RESEARCH ARTICLE

Figla Favors Ovarian Differentiation by Antagonizing Spermatogenesis in a Teleosts, Nile Tilapia (Oreochromis niloticus)

Yongxiu Qiu1☯, Shaohua Sun1☯, Tapas Charkraborty2☯, Limin Wu1☯, Lina Sun1, Jing Wei1, Yoshitaka Nagahama2, Deshou Wang1*, Linyan Zhou1*

1 Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), School of Life Science, Southwest University, Beibei, Chongqing, China, 2 South Ehime Fisheries Research Center, Ehime University, Funakoshi, Ainan, Ehime, Japan

☯ These authors contributed equally to this work.
* wdeshou@swu.edu.cn (DSW); yanlinzhou916@126.com (LYZ)

Abstract

Figla (factor in the germ line, alpha), a female germ cell-specific transcription factor, had been shown to activate genetic hierarchies in oocytes. The ectopic expression of Figla was known to repress spermatogenesis-associated genes in male mice. However, the potential role of Figla in other vertebrates remains elusive. The present work was aimed to identify and characterize the functional relevance of Figla in the ovarian development of Nile tilapia (Oreochromis niloticus). Tissue distribution and ontogeny analysis revealed that tilapia Figla gene was dominantly expressed in the ovary from 30 days after hatching. Immunohistochemistry analysis also demonstrated that Figla was expressed in the cytoplasm of early primary oocytes. Intriguingly, over-expression of Figla in XY fish resulted in the disruption of spermatogenesis along with the depletion of meiotic spermatocytes and spermatids in testis. Dramatic decline of sycp3 (synaptonemal complex protein 3) and prm (protamine) expression indicates that meiotic spermatocytes and mature sperm production are impaired. Even though Sertoli cell (dmrt1) and Leydig cell (star and cyp17a1) marker genes remained unaffected, hsd3b1 expression and 11-KT production were enhanced in Figla-transgene testis. Taken together, our data suggest that fish Figla might play an essential role in the ovarian development by antagonizing spermatogenesis.

Introduction

In mammals, primary sex determination is commenced by the presence or absence of the Y chromosome, which controls the fate of the bipotential gonad. [1, 2]. Numerous studies have shown that sex determining signaling molecules in gonadal somatic cells controls the primordial germ cell (PGC) differentiation and gonad formation. Various studies have also considerably promoted the contributions of germ cells in sex differentiation and gonad formation [3–5]. Loss of germ cells in the XX gonad preferably activates the postnatal transdifferentiation of
granulosa cell lineage to Sertoli cells [6], which indicates the involvement of germ cells in suppressing the male pathway in females mice. *Figla* (factor in the germ line, alpha), which encodes a germ cell-specific basic helix-loop-helix transcription factor, is also known to express more abundantly in female germ cells than male [7]. It was reported that mutation of *Figla* disrupts folliculogenesis and further imposes sterility in females [8, 9]. On the contrary, the ectopic expression of *Figla* in male germ cells has also resulted in the down-regulation of a subset of testis-associated genes, sterility with impaired meiosis and germ cell apoptosis. These aforementioned data demonstrate that *Figla* plays dual roles in both the activation of oocyte-associated genes and the repression of sperm-associated genes during normal postnatal oogenesis [10].

Teleosts are characterized by diverse sex determination mechanisms [11]. However, the majority of downstream sex-related factors in somatic cells of gonads remain conserved throughout the vertebrate kingdom. In teleosts, germ cells development in ovary undergo three main processes: (i) migration/patterning of PGCs, (ii) proliferation of germ cells, and (iii) meiotic initiation at the critical timing of sex determination [12]. Genes related to PGC migration and maintenance has been extensively investigated for their involvements in sex determination and differentiation in fish. Similar to mouse, depletion of PGCs in genetic female individual has also resulted in the development of sterile testis in zebrafish [13, 14], medaka [15] and tilapia [16]. Rodriguez-Marí et al. [17] reported that meiotic failure or excessive apoptosis of oocytes in adult zebrafish gonad impaired the fertility, suggesting that oocytes-derived signals are essential in promoting ovarian differentiation. Previous studies have detected robust expression of *Figla* in the early primary oocytes in several teleosts [18–20]. Moreover, in black porgy (*Acanthopagrus schlegeli*), *Figla* is even suspected of playing a critical role in controlling the transformation of Sertoli cell into follicle-like cells during the sex-reversal from male to female [20].

