The highly overlapping actions of Lh signaling and Fsh signaling on zebrafish spermatogenesis

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Abstract

Gonadotropin signaling plays a pivotal role in the spermatogenesis of vertebrates, but exactly how gonadotropins regulate the process in non-mammalian species remains elusive. Using a gene knockout approach in zebrafish, we have previously demonstrated the non-canonical action of gonadotropin signaling on spermatogenesis by analyzing four single mutant lines (*lhb*, *lhr*, *fshb* and *fshr*) and three double mutant lines (*lhb*;*fshb*, *lhr*;*fshr* and *fshb*;*lhr*). In this study, we further investigated the actions of gonadotropins on the testis by establishing three other double-mutant zebrafish lines (*lhb*;*lhr*, *fshb*;*fshr* and *lhb*;*fshr*). All *lhb*;*lhr* and *fshb*;*fshr* mutant males were fertile. Analysis on the gonadosomatic index and testicular histology in these *lhb*;*lhr* and *fshb*;*fshr* mutants demonstrated that Lh signaling and Fsh signaling could functionally compensate each other in the testis. Intriguingly, it was found that the *lhb*;*fshr* mutant male fish were also morphologically and histologically normal and functionally fertile, a phenomenon which could be explained by the cross-activation of Lhr by Fsh. We have demonstrated this cross-reactivity for the first time in zebrafish. Fsh was shown to activate Lhr using three different assay systems, in which Lh-Fshr activation was also confirmed. Taken together, we conclude that the action of Lh signaling and Fsh signaling is redundant in that either alone can support zebrafish spermatogenesis based on two observations. First, that either Lh signaling or Fsh signaling alone is sufficient to support male fertility. Second, that the two gonadotropin ligands could promiscuously activate both receptors. Apart from revealing the complexity of gonadotropin signaling in controlling male reproduction in zebrafish, this study also shed light toward a better understanding on the evolution of gonadotropin signaling in vertebrates from fish to mammals.

Key Words

- Fsh
- Lh
- testis
- spermatogenesis
- zebrafish

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Introduction

Spermatogenesis is a complex and highly coordinated process for the continued production of a large number of spermatozoa. This process is highly conserved in vertebrates. The differentiating spermatogonia give

rise to spermatocytes, which will then undergo meiosis resulting in haploid spermatids. Without further proliferation, mature motile spermatozoa emerging from the spermatids acquire the ability to fertilize. All these

phases of spermatogenesis are under the strict control of gonadotropic hormone stimulation of their receptors in the testes (Pudney 1995, Schulz *et al.* 2010).

More specifically, both pituitary gonadotropins including luteinizing hormone (LH) and follicle-stimulating hormone (FSH) exert their actions through binding to specific receptors in the testes, namely luteinizing hormone receptors (LHR) and follicle-stimulating hormone receptor (FSHR). The structure, expression and function of gonadotropins and their receptors have been studied in a number of vertebrates (Fan & Hendrickson 2005, Levavi-Sivan *et al.* 2010, Menon & Menon 2012). Most of the information though comes from studies in mammals. Both LH and FSH regulate steroidogenesis and spermatogenesis by activating receptors expressed in Leydig cells (LHR) and Sertoli cells (FSHR), respectively. LH regulates the activity of Leydig cells by stimulating the synthesis and secretion of testosterone, while FSH regulates the function of Sertoli cells to support many aspects of sperm cell maturation (Themmen & Huhtaniemi 2000, Huhtaniemi & Themmen 2005). However, how spermatogenesis is controlled by gonadotropin signaling in non-mammalian species is still largely unclear. In the primitive ancestral agnathan (jawless) vertebrates species such as Hagfish and lampreys, only one single gonadotrophin without specific Lh- or Fsh-like function and two receptors have been identified (Sower *et al.* 2009, Uchida *et al.* 2010, Nozaki 2013). During genome duplication from jawless teleost to jawed fish, two gonadotropins, namely Lh and Fsh akin to those in mammals, have emerged (Huhtaniemi 2015). Thus, studies on the actions of the more primitive gonadotropins and their receptors in fish are important in understanding the evolution of gonadotropin signaling in vertebrates. Comparing with the mammalian counterparts, differences on the structure, expression and regulation of fish gonadotropins have been reported. For example, marked divergence exists with regard to the specificity of the receptors toward the gonadotropins in fish. In mammals, interactions between the gonadotropins and their receptors are highly specific (Moyle *et al.* 1994), despite the fact that the equine chorionic gonadotropin (eCG) possessing equine LH activity has been reported to stimulate FSHR in several species (Camp *et al.* 1991, Cahoreau *et al.* 2015, Chakraborty & Roy 2015, Wei *et al.* 2016). On the other hand, however, interactions between fish gonadotropins and their receptors appear to be less discriminatory. Fshr could be activated by both Fsh and Lh in a number of fish

species (Yan *et al.* 1992, Vischer *et al.* 2003, Bogerd *et al.* 2005, Sambroni *et al.* 2007). However, reports about the activation of Lhr by Fsh are limited. Currently, it has only been reported in very few species such as rainbow trout (Sambroni *et al.* 2007). These differences imply functional differentiation of gonadotropins and their receptors from fish to mammals.

