The *kiss/kissr* Systems Are Dispensable for Zebrafish Reproduction: Evidence From Gene Knockout Studies

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The *kiss1/gpr54* signaling system is considered to be a critical regulator of reproduction in most vertebrates. However, this presumption has not been tested vigorously in nonmammalian vertebrates. Distinct from mammals, multiple *kiss1/gpr54* paralogous genes (*kiss/kissr*) have been identified in nonmammalian vertebrates, raising the possibility of functional redundancy among these genes. In this study, we have systematically generated the zebrafish *kiss1*$^{-/-}$, *kiss2*$^{-/-}$, and *kiss1*$^{-/-}$;*kiss2*$^{-/-}$ mutant lines as well as the *kissr1*$^{-/-}$, *kissr2*$^{-/-}$, and *kissr1*$^{-/-}$;*kissr2*$^{-/-}$ mutant lines using transcription activator-like effector nucleases. We have demonstrated that spermatogenesis and folliculogenesis as well as reproductive capability are not impaired in all of these 6 mutant lines. Collectively, our results indicate that *kiss/kissr* signaling is not absolutely required for zebrafish reproduction, suggesting that the *kiss/kissr* systems play nonessential roles for reproduction in certain nonmammalian vertebrates. These findings also demonstrated that fish and mammals have evolved different strategies for neuroendocrine control of reproduction. (*Endocrinology* 156: 589–599, 2015)

The hypothalamus-pituitary-gonad axis plays a critical role in the control of reproduction in vertebrates. GnRH occupies a central position in the reproductive axis, but the signals that regulate GnRH release are not well understood. In 2003, 2 independent groups reported loss-of-function mutations in the G protein-coupled receptor 54 (*GPR54*), the kisspeptin receptor, leading to hypogonadotrophic hypogonadism in human (1, 2). In mouse, knockout of either the kisspeptin-encoding gene (*Kiss1*) or the *Gpr54* gene led to the lack of puberty and infertility (3, 4). It is now commonly accepted that the *Kiss1/Gpr54* system plays a central role in regulating key aspects of reproduction such as puberty onset, gonadotropin secretion, and sex steroids feedback by stimulating GnRH release in mammals (5–7).

The *kiss1/gpr54* system has also been identified in nonmammalian vertebrates. Different from the mammalian *Kiss1/Gpr54* system, multiple *kiss1/gpr54* paralogs are present in nonmammalian vertebrates. Two *kiss1* genes (*kiss*), namely *kiss1* and *kiss2*, have been cloned in zebrafish and several other teleosts (8–14). Two kisspeptin receptor genes (*kissr*, the *Gpr54* homologs), namely *kissr1* and *kissr2*, have also been identified in zebrafish and other teleosts (8–10, 14). In the coelacanth genome, 4 *kissr* genes have been reported (15). In frogs, 3 *kiss* and

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Abbreviations: dpf, days postfertilization; *GPR54*, G protein-coupled receptor 54; GSI, gonad-somatic index; IHC, immunohistochemistry; ISH, in situ hybridization; kiss, kiss1 or kiss2; kissr, kiss receptor; ORF, open reading frame; TALEN, transcription activator-like effector nuclease; TM, transmembrane; WT, wild type.

3 *kissr* genes have been identified (9). In birds, a putative *kiss2-like* gene has been identified recently (16). Genomic synteny analysis indicated that these *kiss/kissr* genes originate from the 2 rounds of genome duplications that happened during the early evolution of vertebrates (15, 17).

The potential roles of the *kiss/kissr* systems in the control of the reproductive axis have also been investigated in nonmammalian vertebrates. In zebrafish, the *kiss1* and *kissr1* are mainly expressed in the habenular nucleus, suggesting that the *kiss1/kissr1* system might play nonreproductive functions in this species (18, 19). The *kiss2* neurons are located at the lateral preoptic area and the ventral hypothalamus where *kissr2* is also expressed, indicating that the *kiss2/kissr2* system is involved in the regulation of reproduction (18, 20). In goldfish, *kiss2* is expressed in the preoptic area region, and both *kiss1* and *kiss2* are responsive to estrogen treatment (21, 22). In medaka, however, *kiss1* but not *kiss2* is sensitive to estrogen treatment (23). Injection of Kiss2 but not Kiss1 could stimulate *lhβ* and *fshβ* gene expression in zebrafish (11), sea bass (12), and chub mackerel (24). In sea bass, injection of both Kiss1 and Kiss2 could stimulate gonadotropin release, with the Kiss2 exhibiting greater efficacy (12). In goldfish, injection of Kiss1 but not Kiss2 could significantly stimulate LH release (8). In several teleost, the expression of *kiss* and *kissr* genes peaks around puberty and chronic administration of kisspeptins could advance gonad development (9, 10, 25–28). Taken together, these results suggest that different ligand/receptor subtypes of the *kiss/kissr* systems may be involved in the regulation of reproduction in nonmammalian vertebrates in a species-dependent manner.

