Targeted Gene Disruption in Zebrafish Reveals Noncanonical Functions of LH Signaling in Reproduction

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The pivotal role of gonadotropin signaling in regulating gonadal development and functions has attracted much research attention in the past 2 decades. However, the precise physiological role of gonadotropin signaling is still largely unknown in fish. In this study, we have established both LH β-subunit (lhb) and LH receptor (lhr) knockout zebrafish lines by transcription activator-like effector nucleases. Intriguingly, both homozygous lhb and lhr mutant male fish are fertile. The fertilization rate, sperm motility, and histological structure of the testis were not affected in either lhb or lhr mutant males. On the contrary, homozygous lhb mutant females are infertile, whereas homozygous lhr mutant females are fertile. Folliculogenesis was not affected in either lhb or lhr mutants, but oocyte maturation and ovulation were disrupted in lhb mutant, whereas only ovulation was affected in lhr mutant. Differential expression of genes in the ovary involved in steroidogenesis, oocyte maturation, and ovulation was found between the lhb and lhr mutants. These data demonstrate the essential role of LH signaling in oocyte maturation and ovulation, and support the notion that LH acts through the FSH receptor in the absence of LH receptor. Moreover, the defects of lhb mutant could be partially restored by administration of human chorionic gonadotropin. This in vivo evidence in the present study demonstrates, for the first time in any vertebrate species, that LH signaling is indispensable in female reproduction but not in male reproduction. LH signaling is demonstrated to control oocyte maturation and ovulation in the ovary. (Molecular Endocrinology 28: 1785–1795, 2014)
males and folliculogenesis at the antral stage in females. Thus, both males and females of either LH or LHR knockout mice are infertile (6–8). Such information on teleost fish, being the largest and most diverse group of vertebrates, lags far behind that for mammals, particularly regarding the in vivo functions of the gonadotropins and their receptors. Currently, most studies on fish LH and LHR mainly focus on their regulation and expression as well as ligand-binding specificity. The spatial and temporal distribution of LH in the pituitary (9–15) and LHR in the gonads (16–22) was established in several fish species. Although most reports suggest a conserved role of LH signaling from fish to mammals, the physiological functions of LH signaling in controlling fish reproduction still lack in vivo evidence. In addition, marked differences exist between mammals and fish with regard to the specificity of the gonadotropins toward their receptors (1, 23, 24). It has been demonstrated in several fish species including zebrafish that FSH stimulates only FSHR, whereas LH stimulates both FSHR and LHR (11, 25–28). This difference implies functional differentiation of LH signaling from fish to mammals. So far, the most information on the specificity of fish gonadotropins in recognizing their receptors has come from in vitro studies performed in mammalian cell lines. Because of the lack of efficient in vivo gene manipulation methods in fish, the physiological importance of this ligand/receptor differentiation is still unknown.

Recently, a new method has emerged to achieve targeted gene knockout in zebrafish by using the transcription activator-like effector nucleases (TALENs) (29, 30). Transcription activator-like-repeat sequences recognize their target sequences in a context-independent modular fashion (31). Therefore, once the target sites are chosen, transcription activator-like effector arrays for these sites can be predicted and assembled. So far, TALENs have been used to edit specific genomic loci in yeast (31), worms (32), plants (33), zebrafish (29, 30, 34, 35), mice (36), rats (37), frogs (38), and pigs (39). Previously, we adapted the original TALEN plasmids and successfully modified the expression vector to make it suitable for gene knockout studies in animals (38, 40). The emergence of this method provides a convenient and robust tool to study in vivo gene functions in fish. In the present study, using zebrafish as the model animal, the TALEN system was used to disrupt lhb and lhr, respectively, and noncanonical functions of LH signaling in zebrafish reproduction were revealed.

Materials and Methods

Zebrafish husbandry

AB zebrafish used in this study were maintained at 28°C in the zebrafish facility of The Chinese University of Hong Kong.
of the fertilization rate. The above experiment was repeated 3 to 5 times.

### Sperm motility assessment

Male fish (4 months postfertilization, 4 fish in each group of wild-type, heterozygotes, and homozygotes) were anesthetized using Tris-buffered tricaine (170 mg/mL, pH 7.5). About 1.5 μL of fresh semen was collected using a capillary tube and diluted with 20 μL of zebrafish sperm immobilizing solution. To activate the sperm, 1 μL of semen suspension in zebrafish sperm immobilizing solution was mixed with 20 μL of aged tap water. Approximately 0.5 μL of the activated sperm was quickly applied to a single well of a 12-well multitest slide (MP Biomedicals). The slide and coverslip were coated with 1% (w/v) polyvinyl alcohol to reduce sticking of the sperm. Sperm motility was assessed using computer-assisted sperm analysis by following the method of McAllister and Kime (44).