Tilapia, an economically important species for aquaculture with XX/XY sex determination system, is known to be an ideal model to study fish sex determination/differentiation due to the availability of monosex larvae, genome information and well-documented sexual development processes. Recently, several reports have demonstrated the essentiality of molecular signaling and estrogen production from somatic cells in tilapia sex determination/differentiation [21–23]. Meanwhile, the timing of meiotic initiation of germ cells in tilapia also demonstrates the evident sexual dimorphism, suggesting that meiotic germ cells might be involved in sex differentiation [24, 25]. However, the exact roles of meiotic germ cells in fish sex differentiation still remain elusive. To elucidate the potential role of meiotic oocytes in ovarian formation and maintenance, we characterized *Figla* and analyzed its expression profile at different stages of gonadal differentiation. Furthermore, over-expression of *Figla* in the XY fish was also carried out. Our data demonstrated that the ectopic expression of *Figla* in XY fish resulted in the depletion of spermatocytes and mature sperm, and down-regulated the spermatogenesis-associated gene expression. Our present work suggests that fish *Figla* might play an important role in ovarian development via repressing the expression of spermatogenesis-associated genes.

**Materials and Methods**

**Fish**

Tilapias were reared in large tanks with a re-circulating aerated freshwater system. Fish were maintained at ambient temperature (26°C) under natural photoperiod. All genetic females (XX) and males (XY) were obtained by artificial fertilization of eggs from normal female (XX) with sperms from either sex reversed male (XX) or super male (YY), respectively. The super males (YY) were obtained by crossing the normal XY-male with the XY-female, which was
sex-reversed hormonally by E2 treatment. All experiments were conducted in accordance with the legal requirements of China. The Committee of Laboratory Animal Experimentation at Southwest University, China, approved all procedures and protocols related to treatment and maintenance of the animals.

**Identification of tilapia Figla gene**

The deduced mRNA sequence including the open reading frame (ORF) and untranslated regions for Figla gene was isolated from the 3-month-old tilapia gonadal transcriptome database [26]. Gene specific primers were designed to amplify the mRNA of Figla gene from the ovary. All PCR products were ligated into the pGEM-T easy vector (Promega, USA) and sequenced at Life Technologies Corporation (Shanghai, China). Gene structure (exon-intron distribution) was deduced by aligning the mRNA sequence and tilapia genomic DNA sequence of Figla gene (retrieved from http://www.ensembl.org/Oreochromis_niloticus/Info/Index).

**Phylogenetic and syntenic analysis**

A phylogenetic tree of Figla proteins was constructed using the tilapia Max (Myc-associated factor X) (XP_005475095.1) as an out-group. In brief, the deduced amino acid sequences of tilapia Figla and its homologous counterparts from other species were aligned using Clustal W. The neighbor-joining method was used to construct the phylogenetic tree using MEGA 5.0 [27]. The values represent bootstrap scores out of 1000 trials, indicating the credibility of each branch. The GenBank accession nos. of the sequences used in this study are as follows: tilapia (KP096546), tetraodon (Tetraodon nigroviridis) (ACH91670.1), medaka (Oryzias latipes) (NP_001098215.1), fugu (Takifugu rubripes) (NP_001177290.1), zebrafish (Danio rerio) (NP_944601.2), Xenopus (Xenopus laevis) (NP_001088667.1), lizard (Anolis carolinensis) (XP_008120436.1), human (Homo sapiens) (NP_001004311.2), lancelet (Branchiostoma floridae) (XP_002586647.1). The gene loci flanking Figla genes in the genomes of different vertebrates were determined using the ensemble genome browser (http://www.ensembl.org/index.html) with tilapia Figla (ENSONIG00000015856) as the query sequence. Then, their genomic location data was used to rebuild the synteny maps surrounding the Figla genes in vertebrates.

**Real-time PCR and statistical analysis**

The primer sets used for real-time PCR were designed using Primer Express software (Applied Biosystems, USA) with at least one primer in each set flanking the intron-exon boundary, in order to prevent amplification from genomic DNA. Linear standard curves were generated with serial 10-fold dilutions with plasmids DNA containing the ORF of respective genes. All real-time PCRs were carried out in an ABI-7500 fast real-time PCR machine (Applied Biosystems, USA) following the manufacturer’s protocol. The PCR reactions were initiated by denaturation at 95°C (5 min), followed by 40 amplification cycles at 95°C (15 s) and 60°C (30 s). Dissociation protocols were used to measure the melting curves. The relative expression level (RNA abundance) was calculated by dividing the copy no. of target gene by the geometric mean of three reference genes (eef1a1, actb and gapdh). Data were expressed as means ± S.E.M. Significant differences (P<0.05) in the results were analyzed using Kruskal-Wallis ANOVA and GraphPad Prism 4 Software (GraphPad, USA).