The neuroendocrine control of reproduction in fish has been extensively investigated. Different from mammals, teleost lack the hypothalamic-hypophyseal portal system (29, 30). The gonadotrophs in the anterior pituitary are directly innervated by numerous neuronal cell types. Indeed, a multitude of neuropeptides and classical neurotransmitters have been identified to directly regulate gonadotropin release in fish independent of GnRH (29, 31). Early work already established the differential roles of multiple GnRHs, amino acid and catecholaminergic neurotransmitters in teleosts compared with mammalian models (32, 33). In many fish, these multiple stimulatory systems are opposed by a potent inhibitory dopaminergic system (29, 31–33). Although the *kiss/kissr* systems are presumed to be critical regulators of reproduction in fish and other nonmammalian vertebrates (34, 35), however, this presumption has not been tested vigorously. Are the *kiss/kissr* systems indispensable for reproduction in nonmammalian vertebrates? Is there any functional redundancy among the *kiss* genes and the *kissr* genes? Because of the lack of a robust gene knockout animal model in nonmammalian vertebrates, these questions remain unanswered. In this study, using zebrafish as a model, we have systematically generated the *kiss1*$^{-/-}$, *kiss2*$^{-/-}$, and *kiss1*$^{-/-}$;*kiss2*$^{-/-}$ mutant lines as well as the *kissr1*$^{-/-}$, *kissr2*$^{-/-}$, and *kissr1*$^{-/-}$;*kissr2*$^{-/-}$ mutant lines. We have subsequently assessed their phenotypes. Our results demonstrate that the reproductive functions are not impaired in the mutant lines of the ligand or receptor genes, indicating that the *kiss/kissr* systems are not absolutely essential for reproduction in certain nonmammalian vertebrates.

Materials and Methods

Zebrafish husbandry

AB zebrafish were maintained at 28°C in the zebrafish facility of the Sun Yat-Sen University and the Chinese University of Hong Kong. All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of the Sun Yat-Sen University and the Chinese University of Hong Kong.

Transcription activator-like effector nucleases (TALENs) preparation

The TALENs for each target gene were assembled using the golden gate method as described previously (36–39). Briefly, TALENs were assembled by 2 digestion-ligation steps. In the first round of digestion-ligation, modular plasmids recognizing each nucleotide were digested and ligated into the backbones of 2 middle array plasmids. In the second round of digestion-ligation, the middle array plasmids and the last repeat plasmid were cloned into the backbones of 2 optimized TALEN expression plasmids (the pCS2-TALEN-ELD and pCS2-TALEN-KKR) developed by our group (36, 37). The final TALEN expression plasmids were linearized by *Not*I restriction enzyme digestion. TALEN mRNAs were transcribed using the mMESSAGE mMACHINE SP6 kit (Ambion) and purified using the RNeasy Mini kit (QIAGEN). For detailed protocol of the TALENs preparation, see reference (39).

Establishment of zebrafish mutant lines

To generate zebrafish mutant lines, TALEN mRNAs (200–500 pg) were microinjected into 1-cell stage zebrafish embryos. Two days after injection, genomic DNA was isolated from 8–10 pooled larvae. The target genomic regions were amplified by PCR and subcloned into the pTZ57R/T vector (Fermentas). Single colonies were genotyped by sequencing. To obtain germline mutations, the TALEN-injected embryos were raised to adulthood and outcrossed with wild-type (WT) fish. The F1 progeny were genotyped by sequencing. To obtain homozygous mutants, heterozygous mutant of the same mutation were obtained and self-crossed. The primers used in this study were listed in Supplemental Table 1.

