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

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Gonadotropins are primary hormones that control vertebrate reproduction. In a recent study, we analyzed the impacts of FSH and LH on zebrafish reproduction by disrupting FSH and LH-β genes (*fshb* and *lhb*) using transcription activator-like effector nuclease (TALEN) technology. Using the same approach, we successfully deleted FSH and LH receptor genes (*fshr* and *lhcgr*) in the present study. In contrast to the deficiency of its cognate ligand FSH, the *fshr*-deficient females showed a complete failure of follicle activation with all ovarian follicles arrested at the primary growth-previtellogenic transition, which is the marker for puberty onset in females. Interestingly, after blockade at the primary growth stage for varying times, all females reversed to males, and all these males were fertile. In *fshr*-deficient males, spermatogenesis was normal in adults, but the initiation of spermatogenesis in juveniles was retarded. In contrast to *fshr,* the deletion of the *lhcgr* gene alone caused no obvious phenotypes in both males and females; however, double mutation of *fshr* and *lhcgr* resulted in infertile males. In summary, our results in the present study showed that Fshr was indispensable to folliculogenesis and the disruption of the *fshr* gene resulted in a complete failure of follicle activation followed by masculinization into males. In contrast, *lhcgr* does not seem to be essential to zebrafish reproduction in both males and females. Neither Fshr nor Lhcgr deficiency could phenocopy the deficiency of their cognate ligands FSH and LH, which is likely due to the fact that Fshr can be activated by both FSH and LH in the zebrafish. **(*Endocrinology* 156: 3747–3762, 2015)**

Gonadotropins, FSH, and LH, control gonadal development and function by binding to their specific cognate receptors, FSH receptor (FSHR) and LH/choriogonadotropin receptor (LHCGR), respectively. In mammals, FSH acts on the granulosa cells in the ovary and Sertoli cells in the testis to stimulate germ cell growth and development, whereas LH acts on steroidogenic theca cells in the ovary and Leydig cells in the testis to stimulate sex steroid biosynthesis (1, 2). FSHR and LHCGR belong to the G protein-coupled receptor family (3), and the knockout of their genes in the mouse could mostly phenocopy the functional loss of their respective ligands FSH and LH. Both FSH- and FSHR-null mutants in female mice display a blockade of folliculogenesis at preantral follicle stage, leading to infertility; however, male mutants of both genes were surprisingly fertile, albeit with significantly reduced testis size and fecundity (4–6). In LH- and LHCGR-deficient mice, the females demonstrate postnatal defects in gonadal growth with the folliculogenesis being arrested at early antral

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Abbreviations: dpf, days after fertilization; FG, full grown; FSHR, FSH receptor; hCG, human chorionic gonadotropin; HMA, heteroduplex motility assay; HRMA, high-resolution melt analysis; LHCGR, LH/choriogonadotropin receptor; MV, midvitellogenic; PG, primary growth; PV, previtellogenic stage; qPCR, quantitative PCR; TALEN, transcription activator-like effector nuclease.

stage, also resulting in infertility. However, in contrast to FSH and FSHR mutants, the males of LH- and LHCGR-deficient mice are also infertile due to the arrest of spermatogenesis at round spermatid stage (7, 8).

In teleosts, it was believed earlier that only one receptor was responsible for gonadotrophic signaling in fish (9–12). The concept of two gonadotropin receptors was proposed later in salmonids (13–15). This was further confirmed by molecular cloning in the amago salmon (16, 17). Up to now, FSHR (Fshr/*fshr*) and LHCGR (Lhcgr/*lhcgr*) have been identified and characterized in large number of species (16–22) (nearly 40 species according to GenBank records in 2014), including the zebrafish (23). Interestingly, unlike their mammalian counterparts with a well-defined receptor specificity, fish gonadotropins often show promiscuous hormone-receptor interactions. In the coho salmon, Fshr does not discriminate FSH and LH binding, whereas Lhcgr binds LH only (14). Similarly, in the African catfish and zebrafish, the cloned Fshr responds to both recombinant FSH and LH with a preference for FSH; however, Lhcgr is specifically activated by LH only (21, 24). In some other fish species, the ligand selectivity of the cloned gonadotropin receptors appears different. For example, the Lhcgr of the amago salmon responds to LH but also slightly to FSH; in contrast, the Fshr is activated by FSH only (16, 17). The physiological relevance of the promiscuous hormone-receptor selectivity in fish remains mysterious.

Most studies on Fshr and Lhcgr in fish have focused on their expression profiles during the reproductive cycle (25–28). In the zebrafish, both *fshr* and *lhcgr* are exclusively expressed in the somatic follicle cells in the ovary (29). During folliculogenesis, the expression of *fshr* and *lhcgr* is very low in the primary growth (PG; stage I) follicles; however, the level of *fshr* mRNA increases significantly when the follicles are activated to enter the previtellogenic stage (PV; stage II) and peaks at the midvitellogenic (MV) stage. In contrast, the expression of *lhcgr* starts to increase after the MV stage and reaches the highest level at the full-grown (FG) stage before final oocyte maturation (23, 30). These observations support the traditional views that FSH and its receptor Fshr are involved in follicle activation to enter vitellogenic growth, whereas LH and its receptor Lhcgr play an important role at oocyte maturation and ovulation (31, 32).

In male fish, it was believed that FSH might act on Fshr-expressing Sertoli cells to drive testis growth and spermatogenesis, whereas LH supported steroidogenesis and gonadal maturation through its receptor Lhcgr on the Leydig cells, similar to those in mammals (14, 33). However, this view has recently been revised. New evidence in the zebrafish and other species showed that Fshr was also expressed in the Leydig cells, and the Sertoli cells also expressed Lhcgr (34–36). The expression of Fshr and Lhcgr in both Leydig and Setoli cells in the zebrafish may be related to the undifferentiated gonochorism of gonadal sex differentiation (35). A recent study in the flatfish showed that Lhcgr was also expressed in the germ cells, and LH could induce spermiogenesis through a cAMP/protein kinase A signaling pathway (37).

Despite these studies, our understanding of Fshr and Lhcgr in fish remains limited as compared with that in mammals. Due to the less selective ligand recognition and unique expression patterns in fish, the physiological roles of Fshr and Lhcgr in gonadal development and function are expected to be more complicated, which is an intriguing issue to explore. In mammalian models, the most conclusive evidence for functional roles of FSHR and LHCGR has been provided by studies using a loss-of-function approach (knockout) in the mouse (5–7, 38, 39) and natural mutants in humans (40–42). Unfortunately, the reverse genetics approach using gene knockout had not been available in fish species, making it difficult to perform comparative genetic studies on gonadotropins and their receptors. This situation has changed recently with the development of new genome editing tools, transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR) (43–47).

Using TALEN, we have recently disrupted the genes of gonadotropins (*fshb* and *lhb*) in the zebrafish. The lack of FSH led to a significant delay in both folliculogenesis and spermatogenesis; however, LH could rescue the lost function of FSH, probably through its promiscuous activation of Fshr. The loss of LH, on the other hand, had no effect on gonadal growth, but the female mutant fish were infertile because of failed oocyte maturation and ovulation (48). This study has led us to hypothesize that through activating Fshr, LH may provide a compensatory support to FSH in initiating puberty onset and promoting gonadal growth; however, LH plays a critical role in controlling final oocyte maturation and ovulation via either Fshr or Lhcgr, and FSH cannot compensate for this function.

As a follow-up study to our report on *fshb* (FSH) and *lhb* (LH) disruption (48), we undertook this project to disrupt both FSH and LH receptor genes (*fshr* and *lhcgr*) in the zebrafish using TALEN. Considering that fish gonadotropins often have promiscuous receptor binding (24), we believe that parallel studies on both the ligands (FSH and LH) and receptors (Fshr and Lhcgr) would provide insight into the roles and importance of the promiscuous ligand-receptor recognition in vivo. Unlike many genes in the zebrafish that have duplicated paralogues, there is only one copy of *fshr* and *lhcgr* genes in the ze-

brafish genome, which reduces the complication of the genetic approach. The most striking discoveries from the present study are that the loss of Fshr and Lhcgr did not phenocopy the knockouts of their cognate ligands (*fshb* and *lhb*) alone, but the double knockouts of ligands and receptors showed similar phenotypes, albeit not to the same extent.

Materials and Methods

Establishment of *fshr* and *lhcgr* mutant zebrafish lines

The zebrafish *fshr* and *lhcgr* mutants were generated by TALEN-induced gene knockout as described in our recent report (48). Briefly, the TALEN sites were designed by using the TAL Effector Targeter (https://tale-nt.cac.cornell.edu), and the TALEN constructs were assembled as reported (49). The 5′-capped mRNAs (50 pg each) generated by in vitro transcription with the mMESSAGE and mMACHINE SP6 kit (Invitrogen) were microinjected into the one- or two-cell stage embryos using the Drummond Nanoject injector (Drummond Scientific).

