytes produce a signal that functions to stabilize female-specific gene expression in the somatic gonad, thereby promoting ovary differentiation in the early gonad, as in medaka and zebrafish [66, 67].

Endocrine hormones, especially estrogens, are involved in controlling fish ovarian differentiation and female sexual maturation [68]. Estrogen is a major driver of follicular growth and its insufficiency results in follicle apoptosis, while androgens enhance ovarian granulosa cell apoptosis [69]. In the ovary, the interstitial, granulosa and theca cells mediate steroidogenesis [2, 70, 71]. Thus, decreased estrogen levels and increased 11-KT levels might drive follicle cell apoptosis in *foxh1*$^{-/-}$ fish. And the increased apoptosis of follicle cells in turn arrests oocyte development in *foxh1*$^{-/-}$ fishes, which are characterized by the absence of cortical alveolar oocytes (phase III oocytes) in the ovary. Expression of Cyp11b2 is undetectable in the ovary of normal XX fish at all stages, whereas the signal of Cyp11b2 is observed in the *foxh1*$^{-/-}$ fish at 180 and 240 dah. Therefore, we believe the differences in hormone and gene expression observed were mostly due to the disruption of *foxh1*. Of course, big differences existed between the WT and mutant fish in ovary structure at 180 dah and later stages. The differences in hormone and gene expression observed between WT and *foxh1*$^{-/-}$ fish from differences in the ovary structure could not be completely excluded. The successfully rescued phenotypes of *foxh1*$^{-/-}$ fish by E2 treatment again supported the critical role estrogen plays in maintaining the follicles and promoting their further commitment to folliculogenesis in tilapia. Taking in conclusion, our results suggest that the *foxh1* activated

oocyte signal is required for estrogen biosynthesis and oogensis, as other oocyte-specific factors *figlα* and *bmp15* does in zebrafish [6, 9].

Aromatase, encoded by *cyp19a1a*, is the key enzyme that produces estrogens and therefore a key factor in controlling oogenesis. Our previous study demonstrated Foxl2 and Sf1 synergistically promote the transcription of *cyp19a1a* expression in tilapia and medaka [72, 73], which was further confirmed by a recent study in rainbow trout [74]. On the other hand, disruption of *foxl2* resulted in upregulation of *dmrt1* and *vice versa* [13, 72, 75, 76]. Two *star* genes were identified in vertebrates, and *star2* was found to be more highly expressed in tilapia ovary than testis [77]. In the present study, disruption of *foxh1*$^{-/-}$ led to a decrease in expression of female-biased genes *foxl2*, *star2* and *cyp19a1a* and upregulated expression of *dmrt1* and *cyp11b2*. The simultaneous downregulation of *foxl2* and upregulation of *dmrt1* again demonstrated the antagonistic role of *foxl2* and *dmrt1* [13, 75]. Oogenesis is a complex process, which is governed by gonadotropins and intraovarian factors in an endocrine or autocrine manner [1, 54]. Considerable progress has been made in basic aspects of oogenesis research at the molecular level. Mutation of other ovary-biased transcriptional factors also leads to ovarian infertility [7, 69, 78, 79]. For example, dysregulation of oocyte-specific *nobox* and *tbp* abolished the transition from primordial to growing follicles and accelerated postnatal oocyte loss [8, 80]. Consistent with this, *nobox* and *tbp* were differentially expressed in *foxh1*$^{-/-}$ tilapia. Besides these transcription factors, expression profiles of other genes involved in ovarian differentiation were also changed in *foxh1*$^{-/-}$ fishes. The downregulation of *connexin37* in *foxh1*$^{-/-}$ ovaries, which affects oocyte-granulosa cell gap junctions duiring folliculogenesis [81], suggested that disruption of *foxh1* might also affect cell junction and formation of cortical alveolar oocytes during oogenesis. However, the precise genomic targets of *foxh1* in ovaries have not yet been fully identified. More work is needed to further define its physiological roles.

In summary, *foxh1* was exclusively expressed in the phase I and II oocytes of the Nile tilapia. Loss of *foxh1* resulted in decreased estrogen level, as well as dysregulation of the bidirectional communication between oocytes and their surrounding somatic cells, followed

by arrest of folliculogenesis and subsequent infertility. Our findings suggest that oocyte-specific *foxh1* is required for oogenesis, laying a foundation for future studies aimed at unraveling the specific mechanisms by which oocytes orchestrate follicular development. And the present study also enrich our understanding of the role that forkhead genes play in gametogenesis in teleosts, and in vertebrates generally.