Recently, the emergence of gene knockout techniques provides the opportunity to dissect the *in vivo* gene functions in fish genetically (Huang *et al.* 2011, Chang *et al.* 2013, Hwang *et al.* 2013). Using an optimized TALENs platform established in our laboratory, we have successfully established the *lhb*, *fshb*, *lhr*, *fshr*, *lhb;fshb*, *lhr;fshr* and *fshb;lhr* mutant zebrafish lines (Liu *et al.* 2013, 2014, Chu *et al.* 2014, 2015). Using these genetic means, we have analyzed the functions of *fshb*, *lhb*, *fshr* and *lhr* in the testis of zebrafish. Intriguingly, all the single-gene mutant lines (*fshb*, *lhb*, *fshr* and *lhr*) and the double mutant line (*fshb;lhr*) are fertile. Both the structural integrity of the testis as revealed by histological examination and functional integrity as revealed by the fertilization rate are not affected. However, the double knockout mutant lines *lhb;fshb* and *lhr;fshr* all produce infertile males (Chu *et al.* 2014, 2015). These results suggest that gonadotropin signaling is essential for spermatogenesis and that Fsh signaling and Lh signaling exhibit some overlapping functions in controlling testicular function in zebrafish. To further unravel the complexity of the cross-activity between gonadotropins and their receptors in fish, we have established three additional double mutant zebrafish lines of *lhb;lhr*, *fshb;fshr* and *lhb;fshr* in the present study. Analysis of these mutant lines has led us to propose a more comprehensive model on how gonadotropins regulate spermatogenesis in zebrafish. The present study also contributes toward a better understanding of the evolution of gonadotropin signaling in vertebrates from fish to mammals.

Materials and methods

Zebrafish husbandry

Zebrafish used in this study were maintained at 28°C in the animal holding facilities of The Chinese University of Hong Kong. Fish were reared at a photoperiod of 16L/8D and fed twice a day with newly hatched brine shrimp (Brine Shrimp Direct, Ogden, UT, USA). All animal experiments were conducted following approval from the University Animal Experimentation Ethics Committee.

Targeted gene disruption by TALENs

The procedures of generating the single-gene knockout mutants were performed as described, including the construction of pCS2-TALEN-ELD/KKR plasmids and microinjection (21). The mutations were ascertained by genomic PCR and sequencing (Supplementary Fig. 1, see section on supplementary data given at the end of this article). The TALENs-injected embryos were raised to adulthood and outcrossed with wild-type fish. F1 mutants were selected by sequencing, and self-crossed to obtain F2 generation. Part of the fin of each F2 zebrafish was cut for genotyping. Double gene knockout heterozygotes were obtained by crossing of two single-gene knockout heterozygotes (e.g. *lhb*^{+/-};*fshr*^{+/-} was generated from crossing *lhb*^{+/-} with *fshr*^{+/-}) (Chu *et al.* 2014, 2015). And these double knockout heterozygotes were then self-crossed to obtain the homozygous mutants (e.g. *lhb*^{+/-};*fshr*^{+/-} self-crossed to obtain *lhb*^{-/-};*fshr*^{-/-}). A similar strategy was adopted to obtain the other double knockout homozygous fish lines (Supplementary Fig. 2).

Fertility test and sex ratio determination

Male zebrafish of different genotypes were kept together with wild-type female fish in a 1 to 1 ratio as described (Chu *et al.* 2014). The number of healthy embryos was recorded to indicate successful fertilization, and the survival rate was calculated as the fertilization ratio. Individual mutant males of each genotype were tested every day for two weeks.

The determination of sex ratios of the offspring was from the mating of 3 different pairs of individuals. In order to determine the sex ratios, all offspring were raised to adulthood first, and then sexed by morphological observation followed by histological confirmation.

Histological analysis of testes and immunohistochemistry

Intact testes were carefully excised from adult male zebrafish (4–7 months post fertilization) after anesthetization and decapitation. After fixation in Bouin's fixative buffer (Sigma) overnight at 4°C, testes were dehydrated and embedded in paraffin and sectioned at 3 µm thickness. Slides were then stained with Harris hematoxylin and eosin (H&E) (Sigma) for histological analysis. The staging of spermatogenesis was based on study by Leal and coworkers (Leal *et al.* 2009).

For immunohistochemistry, after antigen retrieval with 0.01 M citrate buffer (pH 6.0), the slides were washed with PTW (PBS, 0.1% Tween) and blocked for 60 min in 5% serum and 1% BSA in PTW. Samples were incubated with the proliferation marker phosphohistone H3 (PHH3) (1:1500; Cell Signaling Technology) overnight at 4°C before being washed and probed with donkey anti-rabbit Alexa 555 (Invitrogen). Cell nuclei were visualized with 4'-6-diamidino-2-phenylindole (DAPI) (Invitrogen) counterstaining, and then mounted and photographed on a confocal microscope (Olympus FV1000, Japan) (Chu *et al.* 2014).

The number of each stage of sperms per unit area was counted using the Weibel's multipurpose test system (Mandarim-de-Lacerda 2003). Spermatogonia, spermatocytes, spermatids and spermatozoa cells could be clearly distinguished by H&E staining, and sperm proliferation could be ascertained by PHH3 immunostaining.

RNA isolation and quantitative real-time PCR

Total RNA samples were extracted from the testes of zebrafish using TRIzol Reagent (Invitrogen). The amount and purity of the RNA were determined by spectrophotometry. Reverse transcription was performed using the PrimeScript RT Reagent Kit (TaKaRa) and quantitative real-time PCR was carried out on an ABI ViiA 7 Real-Time PCR System (ABI, Foster City, CA, USA) using the SYBR Green PCR Master Mix Kit (ABI) as Li and coworkers (Li *et al.* 2015). The full names of the genes and the primers used in this study are listed in Supplementary Table 1.