**Tissue distribution and ontogenic analysis**

For the tissue distribution analysis, three parallel samples from three adult fish were prepared to evaluate the expression of Figla gene. Briefly, total RNA was extracted from head kidney,
kidney, brain, gill, heart, intestine, liver, ovary and testis of adult tilapia, according to the manufacturer’s instructions (TaKaRa, Japan). Total RNA (200 ng) from each tissue was reverse transcribed into first-strand cDNA using PrimeScript RT Master Mix Perfect Real Time Kit (TaKaRa, Japan) following the manufacturer’s instructions. Real-time PCR was carried out according to the aforementioned method. Preliminary analysis showed no sexual dimorphic expression in tissues other than gonads, hence the non-gonadal tissues collected only from female fish were used in the tissue distribution analysis.

For ontogenic expression analysis, three independent gonadal samples of 5, 10, 20, 30, 40 and 50 days after hatching (dah) were used, and each sample was made from the gonadal pool of 50–100 fish. However, three individual fish gonads were used to prepare three separate samples of 60, 90, 120, 150 and 240 dah. All the procedures for RNA extraction, cDNA synthesis, real-time PCR and statistical analysis were carried out as described above.

Western blotting
Western blotting was performed to analyze Figla expression in the gonads of four separate Figla-transgene XY fish and non-transgenic fish. Briefly, proteins were extracted from testes of both wild type XX/XY and Figla-transgene XY fish at 90 dah. Then, 150 ng of the whole gonad protein was separated by SDS-PAGE and transferred onto PVDF membrane (Amersham, Sweden). Then, the membranes were blocked with 5% low fat milk powder in TBST (20mM Tris-HCl pH7.5, 150 mM NaCl, 0.1% Tween 20) and incubated with primary antibody of Figla, and then with AP-labeled secondary antibody. Finally, the immunoreactive signals were stained with NBT/BCIP substrates and visualized on Fusion FX7 (Vilber Lourmat, France). Meanwhile, the expression level of Figla was normalized using β-Actin as reference protein.

To assess the specificity of antibodies including Figla, Dmrt1, Sycp3 and Cyp17a1 in tilapia gonads, Western blotting was carried out in both ovary and testis according to the same protocol. Characterization of StAR antibody in the ovary and testis had been reported previously [29].

Immunohistochemistry (IHC)
The gonads were dissected from monosex (XX and XY) fish at 90 and 150 dah, Figla-transgene and control XY fish at 90 dah, then Bouin-fixed, paraffin-embedded, and cut into 5μm sections. These sections were deparaffinized, hydrated and processed according to the previous reports [28, 29]. The tissues sections were then treated in a blocking solution (Roche, Germany), incubated with hrGFP (Stratagene, Canada), Figla, Dmrt1, Sycp3, Cyp17a1 and StAR polyclonal antibody (1:1000) overnight at 4°C, respectively, and rinsed with 0.01 M PBS three times for 5 min per wash. Subsequently, the sections were incubated with a second antibody conjugated with horseradish peroxidase (Bio-Rad, USA) at 1:2000 for 30 min, washed with PBS and visualized using diaminobenzidine (Sigma, USA) as the substrate. Sections were counterstained with hematoxylin. Polyclonal antibodies used for IHC were produced by immunizing the New Zealand white rabbits with each of the purified recombinant proteins generated from the partial coding region of tilapia Dmrt1, Sycp3, Cyp17a1 and StAR genes. Sera were collected after immunizing the rabbits three times and purified by affinity chromatography on Sepharose 4B Fast Flow resin coupled with the respective recombinant protein. In this study, all the images for IHC were acquired with a Zeiss Axio Imager Z2 microscope equipped with an AxioCam MRc digital camera.

Over-expression of Figla in XY fish
The ORF and 3'-UTR of Figla was amplified from the ovarian cDNA of adult tilapia by using two gene specific primers with appropriate restriction sites. After double digestions by EcoRI
and XhoI (New England Biolabs, USA), the PCR fragments were purified and ligated to the multiple cloning sites of pIRES-hrGFP-1a vector (Stratagene, Canada) digested by the same restriction enzymes. Subsequently, sequence analysis was carried out to confirm the orientation and accuracy of the insert. Finally, the constructs with correct insert were purified by using a QIAquick gel extraction kit (Qiagen, Germany), linearized by PshAI and used for subsequent microinjection and transgene analysis.

Briefly, linearized plasmid DNA of Figla-pIRES1a-hrGFP was diluted in filtered 1X PBS solution to a final concentration of 80ng/μl, and injected into one-cell stage fertilized eggs under microscope using SYS-PV830 injector (WPI, USA). Subsequently, the genomic DNA was isolated from tail of each fish at 90 dah to check the genome integration. Furthermore, the fragment was sub-cloned into pGEM-T easy vector for sequencing analysis. The integration rate was calculated by dividing the number of individual carrying foreign DNA (Figla-pIRES1a-hrGFP) with total number of fish survived at 90dah. The abnormality was scored by calculating the ratio between the numbers of fish carrying transgene sequences and total number of transgenics with impaired spermatogenesis (depletion of meiotic spermatocytes and spermatids).