In situ hybridization (ISH) and immunohistochemistry (IHC) of zebrafish Kiss1 and Kiss2

Digoxigenin-ISH for *kiss1* and *kiss2* gene was performed as described previously (11). IHC was performed as described pre-

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viously using polyclonal antisera for zebrafish prepro-Kiss1 and zebrafish prepro-Kiss2 (40). Coronal brain sections were incubated with a rabbit antizebrafish prepro-Kiss1 (1:500) or antizebrafish prepro-Kiss2 (1:500) prepared in PBS (pH 7.0) containing 2% normal goat serum and 0.5% Triton X-100, and incubated at 4°C for 36 hours in a closed moist chamber. Diluted biotinylated antirabbit IgG (Vector Laborotories), at 1:200 dilution, was then applied to the sections for 1 hour. Sections were scanned and images captured on a Zeiss MIRAX Slide scanning system (Zeiss, GmBH) using the Mirax Viewer Image Software (3DTech).

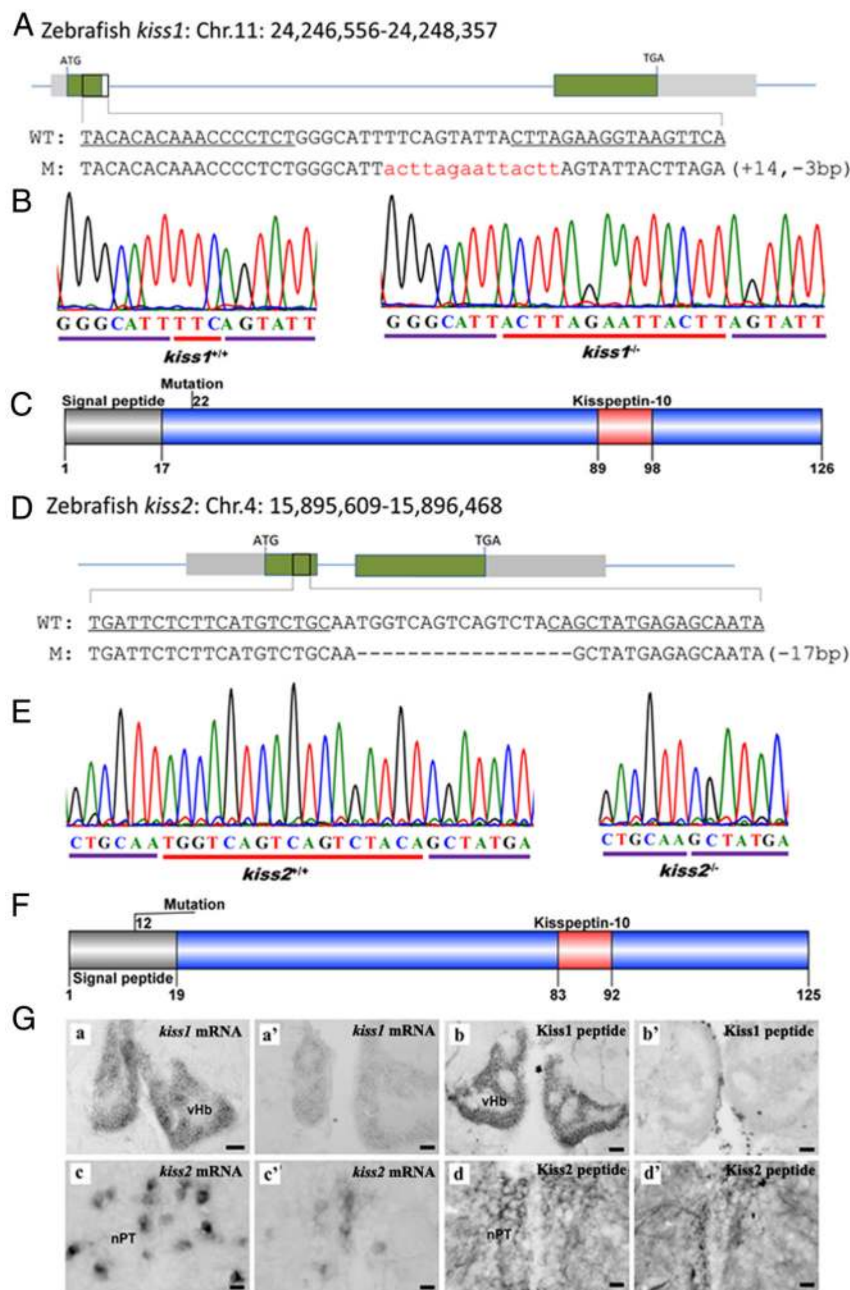

Figure 1. Targeted disruption of zebrafish *kiss1* and *kiss2* genes. A, The location of the TALEN binding sites on the zebrafish *kiss1* gene and the mutated genotype analyzed in this study. The TALEN binding sites are underlined. The inserted nucleotides are shown in lower case letters. B, Sequencing results of *kiss1* locus from the WT ($kiss1^{+/+}$) and homozygous mutant ($kiss1^{-/-}$). C, The position of the mutation in the prepro-kiss1. D, The location of the TALEN binding sites on the zebrafish *kiss2* gene and the mutated genotype analyzed in this study. The TALEN binding sites are underlined. The deleted nucleotides are indicated by dash lines. E, Sequencing results of *kiss2* locus from the WT ($kiss2^{+/+}$) and homozygous mutant ($kiss2^{-/-}$). F, The position of the mutation in the prepro-kiss2. G, ISH and IHC analysis of *kiss* mRNA and protein expression in the WT and *kiss* double mutant. ISH detection of *kiss1* mRNA in the vHB region of the WT (a) and the mutant (a'); IHC detection of Kiss1 protein in the vHB region of the WT (b) and the mutant (b'); ISH detection of *kiss2* mRNA in the nPT region of the WT (c) and the mutant (c'); IHC detection of Kiss2 protein in the nPT region of the WT (d) and the mutant (d'). vHB, ventral habenula; nPT, posterior tuberal nucleus. Scale bar, 20 μm.