**References**


1. Nagahama Y, Yoshikuni M, Yamashita M, Tokumoto T, Katsu Y. Regulation of Oocyte Growth and Maturation in Fish. *Curr Top Dev Biol* 1995, 30:103-145.
2. Lubzens E, Young G, Bobe J, Cerda J. Oogenesis in teleosts: how eggs are formed. *Gen Comp Endocrinol* 2010, 165(3):367-389.
3. Nishimura T, Tanaka M. Gonadal development in fish. *Sex Dev* 2014, 8(5):252-261.
4. Bobe J, Maugars G, Nguyen T, Rime H, Jalabert B. Rainbow trout follicular maturational competence acquisition is associated with an increased expression of follicle stimulating hormone receptor and insulin-like growth factor 2 messenger RNAs. *Mol Reprod Dev* 2003, 66(1):46-53.
5. Gohin M, Bodinier P, Fostier A, Chesnel F, Bobe J. Aromatase Is Expressed and Active in the Rainbow Trout Oocyte During Final Oocyte Maturation. *Molecular Reproduction and Development* 2011, 78(7):510-518.
6. Dranow DB, Hu K, Bird AM, Lawry ST, Adams MT, Sanchez A, Amatruda JF, Draper BW. Bmp15 Is an Oocyte-Produced Signal Required for Maintenance of the Adult Female Sexual Phenotype in Zebrafish. *Plos Genetics* 2016, 12(9).
7. Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R, Galloway SM. Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (*Ovis aries*). *Biology of Reproduction* 2004, 70(4):900-909.
8. Rajkovic A, Pangas SA, Ballow D, Suzumori N, Matzuk MM. NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. *Science* 2004, 305(5687):1157-1159.
9. Qin M, Zhang Z, Song W, Wong QW, Chen W, Shirgaonkar N, Ge W. Roles of Figla/figla in Juvenile Ovary Development and Follicle Formation During Zebrafish Gonadogenesis. *Endocrinology* 2018, 159(11):3699-3722.
10. Carlsson P, Mahlapuu M. Forkhead transcription factors: key players in development and metabolism. *Dev Biol* 2002, 250(1):1-23.
11. Kaestner KH, Knochel W, Martinez DE. Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev* 2000, 14(2):142-146.
12. Castrillon DH, Miao LL, Kollipara R, Horner JW, DePinho RA. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science* 2003, 301(5630):215-218.

13. Li MH, Yang HH, Li MR, Sun YL, Jiang XL, Xie QP, Wang TR, Shi HJ, Sun LN, Zhou LY *et al*. Antagonistic roles of Dmrt1 and Foxl2 in sex differentiation via estrogen production in tilapia as demonstrated by TALENs. *Endocrinology* 2013, 154(12):4814-4825.
14. Mattiske D, Kume T, Hogan BL. The mouse forkhead gene Foxc1 is required for primordial germ cell migration and antral follicle development. *Dev Biol* 2006, 290(2):447-458.
15. Ottolenghi C, Omari S, Garcia-Ortiz JE, Uda M, Crisponi L, Forabosco A, Pilia G, Schlessinger D. Foxl2 is required for commitment to ovary differentiation. *Hum Mol Genet* 2005, 14(14):2053-2062.
16. Zhang XB, Li MR, Ma H, Liu XY, Shi HJ, Li MH, Wang DS. Mutation of foxl2 or cyp19a1a Results in Female to Male Sex Reversal in XX Nile Tilapia. *Endocrinology* 2017, 158(8):2634-2647.
17. John GB, Gallardo TD, Shirley LJ, Castrillon DH. Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth. *Dev Biol* 2008, 321(1):197-204.
18. Uhlenhaut NH, Treier M. Forkhead transcription factors in ovarian function. *Reproduction* 2011, 142(4):489-495.
19. Chen GC, Nomura M, Morinaga H, Matsubara E, Okabe T, Goto K, Yanase T, Zheng H, Lu J, Nawata H. Modulation of androgen receptor transactivation by FoxH1 - A newly identified androgen receptor corepressor. *J Biol Chem* 2005, 280(43):36355-36363.
20. Chen X, Rubock MJ, Whitman M. A transcriptional partner for MAD proteins in TGF-beta signalling. *Nature* 1996, 383(6602):691-696.
21. Nagarajan RP, Liu J, Chen Y. Smad3 inhibits transforming growth factor-beta and activin signaling by competing with Smad4 for FAST-2 binding. *J Biol Chem* 1999, 274(44):31229-31235.
22. Watanabe M, Whitman M. FAST-1 is a key maternal effector of mesoderm inducers in the early Xenopus embryo. *Development* 1999, 126(24):5621-5634.
23. Charney RM, Forouzmand E, Cho JS, Cheung J, Paraiso KD, Yasuoka Y, Takahashi S, Taira M, Blitz IL, Xie X *et al*. Foxh1 Occupies cis-Regulatory Modules Prior to Dynamic Transcription Factor Interactions Controlling the Mesendoderm Gene Program. *Dev Cell* 2017, 40(6):595-607 e594.
24. Halstead AM, Wright CV. Disrupting Foxh1-Groucho interaction reveals robustness of nodal-based embryonic patterning. *Mech Dev* 2015, 136:155-165.
25. Yamamoto M, Meno C, Sakai Y, Shiratori H, Mochida K, Ikawa Y, Saijoh Y, Hamada H. The transcription factor FoxH1 (FAST) mediates Nodal signaling during

anterior-posterior patterning and node formation in the mouse. *Genes Dev* 2001, 15(10):1242-1256.