Finally, the injected XY fish were sacrificed at 90 dah in order to analyze the effects of Figla over-expression by GSI measurement, real-time PCR, histological observation by H.E. staining, Western blotting and IHC according to the protocols aforementioned. To trace the tissue specificity of Figla transgene, real-time PCR analysis was performed to compare the transcription level of Figla transgene in gonads and gonadectomised bodies of Figla-trasngene XY fish.

All the primer sequences used for molecular cloning and PCR amplifications were listed in S1 Table.

Measurement of 11-KT levels by EIA

For EIA, a minimum of 150 μl of blood was drawn from the caudal vein of Figla-transgene and control XY fish at 90 dah. Blood samples were centrifuged at 10 000 g for 5 min at 4°C, and the supernatant was carefully collected. Finally, 11-KT levels in the serum were measured using the 11-KT EIA Kit according to the manufacturer’s instructions (Cayman, USA).

Results

Phylogenetic and syntenic analysis

Tilapia Figla cDNA was identified from the tilapia ovary, and its genomic sequence was obtained from tilapia genome website (http://www.ensembl.org/Oreochromis_niloticus/Info/Index). Comparative analysis of genomic DNA and mRNA revealed that tilapia Figla is located in LG12 and has 5 exons (S1 Fig). Multiple alignments demonstrate a well-conserved DNA binding domain of basic helix-loop-helix of Figla among all vertebrates (S2 Fig). Phylogenetic analysis shows that Figla from fish species cluster into one clade, while the amphibian, reptile and mammalian homologues formed a distinct clade (Fig 1A). Furthermore, synteny analyses reveal the organization of the genomic region surrounding Figla genes in tetrapods and teleosts. Comparative analysis of chromosomal organization shows that, Figla and its upstream gene, HK2 (hexokinase 2), has a conserved synteny in teleosts and Xenopus (Fig 1B). In addition, conserved synteny of the downstream genes of Figla (ADD2 (β-adducing) and MXD1 (MAX dimerization protein 1)) was detected between human, Xenopus and teleosts (Fig 1B). In summary, Figla locus revealed a synteny of evolutionarily conserved genes in vertebrates, despite several local rearrangements and gene insertion in tetrapods.
Tissue distribution

Real-time PCR was used to investigate the expression pattern of Figla gene in different tissues of adult tilapia. In consistence with mice [7], human [30], zebrafish [31] and medaka [32], tissue distribution analysis shows that Figla is dominantly expressed in the ovary, while it is barely detectable in other tissues in adult tilapia (Fig 2A).

Expression of Figla gene during gonadal development

Real-time PCR analysis revealed that mRNA abundance of tilapia Figla gene was remarkably increased from 30 dah, peaked at 90 dah, and decreased from 120 dah in the XX gonads. In contrast, transcript level of Figla gene remained undetectable during all stages in XY gonads (Fig 2B). Consistently, IHC experiments also demonstrate that Figla is expressed in the early
primary oocytes at 90 and 150 dah in ovaries (Fig 3A and 3B). On the other hand, Figla specific signals could not be detected in testes at both stages (Fig 3C and 3D).

Sequence confirmation and morphological changes in Figla-transgene XY tilapia

To clarify the potential functions of fish Figla in gonadal development and differentiation, the ORF and 3'-UTR of Figla gene were sub-cloned into the multiple cloning sites downstream of the CMV promoter in the pIRES-hrGFP-1a vector. After microinjecting into the one-cell stage fertilized egg, a specific band of 947 bp was amplified from the genomic DNA of injected XY fish by genomic PCR (Fig 4A). Sequencing results further confirmed that the amplified DNA fragment contains both pIRES-hrGFP-1a vector and Figla sequence (Data not shown). The integration and abnormality rate induced by over-expression of Figla were approximately 47.62% and 95%, respectively (S2 Table).

Morphologically, the Figla-transgene XY fish had shrunken gonads and significantly reduced GSI values, compared to the control XY fish (Fig 4B). Real-time PCR analysis revealed that expression of Figla gene is dramatically up-regulated in 3-month-old Figla-transgene XY fish compared to control group (Fig 4C). Moreover, real-time PCR results demonstrated a significant difference in Figla transcription between gonad and gonadectomised body of Figla-transgene XY fish, which indicated that over-expression of Figla gene was dominantly expressed in the gonad, but not other tissues (S3 Fig). Western blotting analysis revealed that a specific band around 22.5 kDa for Figla was detected from the total protein in the gonads of control XX and Figla-transgene XY fish, but it was barely detected in the testis of control XY fish (Fig 4D).