The F0 founders carrying mosaic mutations were identified by high-resolution melt analysis (HRMA) (48, 50) and mated with wild-type fish to generate heterozygous F1 offspring. The heterozygous F1 fish were genotyped by fin clip assay, and the individuals with frameshift or nonsense sequence alterations were selected. Male and female siblings of the F1 generation carrying the same mutation were mated to generate the homozygous F2 mutant.

Genotyping of *fshr* and *lhcgr* mutants by HRMA and heteroduplex motility assay (HMA)

For genotyping, the genomic DNA was obtained from a single embryo or caudal fin tissue by the sodium hydroxide (NaOH) method (48, 51) for HRMA and/or HMA (48, 52). HRMA was efficient to detect TALEN-induced mutations (48, 50). Briefly, primers flanking the target site were used to amplify the genomic region on the Bio-Rad CFX96 real-time system and the HRMA analysis was carried out using the Precision Melt Analysis software (Bio-Rad Laboratories). In addition to HRMA, HMA was also used in this study for genotyping, especially for the heterozygous F1. When HRMA data were ambiguous, HMA was useful in providing more definitive genotyping information despite its relatively lower throughput. The same primers for HRMA were used to generate genomic fragment containing the target site, and 5 μL of the PCR was electrophoresed on 20% polyacrylamide gels for HMA.

Sampling and histological examination

All fish sampled were recorded for their body weight, standard body length, and age before processing for histological examination (48). Sibling wild-type (+/+) and/or heterozygous (+/−) fish were used as control for phenotype analysis. Briefly, the fish was anesthetized with MS-222 (tricaine methanesulphonate, 250 mg/L; Sigma-Aldrich) and placed on a petri dish cover to measure body length followed by body weight determination on an analytical balance. After the measurements, the fish were photographed before dissection for histological processing and examination. For whole-body histology, the fish were decapitated and fixed immediately in Bouin's solution for at least 24 hours followed by washing and storing in 70% ethanol. The fixed samples were then dehydrated and embedded in paraffin for sectioning. The sections (7 μm) were stained with hematoxylin and eosin and mounted with Canada balsam (Sigma) for microscopic examination.

To illustrate the effect of Fshr on PG follicle growth, we measured the diameters of follicles at 40 days after fertilization (dpf) in the ovary of *fshr*-deficient fish and the control. Briefly, the longitudinal ovarian section of the largest size in each fish was photographed on the Nikon ECLIPSE Ni microscope followed by diameter measurement of every follicle in the section by SPOT Advanced imaging software (Diagnostic Instruments). Only the follicles with a visible nucleus were included in the analysis.

Primary zebrafish follicle cell culture

The primary follicle cell culture was performed according to our previous report (53). Briefly, zebrafish were anesthetized by ice shock and decapitated before dissection. The ovaries were isolated and dispersed into free ovarian follicles for culture in M199 (Gibco-BRL) with 10% fetal bovine serum (Hyclone) at 28°C in 5% CO_2. After 6 days of incubation, the cultured follicle cells were subcultured in 24-well plates. After 24 hours of incubation for attachment, the cells were washed with serum-free M199 twice before treatment. The cells were treated with human chorionic gonadotropin (hCG; 15 IU/mL) in serum-free M199 medium for 2 hours followed by RNA extraction for real-time quantitative PCR (qPCR) analysis.

RNA extraction and semiquantitative and real-time qPCR

Total RNA was extracted from the ovary, testis, or cultured follicle cells using Tri-Reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's protocol. For ovaries from juvenile fish (40 dpf), the fish were first placed in RNAlater (QIAGEN) and stored at −20°C to harden tissues for easy dissection of the small gonads. All total RNA was converted into cDNA by Moloney murine leukemia virus reverse transcriptase (Invitrogen). Conventional semiquantitative RT-PCR was used to confirm the mutations in mRNA. Real-time qPCR was carried out on the CFX96 real-time system (Bio-Rad Laboratories) using primers listed in Supplemental Table 1. A standard curve was constructed in each real-time qPCR assay using cloned DNA fragment as the template for absolute quantification.

Data analysis

For statistical analysis, the expression levels of target genes in cultured follicle cells or ovaries were normalized to that of the internal control *ef1a* and expressed as fold change relative to the control. All values were expressed as the mean ± SEM, and the data were analyzed by a Student's *t* test using Prism 6 on Macintosh OS X (GraphPad Software).

Results

Establishment of *fshr* and *lhcgr* knockout mutant lines by TALEN

To ensure complete disruption of functional proteins, we chose the TALEN target sites near the translation start

codon (Supplemental Figure 1). The TALE proteins turned out to be extremely efficient to induce somatic mutation in both *fshr* and *lhcgr*. All the embryos sampled after injection of TALEN mRNAs carried mutations at 24 hours after fertilization for both *fshr* (10 of 10) and *lhcgr* (10 of 10) (Supplemental Figure 2). The remaining F0 embryos were raised to adulthood and genotyped by HRMA on genomic DNA isolated from the fin cuts. The mutation rates were 50% (5 of 10) for *fshr* and 60% (6 of 10) for *lhcgr* in the adult F0 fish. The healthy F0 founders carrying somatic mutations were crossed with wild-type fish to obtain F1 offspring. High rates of germline transmission were demonstrated by HRMA in the F1 offspring for both genes (55% for *fshr* and 85% for *lhcgr*) (Supplemental Figure 3).

Although the F0 founder fish are genetically mosaic in both somatic and germ cells, the germline mosaic level was

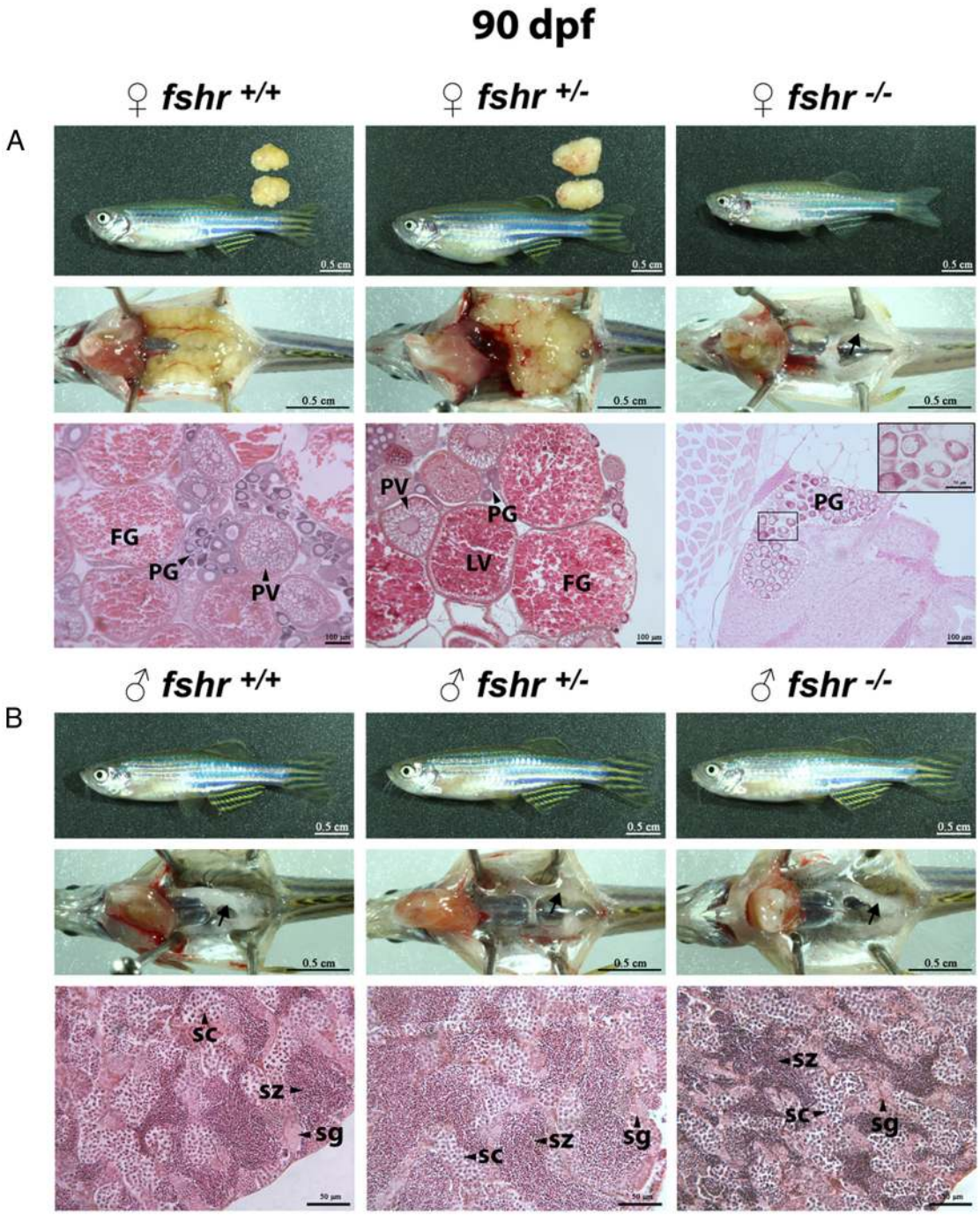

Figure 1. Effects of *fshr* deficiency on ovarian and testis development at 90 dpf. A, Anatomical and histological examination of the ovary in controls ($fshr^{+/+}$ and $fshr^{+/-}$) and *fshr*-deficient fish ($fshr^{-/-}$). B, Anatomical and histological examination of the testis in controls and *fshr*-deficient fish. The arrow in the dissected fish shows the testis. LV, late vitellogenic; sc, spermatocytes; sg, spermatogonia; sz, spermatozoa.

not as high as that in the somatic cells, as suggested in a recent study (50). As shown in Supplemental Figure 3, one *fshr* mutant F0 fish produced two mutations in F1 offspring (Supplemental Figure 3A), and three were produced by one *lhcgr* F0 fish (Supplemental Figure 3B). The low mosaic level and high germline transmission rate make it possible to obtain both male and female sibling fish that carry the same mutation to produce homozygous F2 mutants (Supplemental Figure 3). For *fshr*, an 11-bp deletion (Δ11) leading to frame shifting of the coding sequence was chosen to establish the mutant line for phenotype analysis, and a 14-bp deletion for *lhcgr* (Δ14) was selected (Supplemental Figure 3).