26. Cheng Q, Shi YJ, Li Z, Kang H, Xiang Z, Kong LF. FAST1 promotes the migration and invasion of colorectal cancer cells. *Biochem Biophys Res Commun* 2019, 509(2):407-413.
27. Choi J, Dong L, Ahn J, Dao D, Hammerschmidt M, Chen JN. FoxH1 negatively modulates flk1 gene expression and vascular formation in zebrafish. *Dev Biol* 2007, 304(2):735-744.
28. Pei W, Noushmehr H, Costa J, Ouspenskaia MV, Elkahloun AG, Feldman B. An early requirement for maternal FoxH1 during zebrafish gastrulation. *Dev Biol* 2007, 310(1):10-22.
29. Sirotkin HI, Gates MA, Kelly PD, Schier AF, Talbot WS. fast1 is required for the development of dorsal axial structures in zebrafish. *Curr Biol* 2000, 10(17):1051-1054.
30. Santos EM, Workman VL, Paull GC, Filby AL, Van Look KJ, Kille P, Tyler CR. Molecular basis of sex and reproductive status in breeding zebrafish. *Physiol Genomics* 2007, 30(2):111-122.
31. Yuan J, Tao WJ, Cheng YY, Huang BF, Wang DS. Genome-wide identification, phylogeny, and gonadal expression of fox genes in Nile tilapia, *Oreochromis niloticus*. *Fish Physiol Biochem* 2014, 40(4):1239-1252.
32. Wang G, Liu L, Guo S, Zhang C. Expression and distribution of forkhead activin signal transducer 2 (FAST2) during follicle development in mouse ovaries and pre-implantation embryos. *Acta Histochem* 2016, 118(6):632-639.
33. Kobayashi T, Kajiura-Kobayashi H, Nagahama Y. Differential expression of vasa homologue gene in the germ cells during oogenesis and spermatogenesis in a teleost fish, tilapia, *Oreochromis niloticus*. *Mech Dev* 2000, 99(1-2):139-142.
34. Li MH, Yang HH, Zhao JU, Fang LL, Shi HJ, Li MR, Sun YL, Zhang XB, Jiang DN, Zhou LY *et al*. Efficient and Heritable Gene Targeting in Tilapia by CRISPR/Cas9. *Genetics* 2014, 197(2):591-U219.
35. Conte MA, Gammerdinger WJ, Bartie KL, Penman DJ, Kocher TD. A high quality assembly of the Nile Tilapia (*Oreochromis niloticus*) genome reveals the structure of two sex determination regions. *Bmc Genomics* 2017, 18.
36. Jiang DN, Chen JL, Fan Z, Tan DJ, Zhao J, Shi HJ, Liu ZL, Tao WJ, Li MH, Wang DS. CRISPR/Cas9-induced disruption of wt1a and wt1b reveals their different roles in kidney and gonad development in Nile tilapia. *Developmental Biology* 2017, 428(1):63-73.

37. Coward K, Bromage NR. Histological classification of oocyte growth and the dynamics of ovarian recrudescence in *Tilapia zillii*. *J Fish Biol* 1998, 53(2):285-302.
38. Xie QP, He X, Sui YN, Chen LL, Sun LN, Wang DS. Haploinsufficiency of SF-1 Causes Female to Male Sex Reversal in Nile Tilapia, *Oreochromis niloticus*. *Endocrinology* 2016, 157(6):2500-2514.
39. Kobayashi T, Kajiura-Kobayashi H, Nagahama Y. Induction of XY sex reversal by estrogen involves altered gene expression in a teleost, tilapia. *Cytogenet Genome Res* 2003, 101(3-4):289-294.
40. Zhang XB, Wang H, Li MH, Cheng YY, Jiang DN, Sun LN, Tao WJ, Zhou LY, Wang ZJ, Wang DS. Isolation of Doublesex- and Mab-3-Related Transcription Factor 6 and Its Involvement in Spermatogenesis in Tilapia. *Biology of Reproduction* 2014, 91(6).
41. Tao WJ, Yuan J, Zhou LY, Sun LN, Sun YL, Yang SJ, Li MH, Zeng S, Huang BF, Wang DH. Characterization of Gonadal Transcriptomes from Nile Tilapia (*Oreochromis niloticus*) Reveals Differentially Expressed Genes. *Plos One* 2013, 8(5).
42. Kim D, Landmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* 2015, 12(4):357-U121.
43. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nat Protoc* 2016, 11(9):1650-1667.
44. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 2009, 37(1):1-13.
45. Chen JL, Jiang DN, Tan DJ, Fan Z, Wei YY, Li MH, Wang DS. Heterozygous mutation of eEF1A1b resulted in spermatogenesis arrest and infertility in male tilapia, *Oreochromis niloticus*. *Sci Rep-Uk* 2017, 7.
46. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* 2001, 25(4):402-408.
47. Kofron M, Puck H, Standley H, Wylie C, Old R, Whitman M, Heasman J. New roles for FoxH1 in patterning the early embryo. *Development* 2004, 131(20):5065-5078.
48. Pogoda HM, Solnica-Krezel L, Driever W, Meyer D. The zebrafish forkhead transcription factor FoxH1/Fast1 is a modulator of nodal signaling required for organizer formation. *Curr Biol* 2000, 10(17):1041-1049.
49. Brantley SE, Fuller MT. Somatic support cells regulate germ cell survival through the Baz/aPKC/Par6 complex. *Development* 2019, 146(8).