Hormone assays and ELISA

Blood samples were collected from the caudal vein of the fish as described in Pedroso and coworkers (Pedroso *et al.* 2012). Each fish could provide 5–10 µL blood for the assays. After centrifugation at 5000g for 20 min at 4°C, the supernatants were isolated and purified with ethyl acetate by following the manufacturer's extraction instruction (Cayman Chemical). Competitive enzyme-linked immunosorbent assay (ELISA) kits were used to measure the serum levels of testosterone, 11-ketotestosterone (Cayman Chemical), and serum LH and follicle-stimulating hormone levels were measured by assay kits specific for the zebrafish hormones (MyBioSource, San Diego, CA, USA). All standards and samples were performed in triplicates. The sensitivity, specificity, intra- and inter-assay coefficients

of variation (CV) of the hormone assays according to the suppliers are listed in [Supplementary Table 2](#).

Cell culture, transfection and luciferase assays

Chinese hamster ovary (CHO) cells and African green monkey kidney fibroblast-like (COS-7) cells (ATCC) were used to perform receptor activity assays using transient transfection or stable transfection, respectively. The passage numbers of both cell lines were ranging from 20 to 30. The cDNAs corresponding to the signal peptide and the entire mature zebrafish Lh or Fsh beta domain (117-residues for Lh and 112-residues for Fsh) followed by a 15-residue linker (GGGSGGGSGGGSGGG) and the mature zebrafish gonadotropin alpha domain (92 residues) were synthesized and cloned into pUC57 vector by GenScript (GenScript, Piscataway, NJ, USA). The coding regions of these two plasmids were subsequently subcloned into pcDNA3.1 by us via TA cloning strategy to generate the final pcDNA3.1-*lh* and pcDNA3.1-*fsh* expression plasmids. For construction of the pcDNA3.1-*lhr* and pcDNA3.1-*fshr* expression plasmids, the full coding regions of zebrafish *lhr* and *fshr* were amplified using the primers listed in [Supplementary Table 1](#) and cloned into pcDNA3.1 by TA cloning.

For transient transfections, CHO cells were maintained in Ham's F-12K (Kaighn's) medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PS) and kept at 37°C with 5% CO₂. Cells were seeded at 5 × 10⁴ cells per well on 24-well plates (Corning, Corning, NY, USA) one day prior to transfection. Each well was transfected with 500 ng of a cAMP-responsive reporter construct pCRE-Luc, 50 ng of a pRL-CMV vector containing the Renilla luciferase reporter gene, 200 ng of pcDNA3.1-*lhr* harboring the full coding region of zebrafish *lhr* and different concentrations (50, 100, 200, 500 and 1000 ng per well) of a pcDNA3.1-*fsh* expression plasmid. The mixtures of the constructs were incubated in a total volume of 250 µL serum-free medium containing 1 µL Lipofectamine2000 reagent (Life Technologies) for 30 min before being added into each well. After 6 h, the medium was removed and replaced with 400 µL complete growth medium. For positive controls, cells were transfected with the pcDNA3.1-*lh* expression plasmid (1000 ng per well). For negative control, a frameshift mutation was engineered on the *fsh* beta subunit using two restriction enzymes BspI and XhoI (New England Biolabs, Ipswich, MA, USA), generating a premature stop codon resulting in a defective Fsh. Cells

were transfected with 1000 ng per well of the pcDNA3.1-*fsh*-defective mutant vector in the experiments as negative control. (The mutation site of the negative control is shown in [Supplementary Fig. 3](#) and the primers are listed in [Supplementary Table 1](#).) After 48 h, cells were washed with PBS and lysed by passive lysis buffer (Promega) for 15 min. Firefly and Renilla luciferase activities were measured using the dual luciferase system (Promega) on a luminometer (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity. The same method was performed to test Lh-Fshr activation, using 1000 ng of pcDNA3.1-*fsh* or pcDNA3.1-*fsh*-defective mutant as the positive or negative control, respectively, and different concentrations (50, 100, 200, 500 and 1000 ng per well) of the pcDNA3.1-*lh* expression plasmid as the experimental groups.

For stable transfections, a COS-7-Lhr and a COS-7-Fshr stable cell line were established by following the same steps as described ([Chan & Cheng 2004](#)). Briefly, COS-7 cells were cultured in Dulbecco's Modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% FBS and 1% PS in 100 mm culture plates (Iwaki, Tokyo, Japan) before transfected with 2 µg of the pcDNA3.1-*lhr* or pcDNA3.1-*fshr* plasmid. After transfection, the medium was changed to DMEM supplemented with 10% FBS and 500 ng/mL Geneticin (G418) (Thermo Fisher Scientific) for one week to perform antibiotic selection. A concentration of 1 mg/mL G418 was employed to maintain the COS-7-Lhr or COS-7-Fshr cell line. 1000 ng of the pcDNA3.1-*lh* or the pcDNA3.1-*fsh* plasmid expression plasmid was used as the positive control in the COS-7-Lhr or COS-7-Fshr stable cell line, respectively. For negative controls, 1000 ng per well of the pcDNA3.1-*fsh*-defective mutant construct was transfected in both experiments. To detect the activation of Lhr by Fsh (and Fshr by Lh), different concentrations (50, 200 and 1000 ng) of the pcDNA3.1-*fsh* (or the pcDNA3.1-*lh*) expression plasmid were applied to the COS-7-Lhr (or the COS-7-Fshr) cell line. Luciferase activities were measured as described previously.