Homozygous *fshr* and *lhcgr* mutant individuals ($fshr^{-/-}$ and $lhcgr^{-/-}$) were then obtained in F2 offspring, and their genotypes were confirmed by both HRMA (Supplemental Figure 4) and HMA (Supplemental Figure 3). To confirm the mutation at mRNA level, we performed an RT-PCR analysis for the *fshr* and *lhcgr* mRNAs by using a deletion-specific primer (P3), and the result showed no detection of mRNAs in mutant gonads (Supplemental Figure 4).

Retarded ovarian growth and failed follicle activation in *fshr*-deficient females

Examined at 90 dpf (3 mo) when the zebrafish is expected to be sexually mature and spawning, no homozygous *fshr* mutant fish ($fshr^{-/-}$) showed obvious morphological signs of sexual dimorphism, whereas the genders of the sibling control fish ($fshr^{+/+}$ and $fshr^{+/-}$) could be easily identified with females showing an enlarged abdomen. Dissection and histological analysis showed a full range of follicles from the PG to FG stage in the ovary of the control fish whereas mutant females ($fshr^{-/-}$) had extremely small ovaries, with all follicles being arrested at PG stage with signs of degeneration (shrinking oocytes with condensed basophilic cytoplasm and increased interfollicular space) (54). No PV or stage II follicles could be observed in the mutant ovary, suggesting a complete blockade of folliculogenesis at the PG-PV transition (Figure 1A).

We then examined fish sampled at earlier times covering puberty onset (40 and 45 dpf) and maturation (65 dpf) to find out when the mutant ($fshr^{-/-}$) and control fish ($fshr^{+/-}$) started to differ in their ovarian growth and folliculogenesis. The control and mutant fish examined had similar body size (data not shown). As shown in Figure 2A, the control fish showed normal ovarian development, with large PG follicles at 40 dpf and PV follicles at 45 dpf, a marker for puberty (55, 56). In contrast, the ovaries of mutant fish were extremely small, with only one or two layers of underdeveloped PG follicles in the section. The

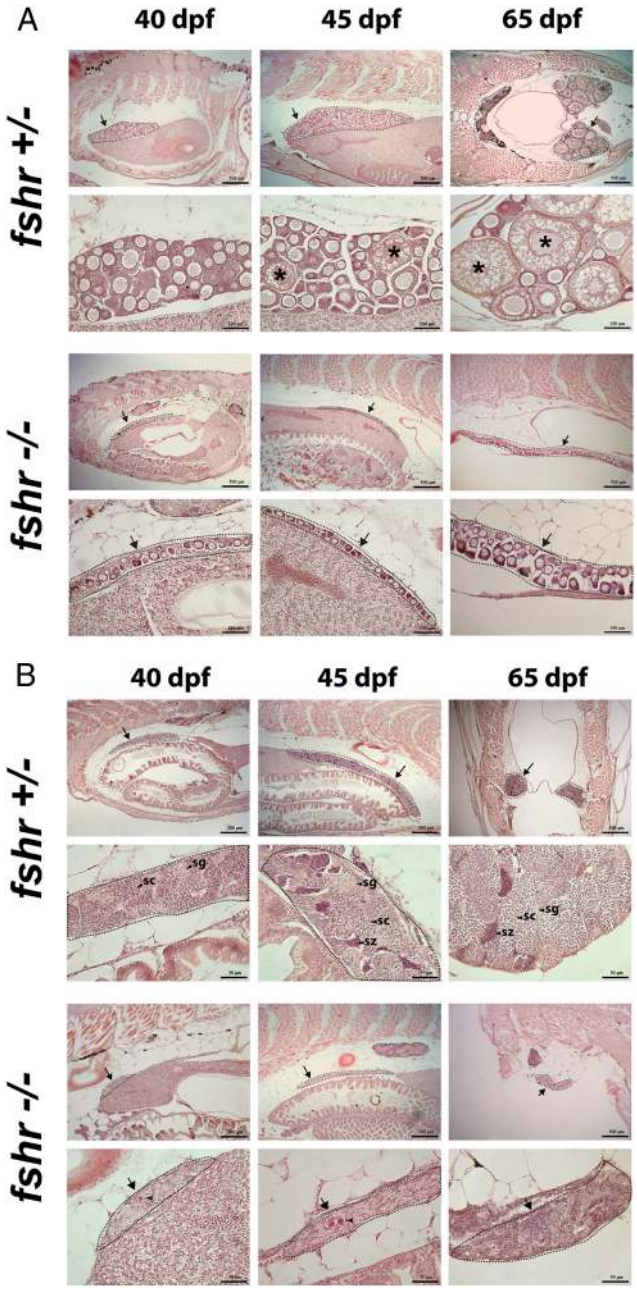

Figure 2. Effects of *fshr* deficiency on ovarian and testis development in juvenile and young zebrafish at 40, 45, and 65 dpf. A, Ovarian growth and follicle development in control ($fshr^{+/-}$) and *fshr*-deficient mutant females ($fshr^{-/-}$). B, Testis development in control ($fshr^{+/-}$) and mutant males ($fshr^{-/-}$). The asterisk indicates the activated PV follicle, and the arrow shows the ovary or testis in each longitudinal section, which is also circled by a dotted line to distinguish from the surrounding organs. The degenerating or apoptotic oocyte-like germ cells in the testis are indicated by the arrowhead. sc, spermatocytes; sg, spermatogonia; sz, spermatozoa.

PG follicles in the mutant ovaries were also small, with increased basophilia and condensation of ooplasm, a sign of degeneration. This situation remained the same at 65 dpf except that the interfollicular space increased further (Figure 2A).