Morphological and histological analyses of the zebrafish mutant lines

To assess the phenotype of each mutant line, gross morphology of adult fish was analyzed at 75 days postfertilization (dpf). Fish were euthanized using tricaine. Images were taken using a digital camera. Body length, body weight, and gonad weight were measured. The gonad-somatic index (GSI) was calculated as (gonad weight/body weight) $\times$ 100%. Gonad histology analyses were performed on fish sampled at 45, 60, and 75 dpf. For gonad histology, the testicular or ovarian samples were fixed in Bouin's solution overnight at room temperature. The samples were dehydrated through a graded series of ethanol and embedded in paraffin wax. The samples were sectioned on a Leica microtome. After rehydration, the sections were stained with hematoxylin and eosin. For each mutant line, gonads from 2–3 fish were sectioned for examination of gonad developmental stages.

Sperm quality assessment

Male fish was anesthetized using tricaine. About 1.5 μL of fresh semen were collected using a capillary tube and diluted with 20-μL zebrafish sperm immobilizing solution (140mM NaCl, 10mM KCl, 2mM $CaCl_2$, and 20mM HEPES titrated to pH 8.5 with 1.0M NaOH). Samples were kept on wet ice and used within 1 hour. To activate the sperms, 1 μL of semen suspension in zebrafish sperm immobilizing was mixed with 20 μL of aged tap water and quickly applied into a single well of a 12-well multitest slide (MP Biomedicals). The slides and coverslips were coated with 1% (wt/vol) polyvinyl alcohol to reduce sticking of sperms. Sperm quality was assessed using computer-assisted sperm analysis.

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Fecundity and fertilization rate assessment

To induce spawning, 1 female was paired with 1 male in a spawning tray. One hour after light on in the morning, spawned eggs were collected and counted. The developing embryos were maintained at 28°C in 30% Danieau's solution. The number of fertilized/unfertilized eggs was discerned under a dissecting microscope at 4 hours postfertilization. For each fish line, egg production and fertilization rate were measured from 3 matured females and 3 males for 3 independent crosses.