In vivo microinjection and luciferase assays

To test the activation of Lhr by Fsh, fertilized AB wild-type zebrafish embryos at one-cell stage were co-injected with 50 ng/µL of the pCRE-Luc plasmid, 0.5 ng/µL of the pRL-CMV construct, 50 ng/µL of the pcDNA3.1-*lhr* and different amounts (200 ng/µL, 500 ng/µL) of the pcDNA3.1-*fsh* plasmid ([Ahmed et al. 2011](#)). For positive and negative controls, the embryos were microinjected

with the pcDNA3.1-*lh* or the pcDNA3.1-*fsh*-defective mutant expression plasmids (500 ng/μL). Three nanoliters of DNA samples were microinjected into the yolk of each zygote at one-cell stage. The embryos were allowed to develop until 24 hpf and then groups of 15 embryos were lysed in passive lysis buffer (Promega), and luciferase activities were measured as described (Alcaraz-Perez *et al.* 2008). Similar procedures were performed to study Lh-Fshr activation using the pcDNA3.1-*fshr* construct, with 500 ng/μL of the pcDNA3.1-*fsh* or pcDNA3.1-*fsh*-defective mutant expression plasmids as the positive control and negative control, respectively. The experimental groups were consisted of 200 or 500 ng/μL of the pcDNA3.1-*lh* expression plasmid.

Statistical analysis

The GraphPad InStat software (GraphPad Software) was utilized for all the statistical analyses. All data were presented as mean ± S.D. Student's *t*-test and one-way ANOVA followed by Tukey's test were used for comparisons between two and more groups, respectively. And $P < 0.05$ was considered statistically significant.

Results

Sex ratio and fertility assessment of the *lhb;lh* and *fshb;fshr* double knockout mutants

The heterozygous and homozygous double knockout mutants of *lhb;lh* and *fshb;fshr* were ascertained by sequencing. A normal ratio of males to females was found in the adult homozygous *lhb;lh* mutants. However, double knockout of *fshb;fshr* led to all male offspring (Fig. 1A and B). This result confirmed that the Fsh signaling is essential for maintaining the female status (Chu *et al.* 2015, Zhang *et al.* 2015a).

The fertility of *lhb;lh* and *fshb;fshr* mutant males was assessed. No significant difference in fertilization rate was found between the homozygous males (*lhb^{-/-};lh^{-/-}* and *fshb^{-/-};fshr^{-/-}*) and their corresponding heterozygous controls (*lhb^{+/-};lh^{+/-}* and *fshb^{+/-};fshr^{+/-}*), indicating that the double mutant *lhb;lh* and *fshb;fshr* males are fertile (Fig. 1C and D).

Spermatogenesis in the *lhb;lh* and *fshb;fshr* double knockout males

To further analyze the phenotypes of the *lhb;lh* and *fshb;fshr* double knockout males, testes of the adult

Figure 1

Sex ratio and fertility assessment of the *lhb;lh* and *fshb;fshr* double mutant zebrafish at four months postfertilization. (A) The sex ratio of the *lhb^{+/-};lh^{+/-}* (no. of offspring used = 54) and *lhb^{-/-};lh^{-/-}* (no. of offspring used = 14) zebrafish. (B) The sex ratio of the *fshb^{+/-};fshr^{+/-}* (no. of offspring used = 68) and *fshb^{-/-};fshr^{-/-}* (no. of offspring used = 22) zebrafish. (C) The fertilization ratio of control (*lhb^{+/-};lh^{+/-}*) and homozygous (*lhb^{-/-};lh^{-/-}*) mutant male zebrafish upon crossing with wild-type females ($n = 4$ from each group of fish). (D) The fertilization ratio of control (*fshb^{+/-};fshr^{+/-}*) and homozygous (*fshb^{-/-};fshr^{-/-}*) mutant male zebrafish upon crossing with wild-type females ($n = 3$ from each group fish). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0079>.

fish were analyzed morphologically and histologically. No significant difference in the gonadosomatic index (GSI) was found between the adult homozygous double mutant males and their heterozygous controls (Fig. 2A and B). Histological analysis by H&E staining showed that different stages of sperm cells could be found in the testes of both the *lhb;lh* and *fshb;fshr* mutants (Fig. 2C). Normal localization of spermatocytes was also confirmed by immunostaining of PHH3, which is a marker for the metaphase of germ cells (Ladstein *et al.* 2010) (Fig. 2D).

The number of different stages of spermatogenic cells including spermatogonia (SG), spermatocytes (SC), spermatids (ST) and spermatozoa (SZ) were further calculated based on the H&E staining and PHH3 immunostaining. In both the *lhb;lh*- and *fshb;fshr*-mutant males, no significant difference was found in the number of the different stages of sperm cells between the homozygotes (*lhb^{-/-};lh^{-/-}* and *fshb^{-/-};fshr^{-/-}*) and the heterozygous controls (*lhb^{+/-};lh^{+/-}* and *fshb^{+/-};fshr^{+/-}*) (Fig. 2E and F). The number of SG appeared to be somewhat decreased in the *lhb^{-/-};lh^{-/-}* mutant group (Fig. 2E), but the difference was not statistically significant. These data demonstrated that the actions of Lh signaling