### Morphological and histological analysis

Intact testes and ovaries from adult zebrafish (4 months postfertilization) were carefully dissected after anesthetization and decapitation, and observed in a 100-mm culture dish containing 60% Leibovitz L-15 medium, or fixed in 4% buffered paraformaldehyde (Sigma-Aldrich) or Bouin’s fixative buffer (Sigma-Aldrich) overnight at 4°C, dehydrated and embedded in paraffin, and sectioned at 7-μm thickness. Sections were stained with Harris hematoxylin and eosin (Sigma-Aldrich). For testis, periodic acid Schiff/ferric hematoxylin/metalin yellow staining and PCNA immunostaining was also used (Supplemental Methods). The staging system adopted for the ovarian follicles was based on the original definition of Selman et al (45) as modified by Wang and Ge (46) and Pang and Thomas (47). The staging system on spermatogenesis was based on Schulz and colleagues (48). The reproducibility of all the morphological data was verified by similar findings in at least 3 individual fish.

### Statistical analysis

All data are expressed as mean values ± SEM. A value of P < .05 was considered statistically significant using one-way ANOVA, followed by the Tukey test for multiple comparisons to determine statistical differences between groups using GraphPad Instat software (GraphPad Software). All experiments were performed at least 2 or 3 times to confirm the results.

### Results

#### Gene targeting of lhb and lhr in zebrafish

Using our established TALENs platform, we have systematically knocked out the LH β-subunit (lhb) and LHR (lhr) genes in zebrafish. Based on the published sequence of zebrafish lhb, the site for lhb chosen for targeting is located at the middle of exon 2, with 15 bp on the left and 16 bp on the right binding sites, respectively (Figure 1A). Primers were designed to detect deletion of the targeted genomic fragment (Supplemental Figure 1A). PCR amplification of gDNA isolated from the pooled P0 embryos indicated the high frequency of somatic mutation occurring in the spacer region (about 31%) (Supplemental Figure 1B), and sequencing results confirmed that deletions correctly occurred at the target site (Supplemental Figure 1C). The ratio among wild-type (lhb+/+), heterozygous (lhb+/m), and homozygous (lhb/m/m) mutants in the F2 zebrafish obeyed Mendelian inheritance. The mutation was confirmed by sequencing gDNA amplified by PCR

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Figure 1. Targeted disruption of the zebrafish lhb gene by TALENS. A, Location of the engineered TALENs binding site on the lhb gene of zebrafish. The sequences of a pair of TALENs binding sites are underlined. WT, wild-type; M, homozygous mutant line of F2 generation zebrafish. B, Sequencing results of lhb from wild-type (lhb+/+), heterozygous (lhb+/m), and homozygous (lhb/m/m) zebrafish. C, Schematic protein structure of lhb from wild-type and mutant zebrafish based on sequencing results.
(Figure 1B). The sequencing results showed that 22 bp were deleted and 8 bp were inserted in targeting sites, causing an open-reading frame shift at amino acid position 28, which produced a truncated Lhb protein mutant (Figure 1C).

With use of a similar approach for targeted disruption of lhr in zebrafish, the TALENs binding sites were chosen at exon 1 (Figure 2A). PCR amplification of gDNA isolated from the pooled P0 embryos indicated the high frequency of somatic mutation occurring in the spacer region (about 80%) (Supplemental Figure 1, D and E). The ratio among wild-type (lhr<sup>+/+</sup>), heterozygous (lhr<sup>+</sup>m), and homozygous (lhr<sup>m/m</sup>) mutants in the F2 also obeyed Mendelian inheritance. For lhr<sup>m/m</sup>, the mutation leads to the generation of a stop codon at amino acid position 34, thus disrupting the biogenesis of the functional LHR (Figure 2, B and C). These results indicate that we have successfully engineered a null mutation at either the lhb or lhr locus that led to LH or LHR deficiency, respectively, in zebrafish.

**Fertility defects in lhb and lhr knockout female but not male zebrafish**

Homozygous lhb or homozygous lhr knockout zebrafish of both sexes were born phenotypically normal, and no distinct differences were noted regarding their survival, embryonic development, and sex differentiation. Mating of lhb or lhr mutant male zebrafish with wild-type females still produced normal numbers of offspring, and no significant difference in the fertilization rate was noticed (Figure 3, A and B). These data indicate that lhb and lhr mutant male zebrafish were fertile. In sharp contrast, mating between homozygous lhb mutant females and wild-type males did not result in any offspring (Figure 3C), indicating that the female lhb-null zebrafish were...
infertile. Mating between heterozygous or homozygous lhr mutant females and wild-type males resulted in some decrease, although not statistically significant, in the number of embryos (Figure 3D), indicating that ovary development in lhr mutant female was somewhat affected.