50. Jalabert B. Production of Fertilizable Oocytes from Follicles of Rainbow-Trout (Salmo-Gairdnerii) Following Invitro Maturation and Ovulation. *Ann Biol Anim Bioch* 1978, 18(Nb2):461-470.
51. Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction* 2001, 122(6):829-838.
52. Eppig JJ. Reproduction: Oocytes Call, Granulosa Cells Connect. *Curr Biol* 2018, 28(8):R354-R356.
53. Emori C, Sugiura K. Role of oocyte-derived paracrine factors in follicular development. *Anim Sci J* 2014, 85(6):627-633.
54. Ge W. Intrafollicular paracrine communication in the zebrafish ovary: the state of the art of an emerging model for the study of vertebrate folliculogenesis. *Mol Cell Endocrinol* 2005, 237(1-2):1-10.
55. Drummond AE, Le MT, Ethier JF, Dyson M, Findlay JK. Expression and localization of activin receptors, Smads, and beta glycan to the postnatal rat ovary. *Endocrinology* 2002, 143(4):1423-1433.
56. Findlay JK, Drummond AE, Dyson ML, Baillie AJ, Robertson DM, Ethier JF. Recruitment and development of the follicle; the roles of the transforming growth factor-beta superfamily. *Mol Cell Endocrinol* 2002, 191(1):35-43.
57. Luo S, Kleemann GA, Ashraf JM, Shaw WM, Murphy CT. TGF-beta and insulin signaling regulate reproductive aging via oocyte and germline quality maintenance. *Cell* 2010, 143(2):299-312.
58. Wang Y, Ge W. Spatial expression patterns of activin and its signaling system in the zebrafish ovarian follicle: evidence for paracrine action of activin on the oocytes. *Biol Reprod* 2003, 69(6):1998-2006.
59. Xu J, Oakley J, McGee EA. Stage-specific expression of Smad2 and Smad3 during folliculogenesis. *Biol Reprod* 2002, 66(6):1571-1578.
60. Zheng S, Long J, Liu Z, Tao W, Wang D. Identification and Evolution of TGF-beta Signaling Pathway Members in Twenty-Four Animal Species and Expression in Tilapia. *Int J Mol Sci* 2018, 19(4).
61. Li Q, Pangas SA, Jorgez CJ, Graff JM, Weinstein M, Matzuk MM. Redundant roles of SMAD2 and SMAD3 in ovarian granulosa cells in vivo. *Mol Cell Biol* 2008, 28(23):7001-7011.
62. Pangas SA, Li X, Robertson EJ, Matzuk MM. Premature luteinization and cumulus cell defects in ovarian-specific Smad4 knockout mice. *Mol Endocrinol* 2006, 20(6):1406-1422.

63. Peng J, Li Q, Wigglesworth K, Rangarajan A, Kattamuri C, Peterson RT, Eppig JJ, Thompson TB, Matzuk MM. Growth differentiation factor 9:bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. *Proc Natl Acad Sci U S A* 2013, 110(8):E776-785.
64. Attisano L, Silvestri C, Izzi L, Labbe E. The transcriptional role of Smads and FAST (FoxH1) in TGFbeta and activin signalling. *Mol Cell Endocrinol* 2001, 180(1-2):3-11.
65. Pang Y, Ge W. Epidermal growth factor and TGFalpha promote zebrafish oocyte maturation in vitro: potential role of the ovarian activin regulatory system. *Endocrinology* 2002, 143(1):47-54.
66. Kurokawa H, Saito D, Nakamura S, Katoh-Fukui Y, Ohta K, Baba T, Morohashi KI, Tanaka M. Germ cells are essential for sexual dimorphism in the medaka gonad. *P Natl Acad Sci USA* 2007, 104(43):16958-16963.
67. Slanchev K, Stebler J, de la Cueva-Mendez G, Raz E. Development without germ cells: The role of the germ line in zebrafish sex differentiation. *P Natl Acad Sci USA* 2005, 102(11):4074-4079.
68. Guiguen Y, Fostier A, Piferrer F, Chang CF. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. *Gen Comp Endocrinol* 2010, 165(3):352-366.
69. Billig H, Furuta I, Hsueh AJ. Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. *Endocrinology* 1993, 133(5):2204-2212.
70. Nagahama Y, Yamashita M. Regulation of oocyte maturation in fish. *Dev Growth Differ* 2008, 50 Suppl 1:S195-219.
71. Nagahama Y. Endocrine regulation of gametogenesis in fish. *Int J Dev Biol* 1994, 38(2):217-229.
72. Wang DS, Kobayashi T, Zhou LY, Paul-Prasanth B, Ijiri S, Sakai F, Okubo K, Morohashi K, Nagahama Y. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor 1. *Mol Endocrinol* 2007, 21(3):712-725.
73. Watanabe M, Tanaka M, Kobayashi D, Yoshiura Y, Oba Y, Nagahama Y. Medaka (*Oryzias latipes*) FTZ-F1 potentially regulates the transcription of P-450 aromatase in ovarian follicles: cDNA cloning and functional characterization. *Mol Cell Endocrinol* 1999, 149(1-2):221-228.
74. Bertho S, Herpin A, Branthonne A, Jouanno E, Yano A, Nicol B, Muller T, Pannetier M, Pailhoux E, Miwa M *et al*. The unusual rainbow trout sex determination gene hijacked

the canonical vertebrate gonadal differentiation pathway. *P Natl Acad Sci USA* 2018, 115(50):12781-12786.