Figure 2

Testicular development of the *lhb;lhr* and *fshb;fshr* double mutant males. (A) GSI of *lhb*^{+/+};*lhr*^{+/+} and *lhb*^{-/-};*lhr*^{-/-} male zebrafish. (B) GSI of *fshb*^{+/+};*fshr*^{+/+} and *fshb*^{-/-};*fshr*^{-/-} male zebrafish. (C) Histology of testes from *lhb*^{+/+};*lhr*^{+/+} (a), *lhb*^{-/-};*lhr*^{-/-} (b), *fshb*^{+/+};*fshr*^{+/+} (c) and *fshb*^{-/-};*fshr*^{-/-} (d) male zebrafish. Bars=50 µm. SC, spermatocytes; ST, spermatids; SZ, spermatozoa; spermatogonia were marked by arrows. (D) Testes from *lhb*^{+/+};*lhr*^{+/+} (a), *lhb*^{-/-};*lhr*^{-/-} (b), *fshb*^{+/+};*fshr*^{+/+} (c) and *fshb*^{-/-};*fshr*^{-/-} (d) male zebrafish stained with PHH3 (red) and DAPI (blue). Bars=100 µm. (E) The number of the different stages of spermatogenic cells in *lhb*^{+/+};*lhr*^{+/+} and *lhb*^{-/-};*lhr*^{-/-} males. (F) The number of the different stages of spermatogenic cells in *fshb*^{+/+};*fshr*^{+/+} and *fshb*^{-/-};*fshr*^{-/-} males. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0079>.

and Fsh signaling on spermatogenesis in zebrafish are highly overlapping and could functionally compensate each other.

Androgen levels and gene expression profile in *lhb;lhr* and *fshb;fshr* double knockout males

To further assess the effects of gene knockout on testicular function, the concentration of androgens in the serum was measured by ELISA. Testosterone (T) and 11-ketotestosterone (11-KT) are the two dominant androgens in zebrafish (Schulz *et al.* 2010). The level of 11-KT was not significantly affected in both homozygous mutants (*lhb^{-/-};lhr^{-/-}* and *fshb^{-/-};fshr^{-/-}*) comparing with their corresponding heterozygous controls (*lhb^{+/-};lhr^{+/-}* and *fshb^{+/-};fshr^{+/-}*). However, the level of T was significantly lower in the *fshb^{-/-};fshr^{-/-}* mutant than its control (Fig. 3A and B). The expression of a number of key enzymes involved in steroidogenesis (*star*, *p450scc*, *3b-hsd* and *17b-hsd3*) was further assessed by quantitative real-time PCR. In the *lhb^{-/-};lhr^{-/-}* mutant, the expression of *p450scc* and *17b-hsd* was significantly decreased, while that of *star* was increased as compared to the control. And in the *fshb^{-/-};fshr^{-/-}* mutant, the expression of all these genes was significantly decreased (Fig. 3C and D). These results suggest that the shutting down of either the Lh signaling or the Fsh signaling pathway did produce some changes on the gene expression of the steroidogenic enzymes in

the testis, leading to some minor changes in the androgen levels in the mutants. However, these changes were not sufficient to alter the phenotype of the mutants, which are fully fertile morphologically, histologically and functionally. These findings lend strong support that Lh signaling and Fsh signaling could essentially compensate each other to regulate spermatogenesis in zebrafish.

The possibility that compensation of Lh signaling and Fsh signaling in the homozygous mutants was due to the increase in concentration of the remaining intact undisrupted gonadotropin was also considered. Thus, plasma Fsh level in the *lhb;lhr* mutant males and plasma Lh level in the *fshb;fshr* mutant males were also measured by ELISA. The results indicate that the level of the intact undisrupted gonadotropin remained normal in both the *lhb;lhr* and *fshb;fshr* mutant males (Fig. 3E and F). These data further substantiate the existence of functional redundancy between Lh signaling and Fsh signaling in zebrafish spermatogenesis.

Double knockout of *lhb* and *fshr* leads to all fertile males

Cross-activation of Fshr by Lh has been shown in zebrafish previously, but cross-activation of Lhr by Fsh has never been demonstrated before, thus prompting us to further establish the *lhb;fshr* double knockout mutant line. All adult homozygous *lhb;fshr* mutants (*lhb^{-/-};fshr^{-/-}*) turned out to be all males (Fig. 4A). This result was anticipated

Figure 3

Serum androgen level and gene expression profile in the testes of the *lhb;lhr* and *fshb;fshr* double mutant males. (A) Serum concentration of T and 11-KT in *lhb^{+/-};lhr^{+/-}* and *lhb^{-/-};lhr^{-/-}* males. (B) Serum concentration of T and 11-KT in *fshb^{+/-};fshr^{+/-}* and *fshb^{-/-};fshr^{-/-}* males. (C) The expression of *star*, *p450scc*, *3b-hsd* and *17b-hsd3* (relative to *ef1a*) in the testes of *lhb^{+/-};lhr^{+/-}* and *lhb^{-/-};lhr^{-/-}* males. (D) The expression of *star*, *p450scc*, *3b-hsd* and *17b-hsd3* (relative to *ef1a*) in the testes of *fshb^{+/-};fshr^{+/-}* and *fshb^{-/-};fshr^{-/-}* males. (E) Serum concentration of Fsh in *lhb^{+/-};lhr^{+/-}* and *lhb^{-/-};lhr^{-/-}* males. (F) Serum concentration of Lh in *fshb^{+/-};fshr^{+/-}* and *fshb^{-/-};fshr^{-/-}* males. **P*<0.05, ***P*<0.01, ****P*<0.005, *****P*<0.001. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0079>.

Figure 4

Sex ratio and fertility assessment of the *lhb;lhr* and *fshb;fshr* double mutant zebrafish. (A) The sex ratio of the *lhb*^{+/-};*fshr*^{+/-} (no. of offspring used=55) and *lhb*^{-/-};*fshr*^{-/-} (no. of offspring used=12) zebrafish. (B) The fertilization ratio of control (*lhb*^{+/-};*fshr*^{+/-}) and homozygous (*lhb*^{-/-};*fshr*^{-/-}) mutant male zebrafish upon crossing with wild-type females (*n*=3 from each group of fish). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0079>.

because disruption of Fsh signaling could not sustain the female phenotype. Surprisingly, however, these double mutant homozygous males were fertile, as demonstrated by mating of the homozygous *lhb;fshr* double mutants males (*lhb*^{-/-};*fshr*^{-/-}) with the wild-type females in producing normal offspring. The fertilization ratio of the homozygotes (*lhb*^{-/-};*fshr*^{-/-}) was not significantly different

from the heterozygous control (*lhb*^{+/-};*fshr*^{+/-}), suggesting that simultaneous knockout of *lhb* and *fshr* does not affect male fertility (Fig. 4B).