Quantitative RT-PCR

Total RNAs were isolated from the brain and pituitary. One microgram of total RNA was reverse transcribed into cDNA using the ReverTra Ace-α first-strand cDNA Synthesis kit (TOYOBO). Quantitative RT-PCR was performed on a Roche LightCycler 480 real-time PCR system using SYBR Premix Ex Taq (TAKARA). Quantitative RT-PCR conditions were as follows: denaturation at 95°C for 1 minute, followed by 40 cycles of 95°C for 15 seconds, 58°C for 15 seconds, 72°C for 30 seconds, and 84°C for 10 seconds (fluorescent data collection). Standard curves were generated by serial dilutions of plasmid constructs. After amplification, fluorescent data were converted to threshold cycle values. The concentration of the template in the sample was determined by relating the threshold cycle value to the standard curve. The target gene transcript levels were normalized against *ef1a* transcript level.

Statistical analyses

All data are expressed as mean values ± SEM. Statistical analyses were performed using one-way ANOVA, followed the Fisher's least significant difference test. $P < .05$ was considered statistically significant.

Results

Establishment of the zebrafish *kiss1*$^{-/-}$, *kiss2*$^{-/-}$, and *kiss1*$^{-/-}$;*kiss2*$^{-/-}$ mutant lines

To assess the in vivo functions of the *kiss* genes in zebrafish reproduction, we have designed TALENs for the