75. Wang DS, Zhou LY, Kobayashi T, Matsuda M, Shibata Y, Sakai F, Nagahama Y. Doublesex- and Mab-3-Related Transcription Factor-1 Repression of Aromatase Transcription, a Possible Mechanism Favoring the Male Pathway in Tilapia. *Endocrinology* 2010, 151(3):1331-1340.
76. Uhlenhaut NH, Jakob S, Anlag K, Eisenberger T, Sekido R, Kress J, Treier AC, Klugmann C, Klasen C, Holter NI *et al*. Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell* 2009, 139(6):1130-1142.
77. Yu X, Wu L, Xie L, Yang S, Charkraborty T, Shi H, Wang D, Zhou L. Characterization of two paralogous StAR genes in a teleost, Nile tilapia (*Oreochromis niloticus*). *Mol Cell Endocrinol* 2014, 392(1-2):152-162.
78. Zhang Z, Lau SW, Zhang L, Ge W. Disruption of Zebrafish Follicle-Stimulating Hormone Receptor (fshr) But Not Luteinizing Hormone Receptor (lhcgr) Gene by TALEN Leads to Failed Follicle Activation in Females Followed by Sexual Reversal to Males. *Endocrinology* 2015, 156(10):3747-3762.
79. Jagarlamudi K, Rajkovic A. Oogenesis: Transcriptional regulators and mouse models. *Mol Cell Endocrinol* 2012, 356(1-2):31-39.
80. Gazdag E, Santenard A, Ziegler-Birling C, Altobelli G, Poch O, Tora L, Torres-Padilla ME. TBP2 is essential for germ cell development by regulating transcription and chromatin condensation in the oocyte. *Gene Dev* 2009, 23(18):2210-2223.
81. Kidder GM, Mhawi AA. Gap junctions and ovarian folliculogenesis. *Reproduction* 2002, 123(5):613-620.

## Figure legends

**Fig. 1 The establishment of *foxh1* homozygous mutation line.**

(A) The target site was located in the second exon of *foxh1*. Underlined bases indicate the *HaeIII* cleavage site. F and R indicate the locations of the forward and reverse PCR primers. (B) In vitro-synthesized 250 ng/μl Cas9 and 500 ng/μl gRNA were co-injected into one-cell-stage embryos. Two cleavage bands were detected in the control group, while an additional intact DNA fragment was observed in embryos injected with both Cas9 mRNA and target gRNA. (C) F1 mutant fish were obtained by crossing a wild-type XX female with a F0 mosaic XY male. The F2 mutant fish were obtained by crossing the *foxh1*$^{+/-}$ XX and XY fish, which was obtained by crossing *foxh1* F0 mosaic XY fish with wild-type XX fish. (D) A heteroduplex motility assay identified the *foxh1*$^{+/-}$ individuals as those possessing both heteroduplex and homoduplex amplicons versus the *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ individuals with only homoduplex amplicons. (E) Identification of F2 genotypes by sequencing. *foxh1*$^{-/-}$ XX and XY fish carried the 4 bp insertion (an 11 bp deletion and 15 bp insertion). (F) The frame-shift mutation resulted in the premature termination before Forkhead domain in *foxh1*$^{-/-}$ fish based on *in silico* predication. The symbols $^{+/+}$, $^{+/-}$ and $^{-/-}$ indicate wild-type, heterozygous and homozygous fish. "*" indicates the stop codon. FH, Forkhead domain. WT, wild type.