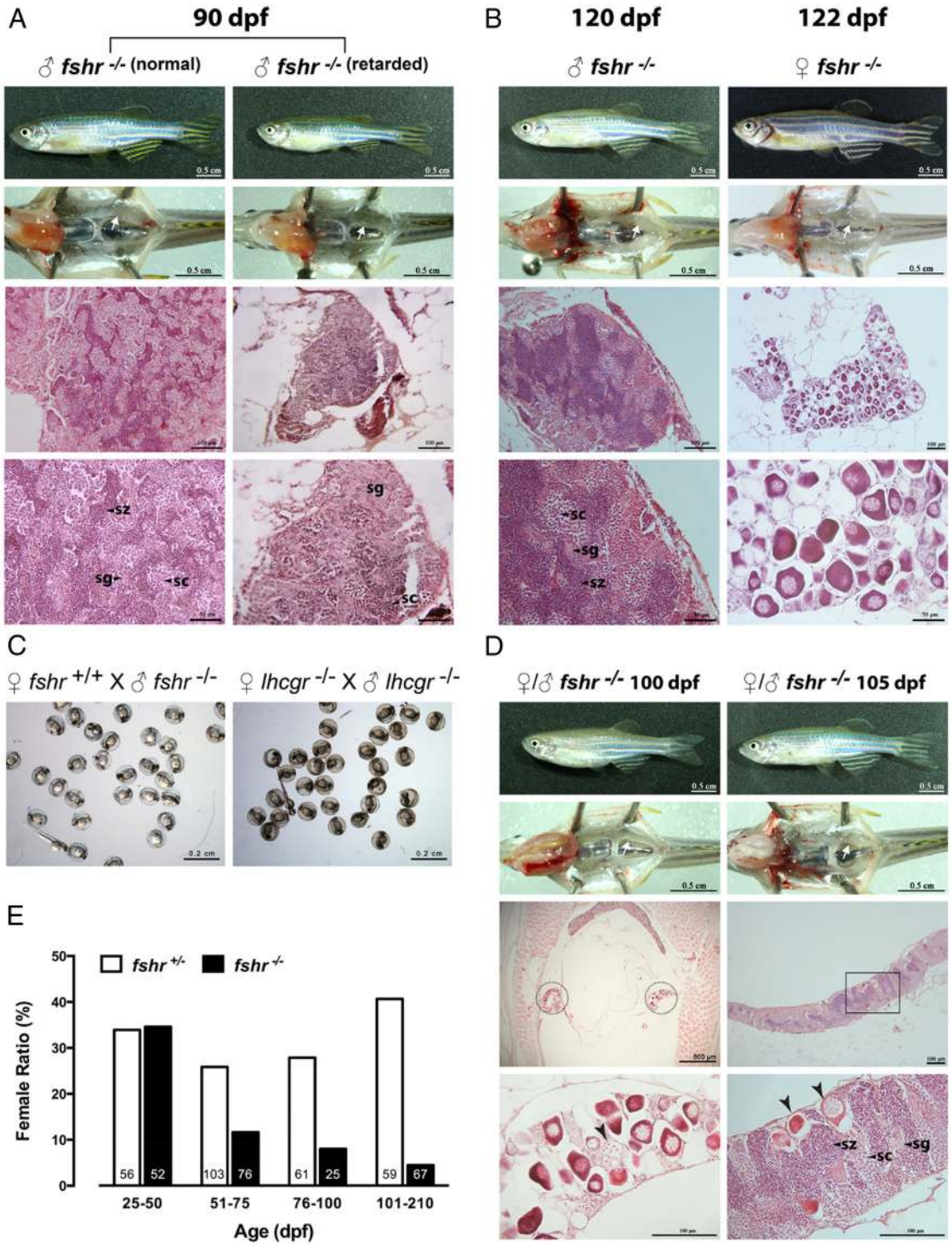

Figure 3. Evidence for blockade of follicle activation, sex reversal, and delayed spermatogenesis in *fshr*-deficient adult zebrafish. A, Testis development in mutant fish (*fshr*$^{-/-}$) at 90 dpf. Some individuals had underdeveloped testis (right panel), which might have developed from females through sex reversal. The arrow in the dissected fish shows the gonads. B, Normal testis development in *fshr*-deficient fish sampled at 120–122 dpf (left panel, 32 in total). Only one female was found at 122 dpf (right panel) in this group with all follicles arrested at the PG stage. C, Fertility assay for *fshr* and *lhcgr* mutants at 90 dpf. The fertility of homozygous mutant fish was assessed by natural breeding in a breeding box (Techniplast). Male *fshr* mutant was crossed with wild-type female because mutant adult females were rare and infertile. D, Evidence for sex reversal in *fshr*-deficient adult zebrafish. Two fish at 100 and 105 dpf were identified with ovotestis. The arrow shows meiotic spermatogenic cells among degenerating PG follicles in the ovary (left panel, circled) at 100 dpf and degenerating follicles among spermatogenic tissue at 105 dpf (right panel). E, Temporal change of female ratio from 25 to 210 dpf. The number in each column is the sample size. sc, spermatocytes; sg, spermatogonia; sz, spermatozoa.

Delayed initiation of spermatogenesis in *fshr*-deficient males

To investigate whether *fshr* deficiency affects early spermatogenesis, we examined juvenile or young males at 40, 45, and 65 dpf. The control fish ($fshr^{+/-}$) showed normal progress of spermatogenesis with spermatocytes at all sampling times and mature spermatozoa present at 45 and 65 dpf. In contrast, the testes in mutant males showed significant retardation in growth with delayed spermatogenesis, and all germ cells were arrested at spermatogonial stage with apoptotic oocyte-like germ cells (Figure 2B).

Despite a delayed initiation of spermatogenesis, the testis of most mutant males showed normal development as compared with the controls at 90 dpf when the zebrafish is supposed to be sexually mature (Figure 1B). When tested for fertility at 90 dpf, all control males ($fshr^{+/-}$) could induce female spawning with normal fertilization. Interestingly, most *fshr*-deficient males ($fshr^{-/-}$) were also fertile (7 of 10); however, some individuals failed to induce wild-type females to spawn (three trials for each individual with a 2 d interval). Further histological analysis showed that the mutant males that could spawn successfully had normal spermatogenesis in the testis (Figure 3A, left panel). However, the mutant males that failed to spawn showed retarded spermatogenesis in the testis, which contained fewer spermatocytes and more abundant spermatogonia (Figure 3A, right panel). These individuals might have developed from females through sex reversal (see below), resulting in delayed spermatogenesis as compared with that of controls as well as genotypic male mutants of the same age.

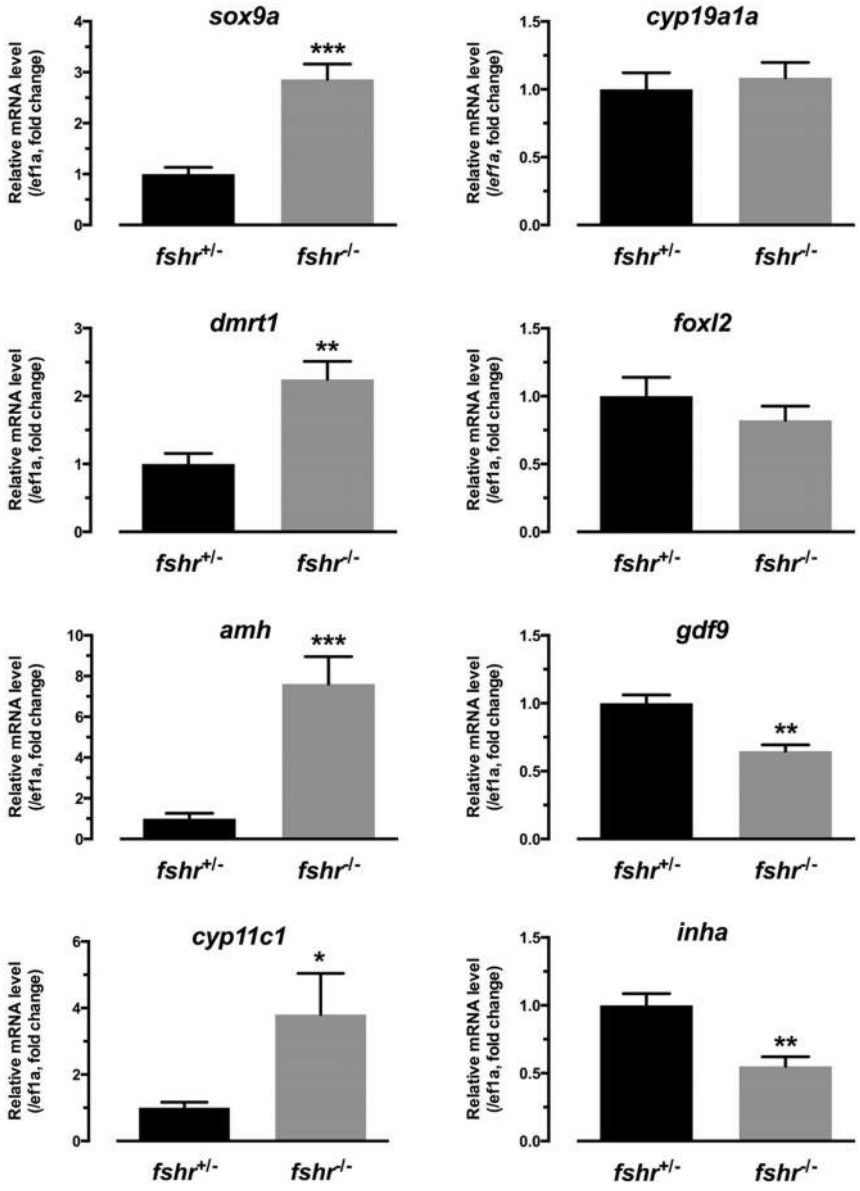

Figure 4. Expression of *sox9a*, *dmrt1*, *amh*, *cyp11c1*, *cyp19a1a*, *foxl2*, *gdf9*, and *inha* in the ovary of *fshr*-deficient ($fshr^{-/-}$) and control ($fshr^{+/-}$) fish at 40 dpf. Total RNA was extracted from the ovary after pretreatment of the fish with RNAlater (QIAGEN) followed by reverse transcription and real-time qPCR analysis. The data are mean ± SEM (n = 10). *, $P < .05$, **, $P < .01$, ***, $P < .001$ vs control. *amh*, anti-Müllerian hormone; *cyp11c1*, cytochrome P450, family 11, subfamily C, polypeptide 1; *cyp19a1a*, aromatase; *dmrt1*, doublesex and mab-3 related transcription factor 1; *foxl2*, forkhead box L2; *gdf9*, growth differentiation factor 9; *inha*, inhibin-α subunit; *sox9a*, SRY-box 9a.