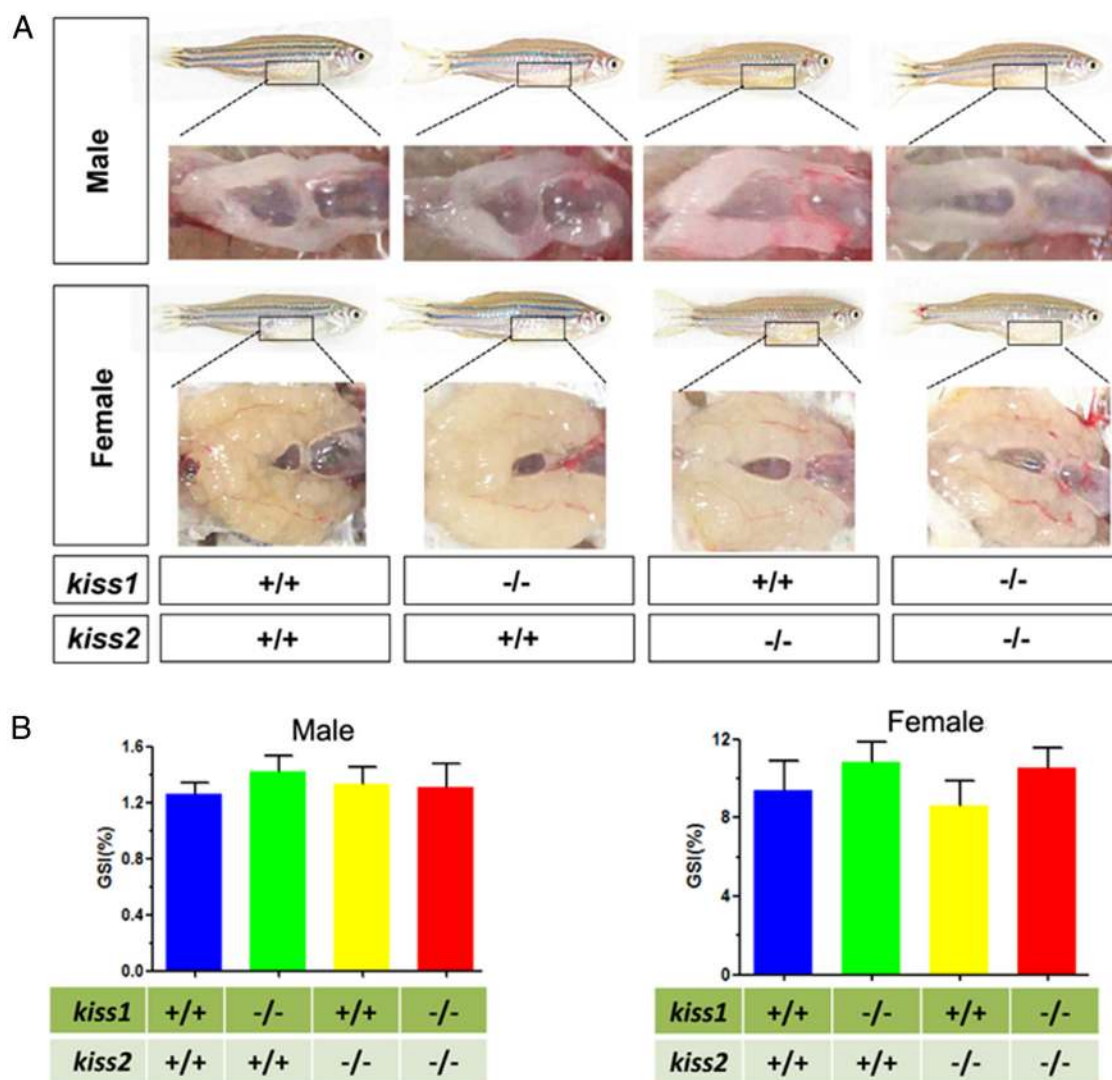

Figure 2. Morphology and GSI of the zebrafish *kiss* mutant lines. A, Overall and gonad morphology of adult zebrafish from the indicated genotypes. B, GSI of adult zebrafish from the indicated genotypes. Data are expressed as mean values ± SEM (n = 6).

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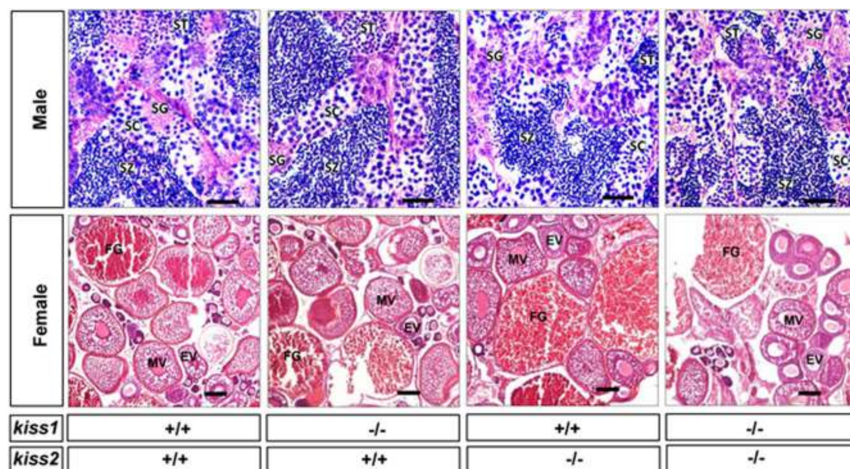

Figure 3. Gonad histology of the zebrafish *kiss* mutant lines. Male and female zebrafish were killed at 75 dpf. SG, spermatogonia; SC, spermatocytes; ST, spermatids; SZ, spermatozoa; EV, early vitellogenic follicle; MV, midvitellogenic follicle, FG, full grown follicle. The scale bars in the testicular sections represent 20 μm and in the ovarian sections represent 100 μm.

zebrafish *kiss1* and *kiss2* genes, respectively. As shown in Figure 1, both *kiss1* and *kiss2* genes contain 2 exons. The TALEN target sites were chosen at the first exon. TALEN mRNAs were injected into zebrafish embryos, and high frequencies of somatic mutation were obtained for both genes (Supplemental Figure 1).

To obtain germline mutations, the injected embryos were raised to adulthood and outcrossed with WT fish. Germline mutations of *kiss1* and *kiss2* were recovered in the F1 progenies. The *kiss1* heterozygote (*kiss1*$^{+/-}$) with a 14-bp insertion and a 3-bp deletion and the *kiss2* heterozygote (*kiss2*$^{+/-}$) with a 17-bp deletion were further used to establish the homozygous mutant line (Figure 1, A and D). These mutations could induce open reading frame (ORF) shifts and thus generating truncated proteins with no functional kisspeptin mature peptides (Figure 1, C and F).