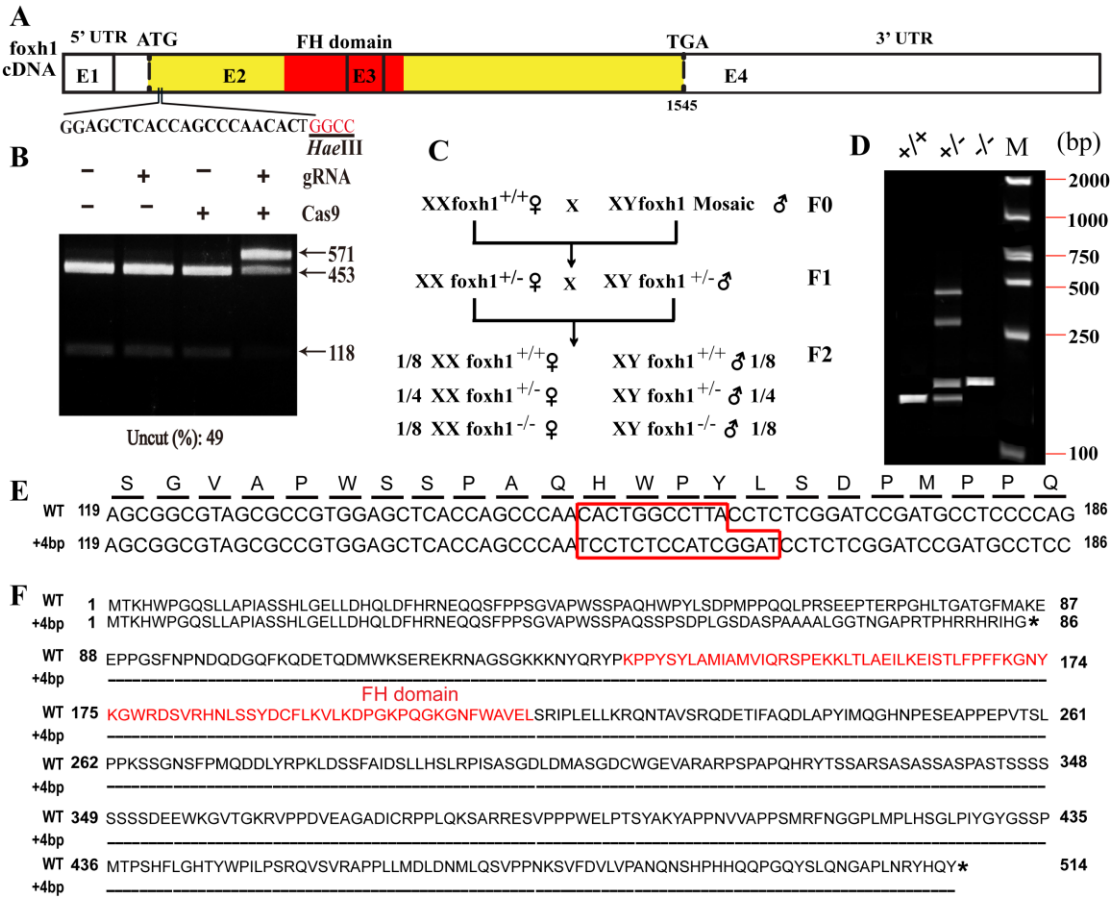

**Fig. 2 Effects of homozygous mutation of *foxh1* on oogenesis in XX fish at 90 dah.**

(a) Morphological observation and histological analysis. (A-B) Ovary morphological observation of *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ fish. (C-D) Histological analysis of *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ fish ovary. Phase I and II oocytes were observed in the ovary of both *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ fish. Phase I and II oocytes were observed in the ovary of both *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ fish. (b) The number of phase I and II oocytes in the *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ fish expressed as the mean ± SD (n=6). (c) No significant differences in GSI were observed between *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ fish, as determined by Student's t test. dah, days after hatching.

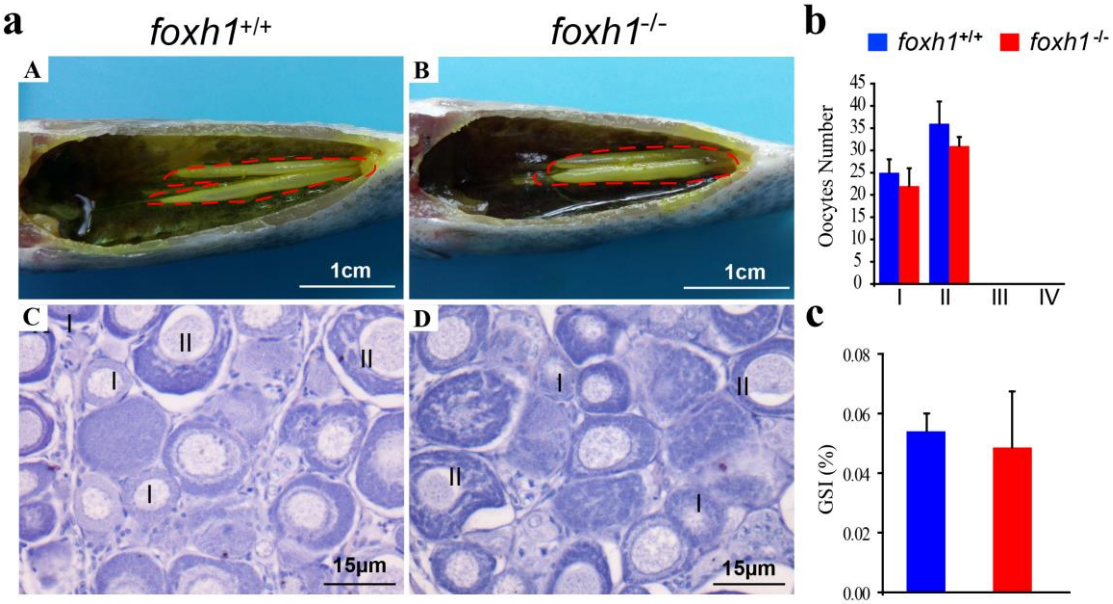

**Fig. 3 Effects of homozygous mutation of *foxh1* on oogenesis in XX fish at 180 dah.**

(a) Morphological observation and histological analysis. (A-B) Ovary morphological observation of *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ fish. (C-D) Histological analysis of *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ fish ovary. Phase I, II, III and IV oocytes were observed in the ovary of *foxh1*$^{+/+}$ fish, while only Phase I and II were found in *foxh1*$^{-/-}$ fish. OC, oocytes; TC, theca cells; GC, granulosa cells; pGC, pre-granulosa cells. (E-F) 4'6-diamidino-2-phenylindole (DAPI) of *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ ovary. (b) Number of phase I-IV oocytes in the *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ XX fish. Data are expressed as the mean ± SD (n=6). Significant differences in GSI and oocyte number were observed between *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ fish, indicated by"*" ($P<0.05$) above the error bar, as determined by Student's t test. (c) GSI of the *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ fish. dah, days after hatching. N.D. indicates not detectable.