**Testicular morphology and histology in lhb and lhr knockout zebrafish**

To further assess the fertility of lhb and lhr mutant male zebrafish, the testes from lhb and lhr knockout males were examined morphologically and histologically. In lhb and lhr mutant males, the testis morphology appeared normal (Figure 4, A and B), and a significant decrease in the gonadosomatic index (GSI) was only found in lhb but not in lhr mutants (Figure 4, C and D). Sperm motility of both lhb and lhr mutants is similar to that of the wild type (Figure 4, E and F). Histological analysis revealed that sperm in different stages, including spermatogonia, spermatids, and spermatozoa appeared normal in both lhb and lhr mutants. These results suggest that testis development was not grossly affected in the absence of either LH or LHR in zebrafish as far as the overall histology and ability to produce viable sperm are concerned. Because a decrease in the GSI was observed in lhb mutant male zebrafish, more subtle effects on spermatogenesis were further analyzed using immunostaining of proliferating cell nuclear antigen (Supplemental Figure 2, A–F) and periodic acid Schiff/ferric hematoxylin/metanil yellow staining (Supplemental Figure 2, G–I). Normal Sertoli cells and Leydig cells could be found in mutants (Supplemental Figure 2, G–I). No significant difference in the number of proliferating cell nuclear antigen–labeled nuclei (type B spermatogonia) (Supplemental Figure 2J) was found between the lhb mutants and the wild type, nor was there any statistically significant decrease in the number of spermatids plus spermatozoa between the lhb mutants and the wild type (Supplemental Figure 2K). However, a significant increase in the number of spermatids plus spermatozoa was observed in lhr mutant male zebrafish (Supplemental Figure 2K).

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**Figure 4.** Morphology and histology of testes of wild-type (lhb<sup>+/+</sup>, lhr<sup>+/+</sup>), heterozygous (lhb<sup>+/−</sup>, lhr<sup>+/−</sup>), and homozygous (lhb<sup>−/−</sup>, lhr<sup>−/−</sup>) mutant male zebrafish. A, Appearance of testes dissected from lhb<sup>+/+</sup> (a), lhb<sup>−/−</sup> (b), and lhb<sup>+/−</sup> (c) male zebrafish. B, Appearance of testes dissected from lhr<sup>+/+</sup> (a), lhr<sup>−/−</sup> (b), and lhr<sup>+/−</sup> (c) male zebrafish. C, GSI of lhb<sup>−/−</sup>, lhr<sup>−/−</sup>, and lhb<sup>+/−</sup> male zebrafish.* P < .05; ** P < .01, compared with lhb<sup>+/+</sup> male zebrafish. The data presented are the mean values ± SEM of measurements from 6 individual fish. D, GSI of lhr<sup>−/−</sup>, lhr<sup>+/−</sup>, and lhr<sup>−/−</sup> male zebrafish. E, Sperm motility in lhb<sup>−/−</sup>, lhr<sup>−/−</sup>, and lhb<sup>+/−</sup> male zebrafish. F, Sperm motility in lhr<sup>−/−</sup>, lhr<sup>+/−</sup>, and lhr<sup>−/−</sup> male zebrafish. G, Histology of testes from lhb<sup>+/+</sup> (a), lhb<sup>−/−</sup> (b), and lhb<sup>+/−</sup> (c) male zebrafish. H, Histology of testes from lhr<sup>+/+</sup> (a), lhr<sup>−/−</sup> (b), and lhr<sup>−/−</sup> (c) male zebrafish. Bars correspond to 50 μm. SC, spermatocytes; ST, spermatids; SZ, spermatozoa; spermatogonia are marked by arrows.
spatiotemporally the process from the meiosis stage to the spermiogenesis stage.