Development of all-male adults in *fshr*-deficient fish due to sex reversal

Although *fshr*-deficient fish showed normal sex differentiation, further gonadal development showed significant abnormalities as described above. The folliculogenesis in females was arrested at the early PG stage without transition into the PV stage, and the spermatogenesis in males was delayed at an early stage. Interestingly, most $fshr^{-/-}$ individuals examined in one experiment at 120–122 dpf (32 in total) were surprisingly males with normal spermatogenesis (Figure 3B, left panel), and only one female was found at 122 dpf with folliculogenesis in the ovary completely halted at the PG stage (Figure 3B). The identification of female individuals became increasingly difficult in older fish, and the last two females were discovered at 210

dpf with all follicles still arrested at the PG stage (data not shown). Different from the mutant males examined at 90 dpf, which included both fertile and infertile individuals, all male individuals examined at 120 dpf (20 in total) could induce female spawning with successful fertilization (Figure 3C). This observation led us to hypothesize that without Fshr, the females observed in juvenile or young fish must have undergone a process of sex reversal to become males. To provide evidence for this hypothesis, we screened fish aged between 100 and 120 dpf by histology with the aim to identify individuals with transitional gonads or ovotestis. All fish examined during this period were males except two individuals identified at 100 and 105 dpf, respectively (Figure 3D). The gonads of the 100-dpf fish were still predominantly ovaries with underdeveloped PG follicles that were well separated with large interfollicular space and enhanced ooplasmic basophilia; however, signs of spermatogenesis could be seen between follicles (Figure 3D, left panel). The 105-dpf fish had a well-developed testis with degenerating oocytes scattered among spermatogenic cells (Figure 3D, right panel).

To provide clues to when sex reversal occurred during gonadal development, we analyzed the temporal change of female ratio at different stages. The samples (499 in total) were divided into four age groups representing gonadal differentiation and puberty (25–50 dpf), sexual maturation (51–75 dpf), early spawning (75–100 dpf), and late spawning (101–210 dpf), respectively. Interestingly, there was no sex ratio difference between the control (*fshr*$^{+/-}$; 34% female) and mutant fish (*fshr*$^{-/-}$; 35% female) during sex differentiation and puberty (25–50 dpf), but the female ratio in the mutant started to drop during sexual maturation (51–75 dpf; 11%) and continued to decrease afterward to less than 5% after 100 dpf (Figure 3E). To provide clues to the molecular mechanism underlying the sex reversal, we determined the expression levels of some genes that are likely involved in gonadal differentiation, including *sox9a*, *dmrt1*, *amh*, *cyp11c1*, *cyp19a1a*, *foxl2*, *gdf9*, and *inha*. The ovarian samples were collected at 40 dpf when both the control and mutant ovaries contained PG follicles only and before sex reversal started. Interestingly, all four testis-promoting genes (*sox9a*, *dmrt1*, *amh*, and *cyp11c1*) significantly increased their expression in the mutant ovaries (*fshr*$^{-/-}$) compared with that in the control (*fshr*$^{+/-}$); in contrast, the expression of oocyte-specific gene *gdf9* decreased significantly in the mutant together with *inha*. Surprisingly, neither aromatase (*cyp191a1*) nor its upstream stimulatory transcription factor *foxl2* showed any significant change (Figure 4).

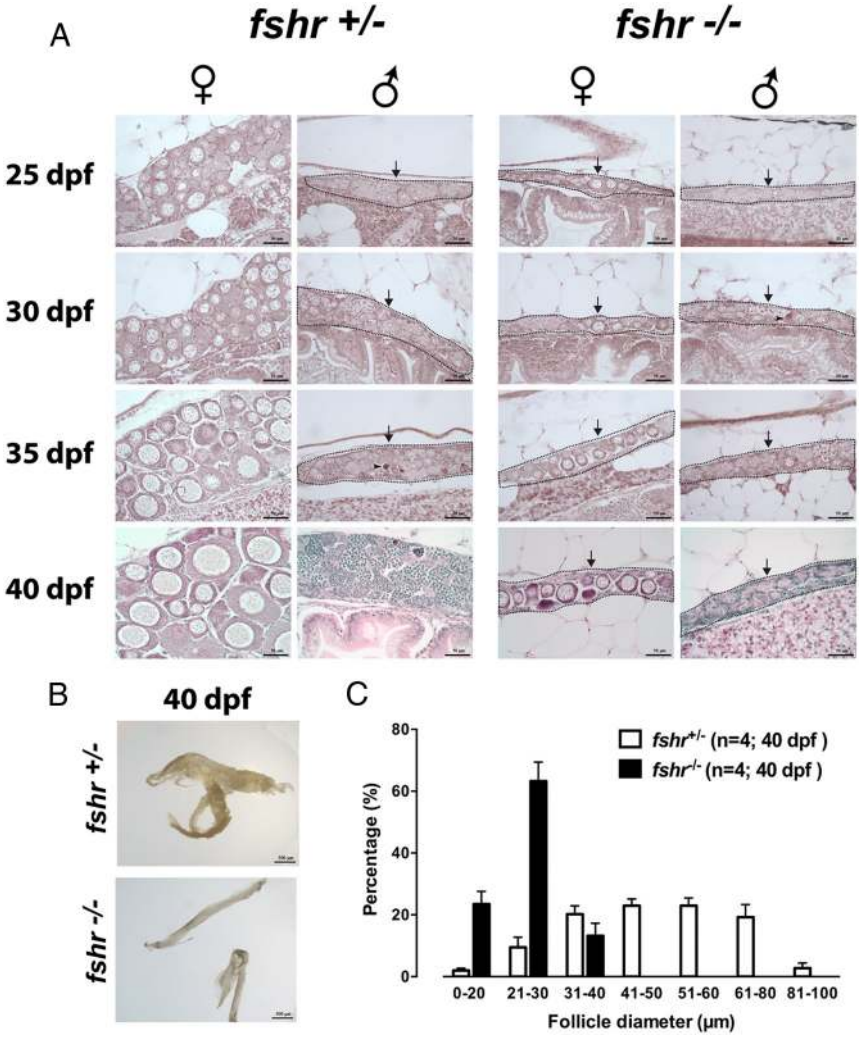

Figure 5. Effects of *fshr* deficiency on gonadal differentiation and ovarian growth in juvenile fish. A, Juvenile control (*fshr*$^{+/-}$) and mutant (*fshr*$^{-/-}$) zebrafish of similar body size were sampled for histological analysis during the period (25–40 dpf) when gonadal differentiation occurs. The lack of Fshr did not seem to affect gonadal differentiation but reduced the size and number of the germ cells, especially in females. The arrow shows the gonads, which are outlined by dotted lines to distinguish from the surrounding tissues, and arrowhead shows degenerating oocyte-like germ cells. B, Ovaries from control (*fshr*$^{+/-}$) and mutant (*fshr*$^{-/-}$) fish. C, Size distribution of PG follicles in the ovary of control and mutant fish at 40 dpf.

Role of Fshr in gonadal development at sex differentiation

After demonstrating delayed spermatogenesis in males and failed pu-

berty onset or PG-PV transition in females, we turned our attention to potential role of Fshr in an even earlier event, ie, gonadal development at sex differentiation, although there was no difference in sex ratio at this stage as shown above (Figure 3E). To this end, we examined fish at 25, 30, 35, and 40 dpf, a critical period when gonadal differentiation occurs in the zebrafish (55, 56).

We did not observe much difference in testis formation and growth between control ($fshr^{+/-}$) and mutant ($fshr^{-/-}$) male fish from 25 to 35 dpf, although the mutant testes seemed to be smaller. Both the control and mutant testes contained primarily spermatogonia during this period. The difference became significant at 40 dpf when the control testes started to grow quickly with active spermatogenesis; in contrast, the mutant testes still remained underdeveloped without any spermatocytes (Figure 5A). In contrast to males, the ovarian formation and growth in females exhibited significant difference between the control and mutant fish during the same period from 25 to 40 dpf. At all four time points, the control fish had a well-formed ovary containing abundant and tightly packed perinucleolar oocytes or PG follicles, which steadily grew in size from 25 to 40 dpf. In contrast, the ovaries of mutant fish were evidently smaller than those of the control (Figure 5, A and B), and they contained much less and smaller follicles, which did not grow much over the period (Figure 5A).

The small follicles in the mutant ovaries suggested that there was a significant blockade of folliculogenesis at early PG stage. To provide quantitative data on the blockage, we measured sizes of follicles in the ovaries of control and mutant fish at 40 dpf (n = 4). Interestingly, the follicles from the control ovaries had a wide range of size distribution up to 100 μm in diameter; in contrast, the mutant ovaries only contained follicles of less than 40 μm, with the majority being in the range of 21–30 μm (Figure 5C).

Normal gonadal development in both females and males without Lhcgr

In contrast to the significant phenotypes of *fshr* deficiency in both females and males, knockout of *lhcgr* surprisingly produced no observable phenotypes in both sexes. Gross anatomical examination at 90 dpf showed normal growth of both the ovary and testis in mutant fish ($lhcgr^{-/-}$) compared with controls ($lhcgr^{+/+}$ and $lhcgr^{+/-}$). Histological analysis demonstrated normal folliculogenesis in the ovary (Figure 6A) and spermatogenesis in the testis (Figure 6B) of all three genotypes. A fertility test showed that both female and male *lhcgr*-deficient zebrafish were fully fertile (Figure 3C).