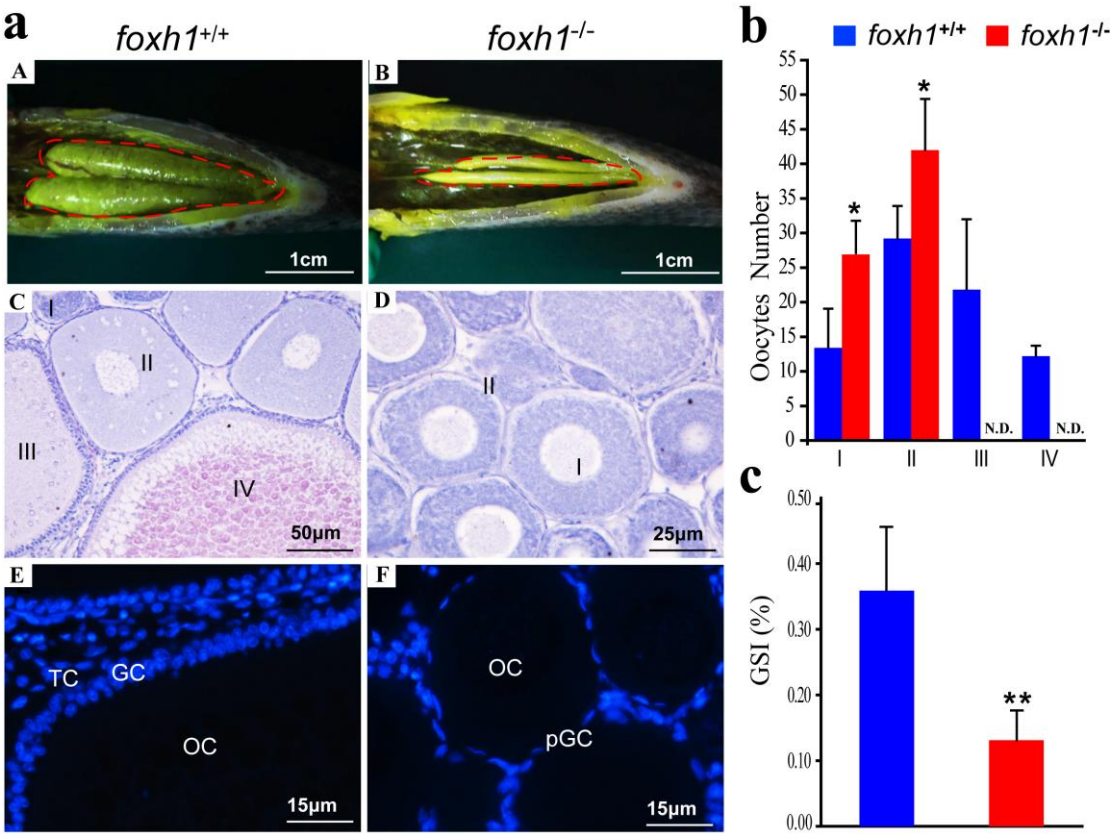

**Fig. 4 Effects of *foxh1* homozygous mutation on E2 and 11-KT synthesis at 180 dah.**

(a) Expression of Cyp19a1a and Cyp11b2 in the gonads of the *foxh1*$^{-/-}$ and *foxh1*$^{+/+}$ XX fish by FIHC. (A-B) At 180 dah, Cyp19a1a is expressed in the interstitial, theca and granulosa cells of the ovary of the *foxh1*$^{+/+}$ fish, while only in the interstitial cells of the *foxh1*$^{-/-}$ fish. (C-D) Expression of Cyp11b2 was not found in normal *foxh1*$^{+/+}$ fish, while it was found to be expressed in the interstitial cells of the ovary in the *foxh1*$^{-/-}$ fish. (b and c) Expression of Cyp19a1a and Cyp11b2 in the gonads of the *foxh1*$^{-/-}$ and *foxh1*$^{+/+}$ XX fish by Western blot. The expression level was normalized using α-tubulin as the internal control protein (n=3). OC, oocytes; TC, theca cells; GC, granulosa cells. (d and e) Serum E2 and 11-KT concentrations. Data were expressed as the mean ± SD (n=6). "**" and "*" above the error bar indicate significant difference at P<0.01 and 0.05 as determined by Student's t test, respectively.