In tilapia, spermatogonia and all phases of spermatogenic cells could be detected in the testis of 3-month-old XY fish (Fig 4E). However, only spermatogonia cells, but not later phase spermatogenic cells, i.e. spermatocytes and spermatids, were detected in Figla-transgene XY testis (Fig 4F), indicating that over-expression of Figla impaired spermatogenesis. Moreover, strong expression of hrGFP (Fig 4H) and Figla (Fig 4J) were detected mainly in the areas of
spermatogonial cells of Figla-transgene XY fish, but not in control XY (Fig 4G and 4I), which suggested the efficacy and specificity of Fig1a over-expression.

Molecular variations in Figla-transgene XY tilapia

Consistent with the histological observations, IHC also shows the absence of Sycp3 expression (Fig 5G) compared to the control group (Fig 5H). However, over-expression of Figla in XY fish didn’t alter the expression of Dmrt1 (Doublesex- and Mab-3-related transcription factor-1) in the Sertoli cells in comparison with control XY fish (Fig 5A and 5B). Meanwhile, no significant difference of both Cyp17a1 (cytochrome P450, family 17, subfamily A, type 1) and StAR (Steroidogenic acute regulatory protein) are detected in the Leydig cells between Figla-transgene (Fig 5C–5E) and control (Fig 5D–5F) groups. The IHC data are further supported by unaltered dmrt1, star and cyp17a1 expression by real-time PCR analysis between the Figla-transgene and
control fish (Fig 6). More interestingly, the expression of prm (protamine), which is specifically found in mature sperm, is significantly down-regulated in the Figla-transgene fish (Fig 6). The specificity in the gonads of Cyp17a1, Dmrt1, Figla, Sycp3 antibodies used for IHC was analyzed by Western blotting analysis. The results demonstrated that each antibody recognized a unique band in ovary and/or testis (S4 Fig).

11-KT level affected by Figla over-expression

11-KT is the major androgen found in the serum of tilapia. To determine whether Figla-transgene could affect androgen production or not, we collected blood samples from both Figla-transgene and control XY fish at 90 dah, and measured the 11-KT levels using EIA. Surprisingly, serum 11-KT levels are significantly elevated in the Figla-transgene XY fish (Fig 7).
Discussion

In this study, we showed that Figla was a female specific gene expressed in the early primary oocytes. The synteny analysis depicted a relatively conserved locus organization surrounding Figla gene throughout vertebrates. Surprisingly, over-expression of Figla in XY fish led to a defect in spermatogenesis. Hence, we provide evidences that Figla is an effective antagonistic factor which represses spermatogenesis, and in turn contributes to ovarian development.

In mammals, sex determination and differentiation is determined by the antagonistic and balancing actions between male and female genes [10, 33]. Meanwhile, coordination between follicles and oocytes are indispensable for ovarian differentiation [34]. Figla encodes a germ cell-specific bHLH transcription factor that was initially identified by its ability to co-ordinate the expression of the oocyte-specific zona pellucida genes and folliculogenesis [7]. The tilapia Figla possesses a conserved bHLH domain, which suggests the similar regulatory mechanisms in fish. Moreover, both phylogenetic and conserved syntenic analysis further indicate tilapia Figla is the homologous gene of its mammalian counterparts, suggesting its conserved role in fish ovarian differentiation.

Previous studies demonstrated that Figla was essential for folliculogenesis in mouse [7], human [35]. Likewise, in our study, tissue distribution analysis also revealed that tilapia Figla gene was dominantly expressed in ovaries, indicating its vital roles in fish ovarian differentiation. In mice, the expression of Figla was first detected at embryonic stage 13, peaked two days postpartum and decreased markedly by 7 and 14 days after birth, while it was barely detectable in adult ovaries [8]. In zebrafish, two peaks of Figla gene were found at 18 day post hatching (dph) and 30 dph [18]. Consistently, we also found significant up-regulation of Figla gene in ovaries around 30 dah (just after the onset of sexual dimorphism), which peaked at 90 dah (the critical period of oogenesis) and declined from 120 dah, which suggests the essentiality of Figla gene in both folliculogenesis and oogenesis in tilapia.

![Fig 7. Comparison of serum 11-KT levels between Figla-transgene and control XY fish. Results were presented as means ± S.E.M. of 6 independent samples, respectively.](doi:10.1371/journal.pone.0123900.g007)
Several reports revealed that Rspo1 and Foxl2 activated pathways in somatic cells are indispensable in promoting and ensuring ovarian differentiation in vertebrates [36, 37]. Meanwhile, cross-talk between oocyte and ovarian follicular is of vital importance for fertility [34]. Previous investigations indicated that germ cells are definitely required to repress the male pathway and promote ovarian differentiation [38]. In mice, loss of germ cells in the XX gonad triggered the postnatal trans-differentiation of granulosa cell to Sertoli cells and promoted the development of testicular cord-like structures [15, 39]. In fish, disruption of PGC population by gene knockdown or chemical treatment resulted in trans-differentiation from pre-follicles into Sertoli and Leydig cells, and further masculinization of female gonad, which highlighted the importance of germ cells in female fertility [13, 15, 25, 40]. Therefore, the interaction between germ cells and somatic cells might also be critical for female sex differentiation in fish [13].