Spermatogenesis in the *lhb;fshr* double knockout males

Morphological and histological examinations including H&E staining and PHH3 immunostaining were carried out on the *lhb;fshr* double knockout fish. The testicular GSI value was not altered (Fig. 5A). Different stages of sperm cells (Fig. 5B), with a significant increase in SC number and a decreased number of ST plus SZ found in the testes of the homozygous *lhb;fshr* mutant (*lhb*^{-/-};*fshr*^{-/-}) (Fig. 5C). Consistent with the H&E staining results, PHH3 immunostaining also revealed a larger amount of spermatocytes, indicating the simultaneous knockout of both *lhb* and *fshr* affects the developmental transition from SC stage to later stages during spermatogenesis (Fig. 5D). These data indicate the *lhb;fshr* mutant males are still capable of undergoing normal spermatogenesis in zebrafish despite some slight defects in the transition of SC to later stages. These changes however were not sufficient to alter the phenotype of the mutants, which are fully fertile functionally.

Figure 5

Testicular histology of the *lhb;fshr* double mutant males. (A) GSI of *lhb*^{+/-};*fshr*^{+/-} and *lhb*^{-/-};*fshr*^{-/-} male zebrafish. (B) Histology of testes from *lhb*^{+/-};*fshr*^{+/-} (a) and *lhb*^{-/-};*fshr*^{-/-} (b) male zebrafish. Bars=50 µm. SC, spermatocytes; ST, spermatids; SZ, spermatozoa; spermatogonia were marked by arrows. (C) The number of the different stages of spermatogenic cells in *lhb*^{+/-};*fshr*^{+/-} and *lhb*^{-/-};*fshr*^{-/-} male zebrafish. (D) Testes from *lhb*^{+/-};*fshr*^{+/-} (c) and *lhb*^{-/-};*fshr*^{-/-} (d) male zebrafish stained with PHH3 (red) and DAPI (blue). Bars=100 µm. **P*<0.05. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0079>.

Androgen levels and gene expression profile in the *lhb;fshr* double knockout males

To further characterize the phenotype of the *lhb;fshr* double knockout males, the concentration of both 11-KT and T in the serum was measured by ELISA. The level of 11-KT in the homozygous *lhb;fshr* double mutant males (*lhb^{-/-};fshr^{-/-}*) is similar to the heterozygous control (*lhb^{+/-};fshr^{+/-}*), while the T level is slightly higher as compared to the heterozygous control (Fig. 6A). Expression of enzymes related to steroidogenesis in the testes was also assessed in the *lhb;fshr* double knockout males by real-time PCR. While the expression of *p450scc* and *3b-hsd* was not affected, the expression of *star* and *17b-hsd3* was significantly decreased in the *lhb;fshr* mutant males (*lhb^{-/-};fshr^{-/-}*) (Fig. 6B). These data suggest that steroidogenesis was affected in *lhb;fshr* mutant males. Interestingly, a much elevated concentration of plasma Fsh was observed in the *lhb;fshr* mutant males (Fig. 6C). This result demonstrates that upon the deletion of Lh in zebrafish, compensation in the increased gene expression of Fsh occurs, and it is conceivable that this increased level of Fsh is exerting its action through Lhr to elicit full reproductive capacity as far as spermatogenesis and male fertility are concerned.

Assessment of cross-activation of Lhr by Fsh and of Fshr by Lh in zebrafish

As indicated in the previous paragraph and in our published findings (Chu *et al.* 2015), an explanation of the fertile phenotype observed in the *lhb;fshr* and *fshb;lhr* double knockout males is the existence of cross-activation between Lh signaling and Fsh signaling. Several assay systems were employed to test the cross-reactivity. To explore the activation of Lhr by Fsh, a cell-based functional assay of Lhr activity was performed using CHO cells co-transfected with zebrafish Lhr and different concentrations of Fsh plasmids in a transient transfection assay system with the proper positive and

negative controls. A dose-dependent increase in CRE promoter activity was observed after transfection with the Fsh plasmid (Fig. 7A), indicating the functional interaction of Fsh with Lhr to elicit the post-receptor signaling event. Meanwhile, an increased CRE promoter activity was also noted after transfection of the Fsh plasmid into a COS-7 cell line stably expressing zebrafish Lhr (Fig. 7B). These results obtained from two different cell systems demonstrate the functional activation of Lhr by Fsh. To further substantiate the occurrence of this cross-activation in an *in vivo* system, both Lhr and Fsh plasmids were microinjected into one-cell stage zebrafish embryos and CRE promoter activity was measured after 24 h post fertilization. The CRE promoter activity was again significantly increased upon co-injection of the Lhr and Fsh plasmids (Fig. 7C). All these results clearly demonstrate that Fsh could activate Lhr in zebrafish.

Similarly, the same three assay systems were utilized to test the activation of Fshr by Lh. Consistent with the previous finding (So *et al.* 2005), zebrafish Lh was capable of stimulating Fshr, resulting in a dose-dependent increase of the CRE promoter activity in the transient- and stable-transfected cells as well as in the zebrafish embryos (Fig. 7D, E and F). These data confirmed the existence of Lh-Fshr activation in zebrafish. Taken together, the cross-activation of the two gonadotropins and the two gonadotropin receptors in zebrafish has been substantiated, further providing evidence for the overlapping actions of Lh and Fsh signaling in zebrafish spermatogenesis.