The lack of observable phenotypes in the *lhcgr* mutant was surprising. To provide further evidence for the successful disruption of the *lhcgr* gene, we performed a functional study by examining the responsiveness of a LH-responsive gene, activin βA2 (*inhbab*), to hCG (LH analog) in cultured follicle cells (53). When treated with hCG, which specifically activates Lhcgr but not Fshr in the zebrafish (23, 24), the expression of *inhbab* increased significantly in cultured follicle cells from both $lhcgr^{+/+}$ and $lhcgr^{+/-}$ ovaries. However, there was no response at all in

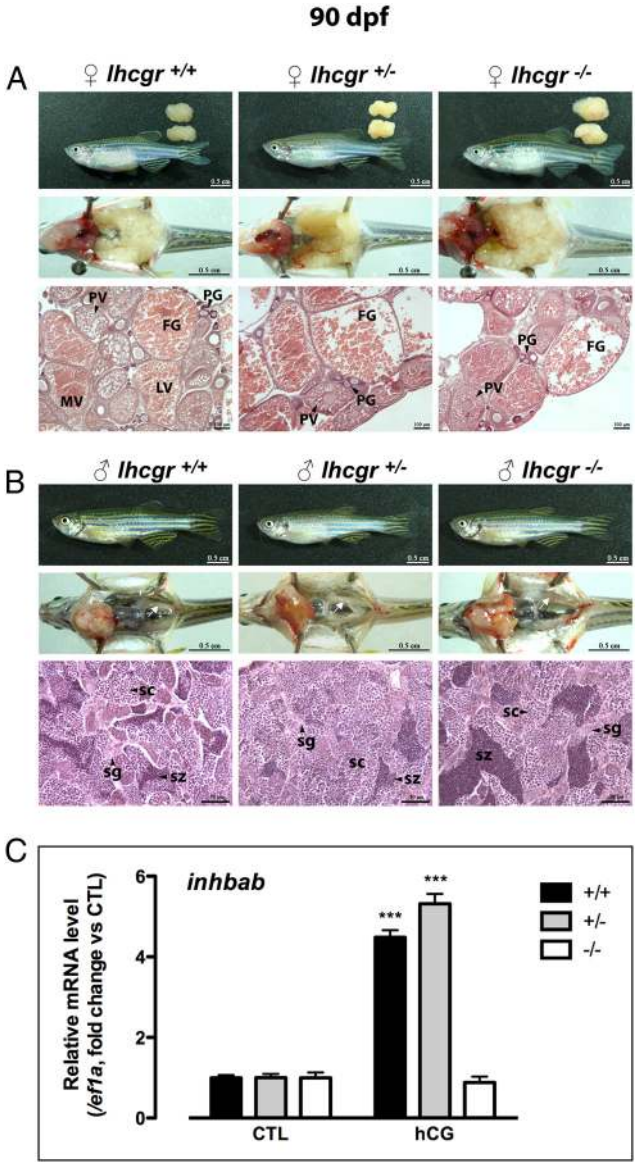

Figure 6. Normal folliculogenesis and spermatogenesis in *lhcgr*-deficient females and males at 90 dpf. Adult zebrafish with similar body size were sampled at 90 dpf for morphological and histological analysis. A, The ovaries from control ($lhcgr^{+/+}$ and $lhcgr^{+/-}$) and mutant ($lhcgr^{-/-}$) females. LV, late vitellogenic. B, The testes (arrow) from control ($lhcgr^{+/+}$ and $lhcgr^{+/-}$) and mutant ($lhcgr^{-/-}$) males. sc, spermatocytes; sg, spermatogonia; sz, spermatozoa. C, Response of *inhbab* expression to hCG (LH analog) in cultured zebrafish ovarian follicle cells from control ($lhcgr^{+/+}$ and $lhcgr^{+/-}$) and mutant ($lhcgr^{-/-}$) zebrafish. The cells were treated with hCG (15 IU/mL) in serum-free M199 medium for 2 hours followed by RNA extraction for real-time qPCR analysis. The data are mean ± SEM (n = 4). ***, $P < .001$. CTL, control.

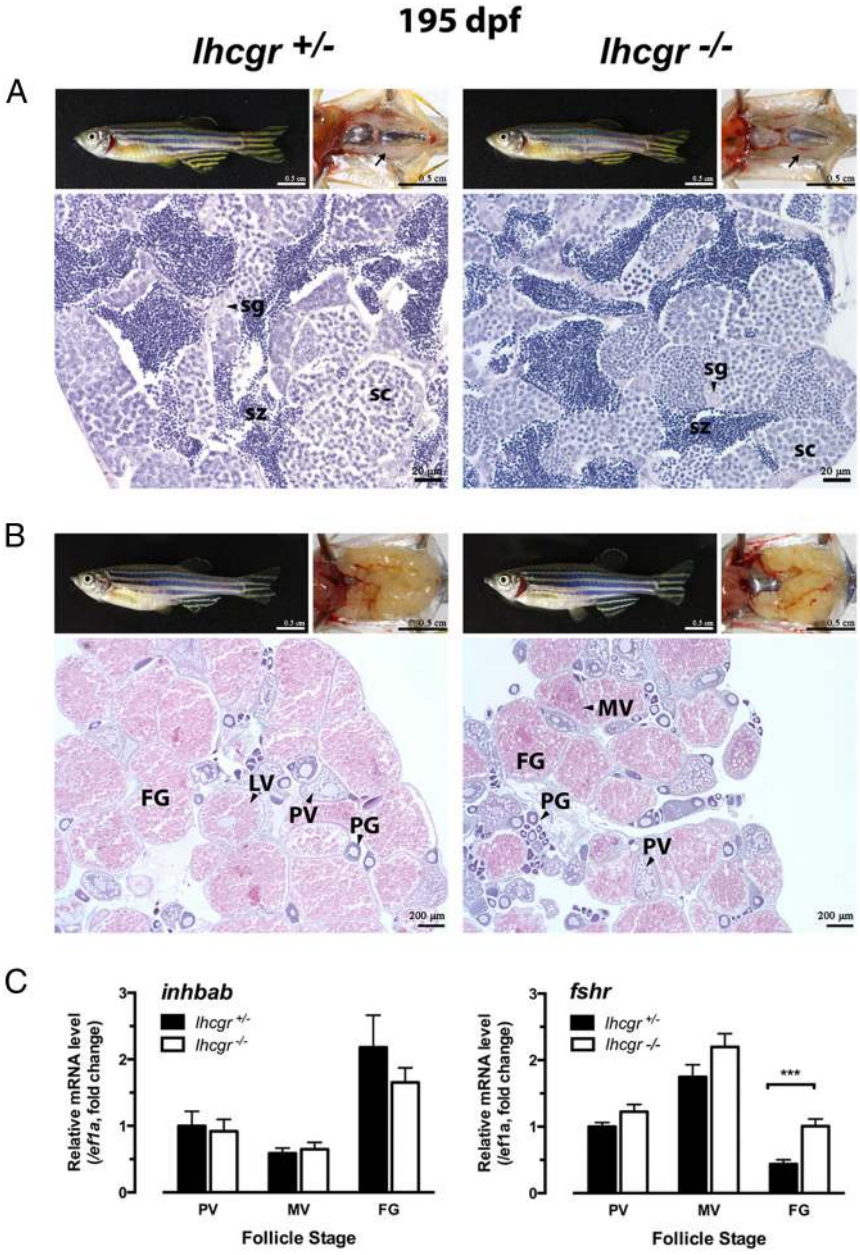

Figure 7. Normal folliculogenesis and spermatogenesis in *lhcgr*-deficient females and males at 195 dpf. A, Spermatogenesis in control ($lhcgr^{+/-}$) and mutant ($lhcgr^{-/-}$) males. sc, spermatocytes; sg, spermatogonia; sz, spermatozoa. B, Folliculogenesis in control and mutant females. LV, late vitellogenic. C, Expression of *inhbab* (LH responsive gene) and *fshr* in the follicles of different stages from control ($lhcgr^{+/-}$) and mutant ($lhcgr^{-/-}$) females.

the cells from mutant fish ($lhcgr^{-/-}$), indicating a complete loss of the functional LH receptor (Figure 6C).

To demonstrate whether there is any decline of gametogenesis in *lhcgr*-mutant fish at late spawning stage, we performed a histological analysis of both testis and ovary at 195 dpf. The result showed no difference in both spermatogenesis and folliculogenesis (Figure 7, A and B). To provide clues to the underlying mechanism for normal gametogenesis in the *lhcgr* mutant, we analyzed the expression of *inhbab* and *fshr* in the follicles of different stages (PV, MV, and FG) from both the control and mutant fish. Interestingly, although *inhbab* is highly responsive to LH in vitro (Figure 6C), its expression in the mutant ovary only decreased slightly but not significantly at FG stage when the expression of *lhcgr* reaches the highest level (23, 30). In contrast, the expression of *fshr* increased significantly in the FG follicles from the mutant ovary (Figure 7C).