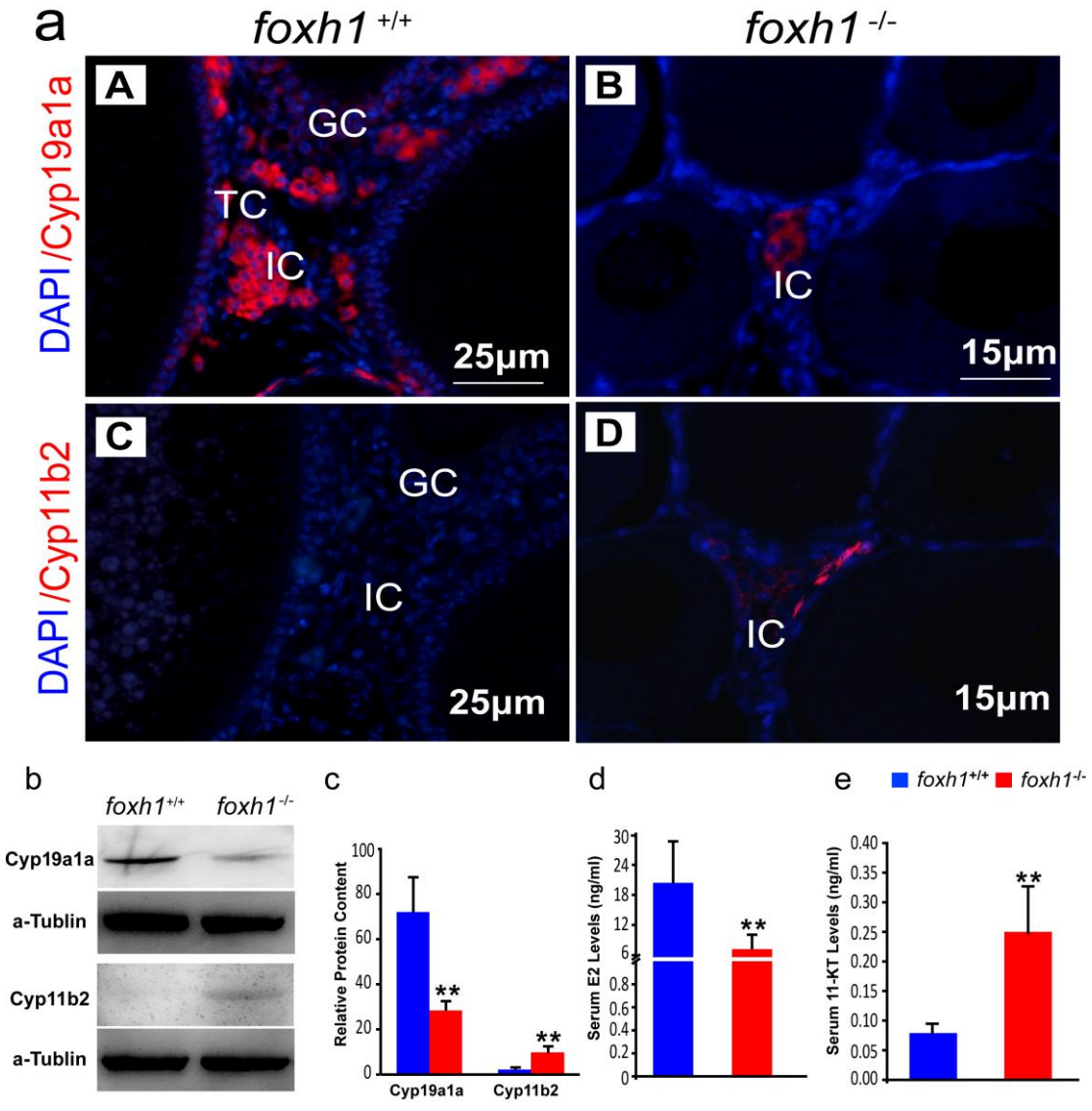

**Fig. 5 Transcriptomic analyses of gene expression profiles in *foxh1*$^{-/-}$ ovaries at 180 dah.** (a) Comparison of gonadal gene expression between *foxh1*$^{+/+}$ and *foxh1*$^{-/-}$ fish. Genes expressed in ovaries were divided into three parts: 472 up-regulated genes, 1,231 down-regulated genes, and 15,503 non-differentially expressed genes. (b) GO enrichment analysis of differentially expressed genes. (c) Validation of the transcriptome data by real-time PCR. All examined genes displayed similar expression profiles to those from the transcriptomic data.

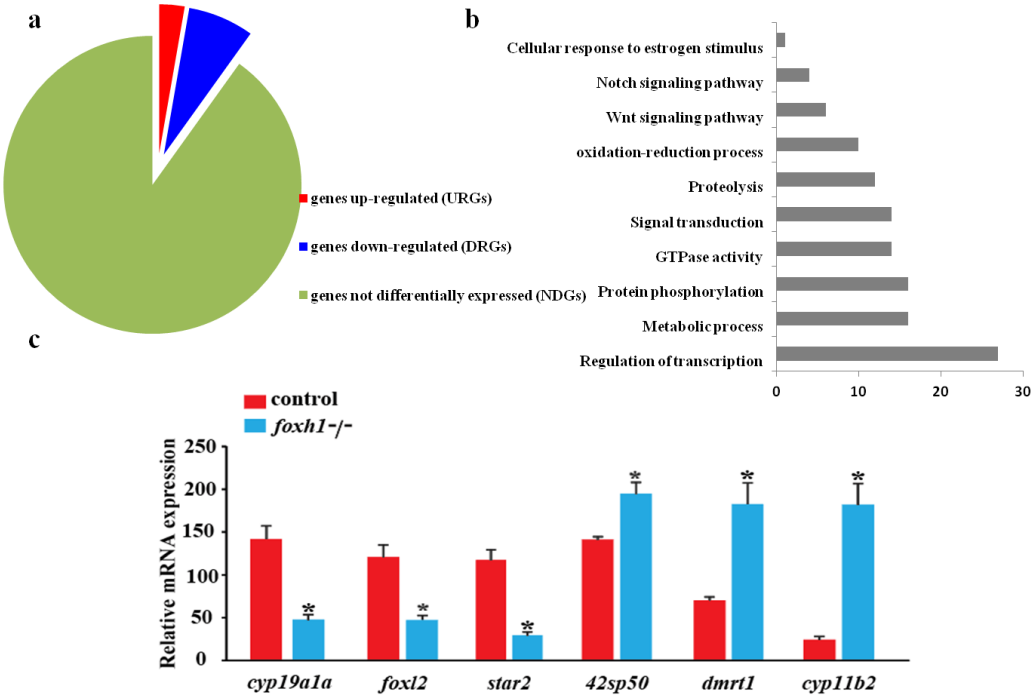

**Fig. 6 Administration of E2 rescued arrest of oogenesis in *foxh1*$^{-/-}$ fish at 240 dah.**

Expression of Cyp19a1a, Cyp11b2 and Caspase3 in the gonads of the *foxh1*$^{+/+}$, *foxh1*$^{-/-}$ and *foxh1*$^{-/-}$ +E2 XX fish by FIHC. The ovaries of *foxh1*$^{+/+}$ fish contained both phase I and II oocytes with only one layer of follicular cells and III and IV oocytes with two layers of follicular cells. In contrast, the gonads of *foxh1*$^{-/-}$ fish remained at early stages of oogenesis, arrested at phase I and II oocytes with one layer of follicular cells. Cyp191a1 was expressed in follicle and interstitial cells (A-C). Cyp11b2 was expressed in interstitial cells in the ovary of *foxh1*$^{-/-}$ fish, but not in the ovary of the *foxh1*$^{-/-}$ + E2 fish and *foxh1*$^{+/+}$ fish (D-F). Homozygous mutation of *foxh1* resulted in the dramatic increase of positive Caspase 3 staining in pre-follicle cells (G-I). GC, granulosa cells; IC, interstitial cells; TC, theca cells.