Discussion

To systematically analyze the functions of gonadotropin signaling in zebrafish reproduction, we have previously established four single mutant lines (*lhb*, *lhr*, *fshb* and *fshr*) and three double mutant lines (*lhb;fshb*, *lhr;fshr*, *fshb;lhr*) using TALENs. All the *lhb*, *lhr*, *fshb* and *fshr*

Figure 6

Serum androgen level and expression profile of key steroidogenic enzymes in the testes of the *lhb;fshr* double mutant males. (A) Serum concentration of T and 11-KT in *lhb^{+/-};fshr^{+/-}* and *lhb^{-/-};fshr^{-/-}* males. (B) The expression of *star*, *p450scc*, *3b-hsd* and *17b-hsd3* (relative to *ef1a*) in the testes of *lhb^{+/-};fshr^{+/-}* and *lhb^{-/-};fshr^{-/-}* males. (C) Serum concentration of Fsh in *lhb^{+/-};fshr^{+/-}* and *lhb^{-/-};fshr^{-/-}* male zebrafish. **P* < 0.05, ***P* < 0.01, ****P* < 0.005. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0079>.

Figure 7

Assessment of the cross-activation of the two gonadotropins and the two gonadotropin receptors in zebrafish. Different amounts of a zebrafish *fsh* expression plasmid were transfected into CHO cells (A), a COS-7-Lhr stable cell line (B) or microinjected into zebrafish embryos (C). The zebrafish *lh* expression plasmid was used as the positive control in Panels (A) to (C). Similarly, different amounts of a zebrafish *lh* expression plasmid were transfected into CHO cells (D), a COS-7-Fshr stable cell line (E) or microinjected into zebrafish embryos (F). The zebrafish *fsh* expression plasmid was used as the positive control in Panels (D) to (F). An expression plasmid harboring the defective mutant zebrafish *fsh* was used as the negative control in all these assays. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$, compared to the negative control group. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0079>.

single mutants and the *fshb;lh* double mutant male fish are fertile and spermatogenesis remains intact. On the other hand, however, both the *lhb;fshb* and *lh;fshr* double mutant male fish are infertile due to impaired spermatogenesis (Chu *et al.* 2014, 2015). All these findings led us to conclude that gonadotropin signaling is essential for spermatogenesis and that Lh can also act through Fshr in a non-canonical manner. In the present study, we have further dissected the actions of gonadotropin signaling in spermatogenesis using three additional double mutant lines (*lhb;lh*, *fshb;fshr* and *lhb;fshr*). There are two major findings from the present study. First, both *lhb;lh* and *fshb;fshr* mutant males are fertile, and this provides unequivocal evidence to the highly overlapping actions of Lh signaling and Fsh signaling in regulating spermatogenesis in zebrafish. Second, analysis of the fertile phenotype in the *lhb;fshr* mutant males revealed the cross-activation of Lhr by Fsh in zebrafish. The cross-activation of Lhr by Fsh and of Fshr by Lh in zebrafish was also substantiated

by a number of transfection and microinjection assays. Based on these genetic and biochemical evidence, we propose a model on the role of gonadotropin signaling in regulating spermatogenesis in zebrafish (Figs 8 and 9).

We have noticed the normal sex ratio between males and females in the adult *lhb;lh* mutants, but all adult *fshb;fshr* and *lhb;fshr* mutants differentiated into males. This result is in fact consistent with our data and that of others on the single mutant (*lhb*, *lh*, *fshb* and *fshr*) and double mutant (*lhb;fshb*, *lh;fshr*, *fshb;lh*) zebrafish (Chu *et al.* 2014, 2015, Zhang *et al.* 2015a,b) as well as a *fshr* mutant medaka (Murozumi *et al.* 2014), that disruption of Fsh signaling could lead to the failure of follicle activation and possibly sex reversal from females to males. All these findings clearly demonstrate that Fsh signaling is pivotal for maintaining the female status in zebrafish. However, it might be worth noting that testicular differentiation in zebrafish goes through an 'ovarian' morphogenic stage, which might therefore be expected to be Fsh independent,

Figure 8

A proposed model of gonadotropin signaling on spermatogenesis in zebrafish. Lh signaling and Fsh signaling can functionally compensate each other in spermatogenesis. The two ligands Lh and Fsh are capable of activating both receptors. SC, spermatocytes; SG, spermatogonia; ST, spermatids; SZ, spermatozoa. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0079>.

even if further ovarian differentiation is dependent on Fsh (Liew & Orban 2014).

Both the *lhb;lhr* and *fshb;fshr* double mutant males are fertile and possess normal GSI and spermatogenesis. These findings are concordant with our recent studies that all *lhb*, *lhr*, *fshb* and *fshr* single mutant male fish are fertile. More interestingly, we found in the present study that the plasma Fsh level in the *lhb;lhr* double mutant male and the plasma Lh level in *fshb;fshr* mutant male were not altered as compared to the controls, providing further support that Fsh signaling or Lh signaling alone is sufficient to maintain normal androgen production and spermatogenesis in zebrafish. This conclusion is also supported by recent studies on the assessment of the action

Figure 9

Comparison of gonadotropin signaling and receptor localization in zebrafish with mouse and human. In zebrafish, the interaction of Lh and Fsh with their receptors is promiscuous. Fshr and Lhr exist on both Leydig cells and Sertoli cells to induce androgen production. Although in mouse and human, LH activates LHR on Leydig cells for androgen production independent of the specific interaction of FSH with FSHR on Sertoli cells. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0079>.