Normal follicle activation or puberty onset in *lhcgr*-deficient females and males

Having demonstrated normal gonadal development in the absence of Lhcgr at 90 and 195 dpf, we further examined whether the *lhcgr* mutation affected the earlier stage of gonadal development, in particular puberty onset when the first cohort of follicles are activated to enter the fast secondary growth stage marked by the appearance of PV follicles (stage II with cortical alveoli in the oocytes). The fish were sampled at 45 dpf when puberty onset is expected. In contrast to the deficiency of Fshr, the lack of Lhcgr had no effect on the timing of follicle activation or puberty onset in females because PV follicles appeared in the ovaries of both control and mutant fish. There was no obvious difference in spermatogenesis as well between the mutant and control at 45 dpf (Figure 8).

Effects of double mutation of *fshr* and *lhcgr* on gonadal development

With *fshr* and *lhcgr* mutants available, we also created zebrafish carrying double mutations of the two receptors. With no observable phenotypes in *lhcgr*-deficient fish, we expected that double mutation of the two genes would phenocopy *fshr* mutation to produce all-male and fertile individuals. Surprisingly, simultaneous disruption of the two receptors led to complete infertility, at least up to 190 dpf. Anatomical and histological analyses revealed that compared with the single mutation control ($fshr^{-/-}/lhcgr^{+/-}$), the gonads of double mutants

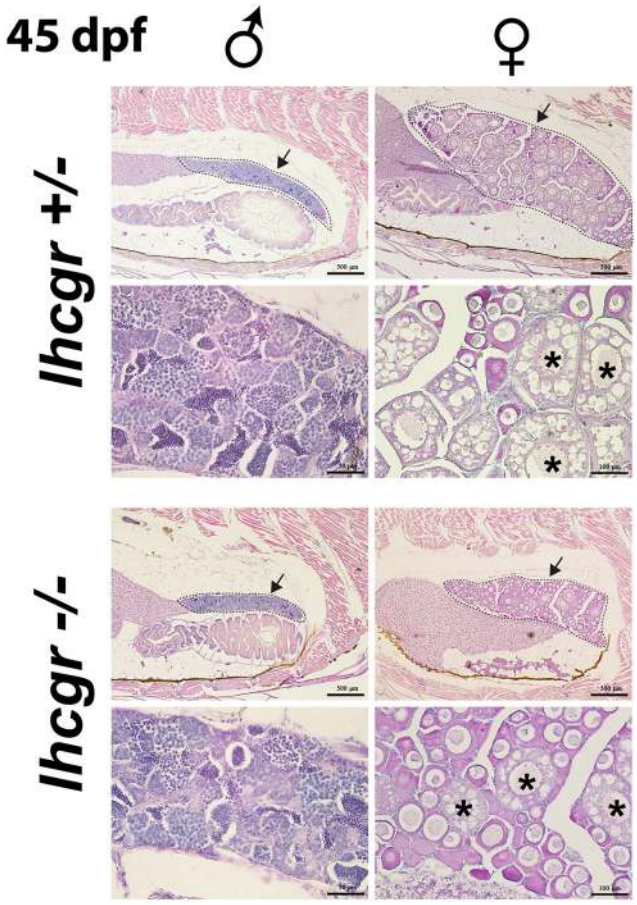

Figure 8. Normal puberty onset in *lhcgr*-deficient females and males. Juvenile zebrafish were sampled for histological analysis at 45 dpf when puberty onset is expected in females. The marker used for puberty onset is the appearance of PV follicles (asterisk) that contain cortical alveoli. The arrow shows the gonads, which are also outlined by dotted lines.

($fshr^{-/-}/lhcgr^{-/-}$) were extremely small, with no growth from 82 to 190 dpf. Unlike the single *fshr* mutation, we did not observe any signs of oocyte formation in the gonads of double mutants. Most gonads were at an undifferentiated state, even at 190 dpf, whereas some individuals showed early signs of spermatogenesis, eg, the fish at 82 and 150 dpf (Figure 9).

Discussion

Using TALEN technology, we have recently deleted the genes coding for FSH- and LH-β subunits (*fshb* and *lhb*) in the zebrafish, creating two mutants that lack FSH or LH hormone, respectively. Despite a significant delay of puberty onset in both females and males, the ovary and testis in *fshb*-deficient zebrafish could still develop to full size, and the fish were fertile in both sexes (48). We hypothesized that the catch-up gonadal growth and fertility in the *fshb*-deficient zebrafish were likely due to the possibility that LH might play a compensatory role for FSH by activating FSH receptor (Fshr/*fshr*) (23, 24). This hypothesis is now supported by the evidence from the present study that the loss of Fshr led to a complete failure of follicle activation or PG-PV transition in the ovary. The activation of Fshr by both FSH and LH in the zebrafish would explain why the deletion of *fshr* could not phenocopy that of *fshb* (48). FSH is the primary ligand of Fshr; however, when FSH is absent, LH may play a compensatory role by activating Fshr to drive folliculogenesis and spermatogenesis in both females and males.

In addition to the blockade of folliculogenesis at the PG-PV transition, the ovaries of *fshr*-deficient fish were extremely small, with much fewer and smaller PG follicles, which could grow to only 40 μm in diameter at 40 dpf compared with 100 μm in the control. This observation strongly suggests that the PG period before follicle activation (PV stage) is not gonadotropin independent, as is usually believed (57), and that the Fshr signaling, triggered by either FSH or LH or both, plays an important role in controlling both the number and growth of PG follicles, especially the late PG stage. In *Fshr*-deficient mouse, the ovaries were also significantly reduced in size with the follicular development being arrested at preantral stage (presumably equivalent to the PG stage in the zebrafish), and the animals cannot initiate puberty for sexual maturation (5, 58). These phenotypes are fully consistent with our observations in the *fshr*-deficient zebrafish. The conserved phenotypes of Fshr/FSHR deficiency in both zebrafish and mammals suggest a conserved role for FSH-FSHR signaling in controlling folliculogenesis across vertebrates.

Very interestingly, although the ovarian growth and follicle activation were completely halted in the absence of Fshr, the follicles did not remain arrested indefinitely. After being blocked at PG stage for some time without Fshr, all female zebrafish eventually turned into males probably by sex reversal, leading to nearly all-male adults after 100 dpf (Figure 10). In support of sex reversal was the evidence that there was no difference in sex ratio between *fshr*-deficient fish and the control from 25 to 50 dpf when gonadal differentiation and puberty occur in the zebrafish and the female ratio decreased significantly during sexual maturation (51–75 dpf). In addition, individuals with ovotestis were observed as late as 105 dpf, which is far beyond the time of sexual differentiation (~30 dpf) (55, 56). Our observation is surprisingly similar to a recent report in the medaka that a mutation in *fshr* gene induced by TILLING (Targeting Induced Local Lesions in Genomes) led to the masculinization of females (59). We also observed similar phenomenon of sex reversal in *fshb*-deficient fish in our recent study; however, the rate of sex

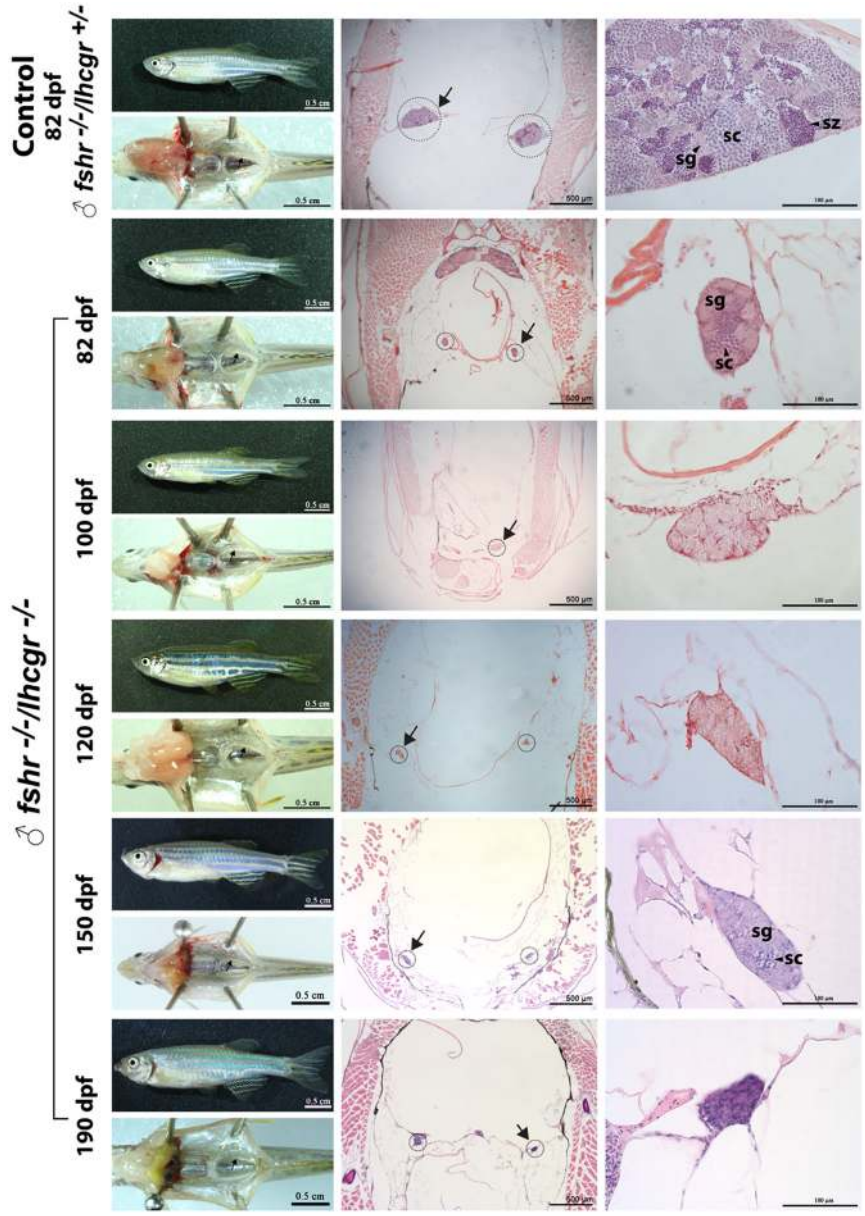

Figure 9. Gonadal development in *fshr* and *lhcgr* double mutants at 82–190 dpf. The control fish ($fshr^{-/-}/lhcgr^{+/-}$) showed normal testis development at 82 dpf, whereas the double mutants ($fshr^{-/-}/lhcgr^{-/-}$) were infertile with extremely underdeveloped testes up to 190 dpf. The testis showed no growth during the sampling period, leading to complete infertility. sc, spermatocytes; sg, spermatogonia; sz, spermatozoa.