Figla, a key factor in the genetic hierarchies of germ cells, was shown to be an activator of oocytes genes during postnatal oogenesis in mammalian species [7]. Mice lacking FIGLA have normal embryonic gonad development, but without primordial follicles and loss of germ cells within days of birth, which resulted in massive depletion of oocytes, shrunken-ovaries and sterility [8]. Ectopic over-expression of Figla in male germ cells in transgenic mice led to defective spermatogenesis and down-regulation of a set of testis-associated genes [10]. Similar to Fig1a over-expressed male mice, our histological data also showed severely disrupted spermatogenesis in male tilapia at 90 dah. In contrast to normal 3-month-old XY fish, the reduced GSI index suggested that over-expression of Figla severely impaired gonad differentiation and development. Noticeable morphological defects further supported that over-expression of Figla arrested the differentiation of spermatocytes and sperm maturation. The defective meiotic progression was further confirmed by the reduced Sycp3 expression. Meanwhile, the reduced prm levels in Fig1a-transgene XY fish were also suggestive of similar phenomenon. In addition, knockout of Figla by TALENs (Transcription activator-like effector nucleases) resulted in gonadal masculinization with the down-regulation of female specific genes and up-regulation of male specific genes (data not shown). Taken together, we hypothesized that Figla might play essential roles in ovarian differentiation and contribute towards female development by both activating the female specific genes and antagonizing the spermatogenesis associated genes.

Previous reports in mice have shown that Figla-transgene disrupted spermatogenesis only from 5 months after birth [10]. In contrast to this report, defective spermatogenesis was observed in Fig1a over-expressed XY fish from 90 dah, when the meiotic initiation just occurred, suggesting a differential regulatory mechanism. In Figla-transgene male mice, the property of somatic cells and the expression of Sox9 in Sertoli cells were normal in the testes [10]. Likewise, no significant difference of dmrt1 gene expression was noticed, indicating that over-expression of Figla didn’t impair the properties of Sertoli cells. In testis, Leydig cells are the major resources for steroidogenesis in fish [41]. Contradictory to the results in mice, we observed enhanced hsd3b1 expression and 11-KT circulation in Fig1a over-expressed XY tilapia, even though the expression of cyp17a1 and Star in Figla-transgene XY fish in the Leydig cells remained constant. We speculate that excess 11-KT production impaired spermatogenesis, as high levels of androgen led to early puberty, shrinkage of testes, and in some cases, infertility in males [42]. Meanwhile, the increased 11-KT level in these fish could also be a compensatory response due to the inhibition of spermatogenesis. Taken together, our data suggests that Fig1a arrests the male gonadal differentiation, possibly by manipulating the steroid production and/or meiotic regulation.

In summary, our particular investigations showed the female specificity of Figla in the early primary oocytes. Over-expression of female specific Figla gene in XY fish eventually arrested spermatogenesis and sperm production. Therefore, fish Figla probably might be an antagonist...
of genetic hierarchies of male, which in turn favors ovarian development. However, further in-depth investigations are required to verify the regulatory mechanisms.

Supporting Information
S1 Fig. Schematic representation of the gene structure of tilapia Figla. (TIFF)
S2 Fig. Multiple alignments of the deduced amino acid sequences of tilapia Figla with its counterparts in other vertebrates. (TIFF)
S3 Fig. Comparison of Figla gene expression in gonad and gonadectomised body in Figla-transgene XY fish by real-time PCR. (TIFF)
S4 Fig. Specificity of Figla (A), Cyp17a1 (B), Dmrt1 (C), Symp3 (D) antibody in the gonads analyzed by Western blotting. (TIFF)
S1 Table. Primer sequences used for molecular cloning and PCR amplification. (DOC)
S2 Table. Integration and abnormality rates of Figla-transgene XY tilapia. (DOC)

Acknowledgments
The authors cordially thank Prof. Ching-Fong Chang from National Taiwan Ocean University, Keelung, Taiwan, for providing the black porgy Figla antibody. The authors are thankful to Dr. Sipra Mohapatra, Ehime University, Japan and Dr. Fengzhu Xiong, Harvard Medical School, USA for their critical reading and English correction of the manuscript.

Author Contributions
Conceived and designed the experiments: LYZ. Performed the experiments: YXQ SHS LMW. Analyzed the data: LNS JW. Contributed reagents/materials/analysis tools: TC. Wrote the paper: YN DSW.