Ovarian morphology and histology in lhb and lhr knockout zebrafish

To further delineate the cause of fertility defects observed in lhb and lhr mutant females, we examined their ovaries morphologically and histologically. In lhb mutant females, the ovary size was increased significantly in the homozygotes compared with that in the heterozygotes and wild type at adult stage (Figure 5A and B). Accordingly, a significant increase in the GSI was found in the homozygous mutants (Figure 5C). Histological analysis revealed that different stages of follicles before maturation could be found in the ovaries of lhb mutant adult fish. Follicles of primary growth (PG), previtelligenic (PV), early vitellogenic (EV), middle vitellogenic (MV), and full grown (FG) stages appeared normal by morphology and histology (Figure 5D). In addition, the ratios of the different stages of follicles between the wild type and mutants were further compared. Because PG stage follicles were too small to be counted directly by morphology, the ratio of the different stages of follicles was assessed by microscopic examination of the sections. The ratio of PG stage follicles decreased, whereas the ratio of FG stage follicles dramatically increased in the ovaries of the mutant fish (Figure 5E). To assess the conditions of the follicles, FG stage follicles were isolated for treatment with 17α,20β-dihydroxy-4-pregnen-3-one (DHP) in vitro. Most of them could enter into the mature stage as indicated by germinal vesicle breakdown (GVBD), and no significant difference was noticed between the mutants and wild type (Figure 5F), indicating that the competency to respond to endogenous hormones was not adversely affected in the follicles of lhb mutant fish.
Somewhat similar phenotypes could be observed in the female lhr mutant zebrafish, with the ovary size and GSI dramatically increased in the homozygotes (Figure 6, A–C). All stages of follicles could be readily identified in the ovary. However, unlike lhb mutants, some mature follicles and degenerating mature follicles could be found in the ovaries of homozygous lhr mutant fish (Figure 6D).

The ratio of follicles beyond the FG stage including the mature stage and the degenerating mature stage has increased in the mutants (Figure 6E). No significant difference in GVBD after treatment with DHP was found between the mutants and wild type (Figure 6F). Collectively, these data indicate that the absence of LH or LHR does not affect folliculogenesis. Yet both oocyte maturation and ovulation were affected in the lhb mutants, whereas only ovulation but not maturation was affected in lhr mutants.

**Gene expression profiles in the ovary of lhb and lhr knockout zebrafish**

To further analyze the molecular mechanism underlying the fertility defects observed in lhb and lhr mutant females as well as the differential phenotype between the 2 mutant lines, a panel of genes involved in ovarian steroidogenesis, oocyte maturation, and ovulation were chosen and their expression profiles were assessed in FG stage follicles from the ovaries of wild-type, heterozygotes, and homozygous lhr mutant female zebrafish by real-time PCR (Table 1 and Supplemental Figure 3). For key enzymes and receptors involved in steroidogenesis, the expression of npr, er2a, star1, p450scc, p450c17a1, and 17β-hsd3 was significantly reduced, whereas only mprb expression was significantly increased in the lhb mutant FG stage follicles. However, the expression of mpra, er2b, cyp19a, star2, 3b-hsd, p450c17a2, 20β-hsd1, 20b-hsd2, and 17b-hsd1 was not significantly changed in the mutants (Supplemental Figure 3). On the other hand, in lhr mutant FG stage follicles, only npr was dramatically decreased. For growth factors reported to be important for ovarian functions, especially oocyte maturation (50–53), the expression of egf, igf3, and inhbβ was greatly decreased in the ovaries of lhb but not lhr mutants, whereas inhbα, fst, and tgbf1 expression remained unchanged in both lhb and lhr mutants.
Defects of oocyte maturation and ovulation in lhb mutant zebrafish

To test whether lhb mutants respond to exogenously given LH, females were intraperitoneally injected with hCG, a hormone commonly used to mimic the action of LH on binding and activating LHR in fish. Mature stage follicles could be observed in the ovary after administration of hCG to homozygous lhb mutant female fish (Figure 7, A and B), and a small number of mature follicles were ovulated (Figure 7C). These results further support the indispensable role of LH in oocyte maturation and ovulation in zebrafish.

Discussion

In this study, TALENs were used to knock out lhb and lhr in zebrafish. We have demonstrated that both lhb and lhr mutant male zebrafish are fertile. On the other hand, lhb-null female zebrafish are infertile, and lhr-null female zebrafish are fertile. These results challenge our conventional view of the essential role of LH signaling in both male and female reproduction (1, 2, 24, 54), where direct evidence coming from mammals shows that both LH- and LHR-null mice are infertile (6–8).