change was not as high as that in *fshr*-deficient fish. This difference could most likely be due to the cross-activation of Fshr by LH in the absence of FSH because the double knockout of *fshb* and *lhb* led to all-male offspring (48). The change from female to male or masculinization without Fshr in both the zebrafish and medaka supports the concept that sex differentiation in fish has greater plasticity as compared with that in mammals.

In addition to genetic factors, both external environmental and internal endocrine conditions may affect sex differentiation in fish. One possible endocrine mechanism for sex reversal after 50 dpf was a lower expression of aromatase because the systemic estrogens have been shown to be important in maintaining female status in the zebrafish (60, 61). A recent study showed that when treated with an aromatase inhibitor, the mature female zebrafish changed sex to male (60). However, our gene expression data did not seem to support this hypothesis. The expression of aromatase (*cyp19a1a*) and its upstream transcription factor *foxl2* (62) in the ovary at 40 dpf showed no difference between *fshr* mutant and the control. In contrast, all four testis-promoting genes (*sox9a*, *dmrt1*, *amh*, and *cyp11c1*) (63–65) significantly increased expression in the *fshr*-deficient ovary, whereas the expression of *gdf9*, an oocyte-specific growth factor (66), decreased. This result suggests that an enhanced testis-promoting activity is likely a major driving force for the sex reversal in the *fshr* mutant. The potential compensatory role of Fshr for Lhcgr is further supported by our observation that the expression of *fshr* was significantly up-regulated in the Lhcgr-deficient follicles, especially at the FG stage, whereas the LH-responsive *inhbab* expression was maintained.

The lack of Fshr did not seem to affect testis development in adults, and all males tested were fertile after 120 dpf. However, examination at earlier stages in juveniles showed that similar to the situation in females, *fshr*-deficient males also demonstrated delayed spermatogenesis. Our observation suggests that Fshr signaling by either FSH or LH plays an important but not determining role in triggering the initiation of spermatogenesis at puberty onset, but its importance declined afterward. This result is consistent with the report in mammals that *Fshr*-deficient male mice showed delayed puberty (6). In some *fshr*-deficient fish, we observed delayed spermatogenesis as late as 90 dpf. These individuals were likely those converted from females. Despite the delay, all males including those converted from genotypic females were fertile as shown by the fertility test.

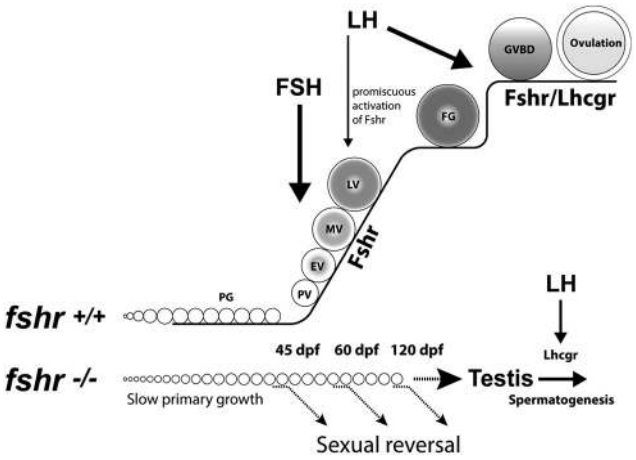

Figure 10. Hypothetical model for roles of gonadotropin receptors, especially Fshr, in ovarian development. Although Fshr does not seem to play a role in gonadal differentiation, it is essential for ovarian growth and folliculogenesis afterward. In the absence of Fshr, both the size and number of the PG follicles in the ovary before puberty are reduced, suggesting a role for Fshr signaling during primary growth stage. Fshr is also indispensable for the activation of PG follicles to enter the fast secondary or vitellogenic growth. After being arrested for some time without Fshr, all female zebrafish eventually turn into males through sex reversal, leading to all-male adults. The spermatogenesis in genetic or sex-reversed males in the absence of Fshr is driven by Lhcgr. Double mutation of *fshr* and *lhcgr* results in male infertility. EV, early vitellogenic.

One of the most striking discoveries of the present study was the lack of obvious phenotypes of Lhcgr deficiency in adult males and females, which were both fertile. This agrees with a similar study published recently (67). Our further experiments on early stages of development revealed no difference between *lhcgr* mutant and the control in terms of follicle activation or puberty onset. In contrast, the knockout of LHCGR in the mouse caused infertility in both males and females with severe abnormalities, including reduced plasma sex steroid levels (estradiol in females and testosterone in males) and reduced gonadal sizes (7). In humans, an inactivating mutation of LHCGR resulted in female infertility with failed ovulation; however, the ovary had normal structure with all stages of follicles present, suggesting normal FSH-FSHR signaling (68). A nonsense mutation of LHCGR in a boy also led to infertility with Leydig cell hypoplasia (69). Different from FSH and its receptor Fshr, which could not fully phenocopy each other when deleted due to promiscuous activation of Fshr by LH, the disruption of the zebrafish *lhcgr* gene seemed to phenocopy that of *lhb* well in terms of gonadal growth. However, the phenotypes of *lhcgr* and *lhb* mutants do not agree in terms of oocyte maturation and ovulation in vivo. The *lhb*-deficient females were infertile due to failed oocyte maturation and ovulation, whereas *lhcgr*-deficient fish were normal in this regard (48).

The lack of obvious phenotypes in Lhcgr-deficient male and female zebrafish poses a challenge to traditional views on the role and importance of the LH-Lhcgr signaling in fish. This signaling pathway does not seem to play any role in puberty onset, gonadal growth, and gamete maturation in both sexes. However, the well-established concept that LH plays an important role in triggering final oocyte maturation and ovulation holds true in the zebrafish because our recent study showed that lack of LH led to infertility because of failed oocyte maturation and ovulation; obviously this role could not be compensated for by FSH (48). The fact that the Lhcgr mutation did not phenocopy LH deficiency in this aspect strongly suggests that LH may most likely signal through an alternative pathway involving Fshr in the absence of Lhcgr. This illustrates a potential safeguarding role of the promiscuous activation of Fshr by LH in the zebrafish and probably other fish species with similar phenomenon as well (21, 70, 71).

The lack of obvious phenotypes in *lhcgr*-deficient zebrafish does not fully exclude the importance of this receptor in reproduction, and its potential role was best illustrated by the double mutation of *fshr* and *lhcgr*. Although the *fshr* and *lhcgr* mutation alone caused limited or little disturbance to spermatogenesis in adult males, simultaneous mutation of both *fshr* and *lhcgr* resulted in a severe retardation of testis development. The testes in mutant fish were so underdeveloped that the fish were all infertile at the time of examination (up to 190 dpf). This suggests that when Fshr alone is absent, the delayed but normal process of spermatogenesis is indeed driven by Lhcgr signaling.

In summary, using TALEN technology to induce gene mutation, the present study provides comprehensive comparative evidence, for the first time in fish, for roles of Fshr and Lhcgr in male and female reproduction. Although our results support some established views in fish models and are consistent with some findings in mammalian models (for comparison, see Supplemental Table 2) such as FSH-Fshr signaling in puberty onset and gonadal growth, some discoveries from the present study are indeed challenging the long-held views in fish reproductive biology. Our major findings are summarized as follows: 1) Fshr signaling is essential for early gonadal growth in both males and females; 2) all *fshr*-deficient females reverse to males after a certain period of blockade at the PG stage (Figure 10); 3) neither Fshr nor Lhcgr alone is indispensable to spermatogenesis; however, the loss of both leads to infertility due to underdeveloped testis and retarded spermatogenesis at adult stage; and 4) the traditional canonical LH-Lhcgr signaling does not seem to be essential for zebrafish fertility, and the role of LH is likely mediated by the LH-Fshr pathway.

Acknowledgments

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