References
1. Swain A, Lovell-Badge R. Mammalian sex determination: a molecular drama. Genes Dev. 1999; 13: 755–767. PMID: 10197976
2. Capel B. The battle of the sexes. Mech Dev. 2000; 92: 89–103. PMID: 10704890
3. McLaren A. Development of the mammalian gonad: the fate of the supporting cell lineage. Bioessays. 1991; 13: 151–156. PMID: 1859392
4. McLaren A. Germ and somatic cell lineages in the developing gonad. Mol Cell Endocrinol. 2000; 163: 3–9. PMID: 10963867
5. Guigon CJ, Magre S. Contribution of germ cells to the differentiation and maturation of the ovary: insights from models of germ cell depletion. Biol Reprod. 2006; 74: 450–458. PMID: 16339043
6. Guigon CJ, Coudouel N, Mazaud-Guittot S, Forest MG, Magre S. Follicular cells acquire Sertoli cell characteristics after oocyte loss. Endocrinology. 2005; 146: 2992–3004. PMID: 15817664
7. Liang L, Soyal SM, Dean J. Figalpha, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. Development. 1997; 124: 4939–4947. PMID: 9362457
8. Soyal SM, Amleh A, Dean J. Figalpha, a germ cell-specific transcription factor required for ovarian follicle formation. Development. 2000; 127: 4645–4654. PMID: 11023867
FIGLA Represses Spermatogenesis in Fish

9. Joshi S, Davies H, Sims LP, Levy SE, Dean J. Ovarian gene expression in the absence of FIGLA, an oocyte-specific transcription factor. BMC Dev Biol. 2007; 7: 67. PMID: 17567914

10. Hu W, Gauthier L, Baibakov B, Jimenez-Movilla M, Dean J. FIGLA, a basic helix-loop-helix transcription factor, balances sexually dimorphic gene expression in postnatal oocytes. Mol Cell Biol. 2010; 30: 3661–3671. doi: 10.1128/MCB.00201-10 PMID: 20479125

11. Devlin RH, Nagahama Y. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture. 2002; 208: 191–364.

12. Nishimura T, Tanaka M. Gonadal development in fish. Sex Dev. 2014; 8: 252–261. doi: 10.1159/000364924 PMID: 25034975

13. 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. Proc Natl Acad Sci U S A. 2005; 102: 4074–4079. PMID: 15728735

14. Siegfried KR, Nusslein-Volhard C. Germ line control of female sex determination in zebrafish. Dev Biol. 2008; 324: 277–287. doi: 10.1016/j.ydbio.2008.09.025 PMID: 18930041

15. Kurokawa H, Saito D, Nakamura S, Katoh-Fukui Y, Ohta K, Baba T, et al. Germ cells are essential for gonadal differentiation in a teleost fish, the Nile tilapia. Proc Natl Acad Sci U S A. 2007; 104: 16958–16963. PMID: 17940041

16. Li M, Yang H, Zhao J, Fang L, Shi H, Li M, et al. Efficient and Heritable Gene Targeting in Tilapia by CRISPR/Cas9. Genetics. 2014; 197: 591–599. doi: 10.1534/genetics.113.163667 PMID: 24709635

17. Rodriguez-Mari A, Canestro C, BreMiller RA, Nguyen-Johnson A, Asakawa K, Kawakami K, et al. Sex Reversal in Zebrafish fancl Mutants Is Caused by Tp53-Mediated Germ Cell Apoptosis. PLOS Genetics. 2010; 6.

18. Jorgensen A, Morthorst JE, Andersen O, Rasmussen LJ, Bjerregaard P. Expression profiles for six zebrafish genes during gonadal sex differentiation. Reprod Biol Endocrinol. 2008; 6: 25. doi: 10.1186/1477-7827-6-25 PMID: 18590525

19. Kanamori A, Toyama K, Kitagawa S, Kamehara A, Higuchi T, Kamachi Y, et al. Comparative genomics approach to the expression of figalpha, one of the earliest marker genes of oocyte differentiation in medaka (Oryzias latipes). Gene. 2008; 423: 180–187. doi: 10.1016/j.gene.2008.07.007 PMID: 18678233

20. Wu GC, Chang CF. Oocytes survive in the testis by altering the soma fate from male to female in the protandrous black porgy, Acanthopagrus schlegeli. Biol Reprod. 2013; 88: 19. doi: 10.1095/biolreprod.112.104398 PMID: 23197163

21. Wang DS, Kobayashi T, Zhou LY, Paul-Prasanth B, Ijiri S, Sakai F, et al. 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. Molecular Endocrinology. 2007; 21: 712–725. PMID: 17912407

22. Wang DS, Zhou LY, Kobayashi T, Matsuda M, Shibata Y, Sakai F, et al. Doublesex- and Mab-3-related transcription factor-1 repression of aromatase transcription, a possible mechanism favoring the male pathway in tilapia. Endocrinology. 2010; 151: 1331–1340. doi: 10.1210/en.2009-0999 PMID: 20056824