of both Lh and Fsh on the fish testis. In zebrafish, both Lh and Fsh could stimulate androgen production *in vitro* and *in vivo* (Garcia-Lopez *et al.* 2010). In rainbow trout, one-third of the regulated transcripts could be similarly regulated by both Fsh and Lh in the testis (Sambroni *et al.* 2013). In big contrast to zebrafish, studies on mutations in human and knockout mice showed the specific actions of both FSH and LH signaling on spermatogenesis, in that FSH regulates Sertoli cell activity to increase the number of spermatogonia and enhances their entry into meiosis (Abel *et al.* 2000, Siegel *et al.* 2013), although the importance of FSH signaling in spermatogenesis of mice and human remains debatable; while LH regulates Leydig cell sex steroid production, which allows the completion of meiosis and spermiogenesis (Kumar 2005, Ramaswamy & Weinbauer 2014). Such difference between zebrafish and mammals could be partially explained by the different tissue expression patterns of the two gonadotropin receptors. In mammals, FSHR and LHR are exclusively expressed in Sertoli cells and Leydig cells, respectively, but these two receptors are co-expressed in both Sertoli cells and Leydig cells in several fish species including zebrafish (Oba *et al.* 2001, Garcia-Lopez *et al.* 2010) (Fig. 9). Our study also raised an interesting question: if either Fsh signaling or Lh signaling alone is sufficient to maintain normal spermatogenesis in zebrafish, why could these two gonadotropin systems be retained in the fish testis during evolution? Noting that the divergent actions of both Lh signaling and Fsh signaling on ovarian functions are conserved from zebrafish to human (Kumar *et al.* 1997, Kumar 2005, Kumar & Sait 2011, Ogiwara *et al.* 2013, Murozumi *et al.* 2014), it is conceivable that the two gonadotropin systems might have evolved primarily to fulfill the needs of ovarian development. Different from that in the ovary, the specialization of Lh signaling and Fsh signaling in the testis occurred gradually during evolution and took a different course in different vertebrates. For example, in some mammals including primates and mice, LH signaling is the dominant gonadotropin system in affecting spermatogenesis; while in other mammals such as hamsters, spermatogenesis is predominantly dependent on FSH signaling (Ramaswamy & Weinbauer 2014, Huhtaniemi 2015).

One of the most striking findings of the present study is the phenotype of the *lhb;fshr* double mutant zebrafish. It has been reported in zebrafish that Fshr could cross-react with Lh but Lhr could not cross-react with Fsh (So *et al.* 2005). Therefore, initially, we expected that the double knockout of both *lhb* and *fshr* would disrupt both Lh and Fsh signaling in zebrafish, leading to an infertile

phenotype similar to that observed in the *lhb;fshb* and *lhr;fshr* double mutants. Surprisingly, we found that all the *lhb;fshr* double adult mutant males are fully fertile. GSI and the ability to produce viable gametes were not significantly affected, demonstrating that the simultaneous double knockout of both *lhb* and *fshr* did not affect male reproduction. This phenotype could not be explained by the constitutive activity of Lhr, because such constitutive activity of either Lhr or Fshr is not capable of supporting full spermatogenesis in zebrafish as demonstrated by the infertile phenotype of the *lhb;fshb* double mutant males (Chu *et al.* 2015). The other explanation is that Fsh exerts its actions by cross-activating Lhr to maintain full functional spermatogenesis in the *lhb;fshr* double mutants. We have substantiated this using three different assay systems. Our results have clearly demonstrated for the first time in zebrafish that Lhr could indeed be cross-activated by Fsh. These strong genetic and biochemical evidence demonstrated unequivocally that Fsh could regulate spermatogenesis through Lhr in the absence of Lh and Fshr in males. The dramatically increased Fsh level in the plasma of the *lhb;fshr* double mutant males could be understood in terms of the attempt by the organism to correct for the genetic defect by upregulating Fsh expression in order to exert a bigger effect in activating Lhr. We have also confirmed the activation of Fshr by Lh using the same assay systems. It thus appears that the interaction of gonadotropins and their receptors in zebrafish is highly promiscuous in that Lh and Fsh are capable of activating both receptors. Compared to the high specificity of ligand–receptor interaction in mammals, our genetic studies in zebrafish clearly demonstrate this much less refinement in ligand–receptor specificity in fish. The physiological significance of this difference is not fully known, but we hypothesize that this could be related to the duration of spermatogenesis. Sperm maturation needs a much longer time in mammals than that in fish (Nobrega *et al.* 2009). In zebrafish, it takes around 6 days for meiosis plus spermiogenesis (Leal *et al.* 2009), while in mice and rats, the time required is over 30 and 50 days, respectively (Franca *et al.* 1998). To guarantee offspring quality, the functions of gonadotropin signaling are more specific in spermatogenesis in mammals, and this entails a highly fine-tuned testicular development process associated with low reproductive efficiency as a price. This evolution of gonadotropins and their receptors represents a prominent example of the co-evolution of ligand–receptor interaction.

In summary, we have established three double knockout zebrafish mutant lines (*lhb;lhr*, *fshb;fshr* and *lhb;fshr*) in

the present study. Analysis of these mutants led us to propose a more comprehensive model on gonadotropin signaling in spermatogenesis of zebrafish. Apart from illustrating the complexity of gonadotropin signaling in fish spermatogenesis, the information obtained from the present genetic analysis also contributes toward a fuller understanding of the evolution of gonadotropin signaling in vertebrates.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-17-0079>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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