Morphological and histological analysis revealed a smaller GSI in lhb mutant males and normal GSI in lhr mutant males compared with that in the wild type, and no differences in sperm motility and testicular structure were observed in lhb and lhr mutants. These results clearly indicate that LH signaling is not crucial for testicular fertility in zebrafish. In mammals, however, both lhb and lhr mutant male mice exhibit testes of reduced size, and spermatogenesis was blocked at the round spermatid stage (6–8). It has been demonstrated in mammals that spermatogenesis is regulated by both FSH and LH. This immense difference in the role of LH signaling in tests between fish and mammals is likely due to the different hormonal interactions in the testis,
mammals prompted us to speculate that FSH signaling might be the dominant factor in regulating fish spermatogenesis. This view is supported by several recent reports in fish (21, 22, 55–60). Most recently, Schulz and colleagues (22) also proposed that FSH serves as the constitutive driving force for fish spermatogenesis. Thus, in conjunction with the data from the present study, a revision of the present concept on the mode of action of gonadotropins in fish testis should be considered. However, it should be noted that although LH signaling is not crucial for male fertility, its involvement in fish testicular development could not be completely excluded because the GSI decreases in lhb mutant male zebrafish, and a significant increase in the number of spermatocytes was observed in homozygous lhb mutant, suggesting that LH signaling still plays a certain role in the regulation of spermatogenesis, especially the process from meiosis stage to spermiogenesis.

In females, folliculogenesis was normal in both lhb and lhr mutants, but oocyte maturation and ovulation were disrupted in lhb mutant, whereas only ovulation was affected in lhr mutant. These findings in fish ovary are very different from those in mammals in which LH and LHR knockout mice largely phenocopy each other, and ovarian folliculogenesis is blocked at the antral stage in either lhb or lhr knockout female mice (6–8). The differential phenotype of LH and LHR knockout on oocyte maturation and ovulation in zebrafish could be explained by the specificity of ligand binding of the gonadotropins to their receptors. The selectivity of gonadotropin ligand/receptor interaction in mammals is well defined: FSH and LH bind to their respective cognate receptors specifically and show little cross-reactivity (0.01%–0.1%) (61, 62). In contrast, the bioactivity of fish gonadotropins seems to be less well separated as a result of promiscuous ligand-receptor interactions (26, 27, 63–66). Most of the evidence has suggested that the wider but still limited functional selectivity of FSHR for both FSH and LH may depend on the fish counterpart, seems to be more specific for its ligand (1). Previously, So et al suggested (11) in zebrafish that FSH specifically activates FSHR, whereas LH could stimulate both FSHR and LHR. Results from the present study provide the first in vivo evidence that LH probably binds and activates FSHR in the absence of LHR in zebrafish. Furthermore, the presence of abnormal mature follicles in the ovary and a low egg production capacity found in the lhr-null fish indicates the importance of LHR in the ovulation process. It is not known whether LHR is physiologically involved in oocyte maturation of zebrafish, because mature follicles were found in lhr mutant ovary. However, the differential expression profile of LHR and FSHR as well as the higher binding affinity of LH to LHR than FSHR in zebrafish (11) lead us to speculate that LH regulates oocyte maturation through LHR under physiological conditions. Future studies to substantiate this are highly warranted.

To understand the molecular mechanisms of LH signaling in the ovary and the differential phenotype between LH and LHR mutant ovary, the expression of a panel of genes involved in steroidogenesis, oocyte maturation, and ovulation was assessed. First, these results supply direct in vivo evidence on the regulation of downstream genes by LH signaling in fish ovary. For example, the absence of lhb leads to decreased expression of star1, p450ccc, and 17b-hsd3 revealed in this study, consistent with the regulation trend from in vitro assays (67). Second, the differential phenotype of lhb and lhr mutant in the ovary could be partially explained by the differential gene expression profiles of the 2 types of mutants. A number of genes related to oocyte maturation such as egf, igf3, and inhhb were down-regulated only in lhb mutant but not in lhr mutant. Finally, several other genes were only changed in the lhb mutant but not in lhr mutant. For example, the expression of mprb, er2a, star1, p450ccc, p450c17a1, and 17b-hsd3 was changed in lhb mutant but not in lhr mutant, suggesting that LH might regulate these genes through FSHR in the absence of LHR.

Collectively, we have demonstrated, for the first time in a vertebrate, the noncanonical role of LH signaling in reproduction, being indispensable for gonadal development in females but not in males. Several lines of evidence in the present study support the crucial role of LH signaling in oocyte maturation and ovulation in female zebrafish. This report, representing the first piece of knock-out studies on fish LH and LHR, contributes toward a fuller understanding of the role of the gonadotropin system in vertebrate reproduction.

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44. McAllister BG, Kime DE. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (Danio rerio). Aquat Toxicol. 2003;65:309–316.
54. Kumar TR. What have we learned about gonadotropin function from gonadotropin subunit and receptor knockout mice? Reproduction. 2005;130:293–302.