23. Li MH, Yang HH, Li MR, Sun YL, Jiang XL, Xie QP, et al. Antagonistic Roles of Dmrt1 and Foxl2 in Sex Determination and Sex Differentiation and Hormone-induced Sex Reversal in the Nile tilapia (Oreochromis niloticus) reveals differentially expressed genes. PLOS ONE. 2013; 8: e63604. doi: 10.1371/journal.pone.0063604 PMID: 23658843

24. Thompson WE, Sanbuissaho A, Lee FY, Anderson E. Steroidogenic acute regulatory (STAR) protein (p25) and prohibitin (p28) from cultured rat ovarian granulosa cells. J Reprod Fertil. 1997; 109: 337–348. PMID: 9155744

25. Liu G, Luo F, Song Q, Wu L, Qiu Y, Shi H, et al. Blocking of progesterin action disrupts spermatogenesis in Nile tilapia (Oreochromis niloticus). J Mol Endocrinol. 2014; 53: 57–70. doi: 10.1530/JME-13-0300 PMID: 24827000

26. Yu X, Wu L, Xie L, Yang S, Charkraborty T, Shi H, et al. Characterization of two paralogous STAR genes in a teleost, Nile tilapia (Oreochromis niloticus). Mol Cell Endocrinol. 2014; 392: 152–162. doi: 10.1016/j.mce.2014.05.013 PMID: 24859646
30. Huntriss J, Gosden R, Hinkins M, Oliver B, Miller D, Rutherford AJ, et al. Isolation, characterization and expression of the human Factor In the Germline alpha (FIGLA) gene in ovarian follicles and oocytes. Mol Hum Reprod. 2002; 8: 1087–1095. PMID: 12468641

31. Onichtchouk D, Aduroja K, Belting HG, Gnugge L, Driever W. Transgene driving GFP expression from the promoter of the zona pellucida gene zpc is expressed in oocytes and provides marker for gonad differentiation in an early zebrafish. Developmental Dynamics. 2002; 228: 393–404. PMID: 14579378

32. Kanamori A. Medaka as a model for gonadal sex differentiation in vertebrates. Tanpakushitsu Kakusan Koso. 2000; 45: 2949–2953. PMID: 11187802

33. Brennan J, Capel B. One tissue, two fates: Molecular genetic events that underlie testis versus ovary development. Nature Reviews Genetics. 2004; 5: 509–521. PMID: 15211353

34. Zamboni L, Upadhyay S. Germ cell differentiation in mouse adrenal glands. J Exp Zool. 1983; 228: 173–193. PMID: 6663256

35. Bayne RAL, da Silva SJM, Anderson RA. Increased expression of the FIGLA transcription factor is associated with primordial follicle formation in the human fetal ovary. Mol Hum Reprod. 2004; 10: 373–381. PMID: 15044608

36. Chassot AA, Gillot I, Chaboissier MC. R-spondin1, WNT4, and the CTNNB1 signaling pathway: strict control over ovarian differentiation. Reproduction. 2014; 148: R97–R110. doi: 10.1530/REP-14-0177 PMID: 25187620

37. Eggers S, Ohnesorg T, Sinclair A. Genetic regulation of mammalian gonad development. Nat Rev Endocrinol. 2014; 10: 673–683. doi: 10.1038/nrendo.2014.163 PMID: 25246082

38. Maatouk DM, Mork L, Hinson A, Kobayashi A, McMahon AP, Capel B. Germ Cells Are Not Required to Establish the Female Pathway in Mouse Fetal Gonads. PLOS ONE. 2012; 7: e47238. doi: 10.1371/journal.pone.0047238 PMID: 23091613

39. Yokoi H, Kobayashi T, Tanaka M, Nagahama Y, Wakamatsu T, Takeda H, et al. Sox9 in a teleost fish, medaka (Oryzias latipes): evidence for diversified function of Sox9 in gonad differentiation. Mol Reprod Dev. 2002; 63: 5–16. PMID: 12211055

40. Fujimoto T, Nishimura T, Goto-Kazeto R, Kawakami Y, Yamaha E, Arai K. Sexual dimorphism of gonadal structure and gene expression in germ cell-deficient loach, a teleost fish. Proc Natl Acad Sci U S A. 2010; 107: 17211–17216. doi: 10.1073/pnas.1007032107 PMID: 20855617

41. Greco TL, Payne AH. Ontogeny of expression of the genes for steroidogenic enzymes P450 side-chain cleavage, 3 beta-hydroxysteroid dehydrogenase, P450 17 alpha-hydroxylase/C17-20 lyase, and P450 aromatase in fetal mouse gonads. Endocrinology. 1994; 135: 262–268. PMID: 8013361

42. Dohle GR, Smit M, Weber RF. Androgens and male fertility. World J Urol. 2003; 21: 341–345. PMID: 14566423