To establish double mutant lines, the *kiss1*$^{+/-}$ and *kiss2*$^{+/-}$ F1 heterozygotes were crossed. The obtained F2 *kiss1*$^{+/-}$;*kiss2*$^{+/-}$ heterozygotes were then intercrossed. In the F3 progenies, the *kiss1*$^{+/+}$;*kiss2*$^{+/+}$, *kiss1*$^{-/-}$;*kiss2*$^{+/+}$, *kiss1*$^{+/+}$;*kiss2*$^{-/-}$, and *kiss1*$^{-/-}$;*kiss2*$^{-/-}$ fish were recovered (Supplemental Figure 2). The genotype of each mutant was confirmed by sequencing (Figure 1, B and E). Sequencing of the cDNA sequences confirmed that the remaining *kiss1* and *kiss2* transcripts were also mutated (Supplemental Figure 3). The *kiss1* and *kiss2* transcript levels in the mutants were significantly lower than that of the WT control (Supplemental Figure 4), suggesting a mechanism of nonsense-mediated mRNA decay (41). Both Kiss1 and Kiss2 mRNA and protein levels were dramatically reduced in the *kiss* double mutant line (Figure 1G). These data indicate that we have successfully generated the *kiss* mutant lines.

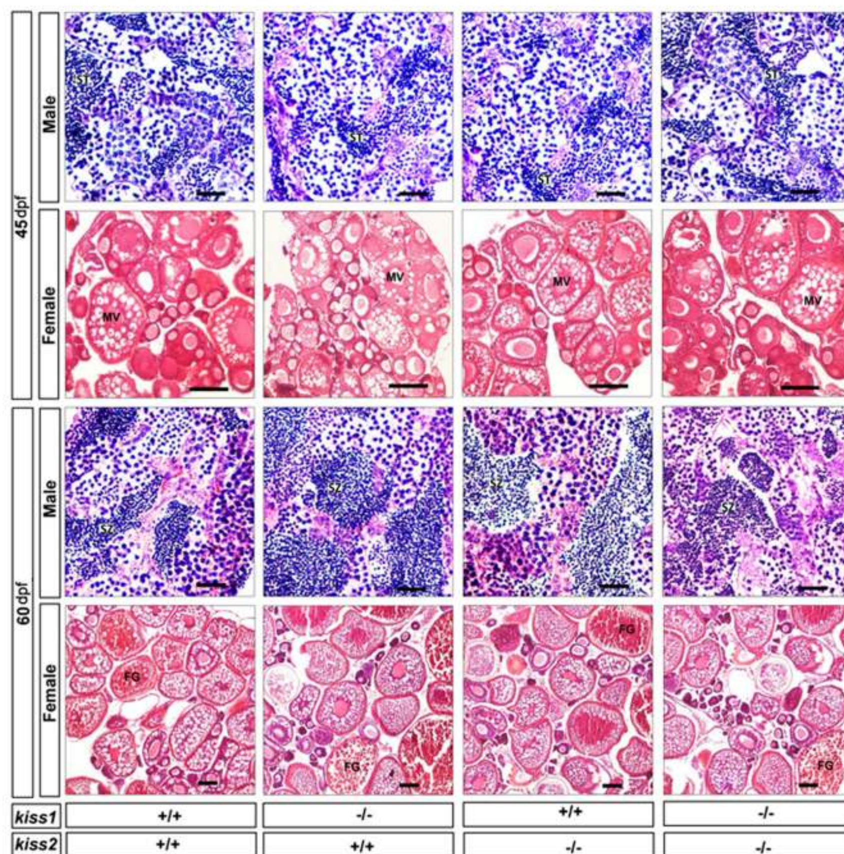

Figure 4. Gonad histology of the zebrafish *kiss* mutant lines during puberty. Male and female zebrafish were killed at 45 and 60 dpf. The appearance of vitellogenic follicles and spermatids were observed at 45 dpf. Spermatozoa and full grown follicles were observed at 60 dpf. ST, spermatids; SZ, spermatozoa; MV, midvitellogenic follicle; FG, full grown follicle. The scale bars in the testicular sections represent 20 μm and in the ovarian sections represent 100 μm.

Puberty onset and sexual maturation are not affected in the zebrafish *kiss1*$^{-/-}$, *kiss2*$^{-/-}$, and *kiss1*$^{-/-}$;*kiss2*$^{-/-}$ mutant lines

All the zebrafish *kiss* mutant lines were viable and developed normally. The genotypes were inherited in the

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expected Mendelian ratio. Body weight and body length were not affected among the genotypes (Supplemental Figure 5). The ovary and testis from each group were fully developed and occupied a large area of the body cavity (Figure 2A). The GSI values among different groups were similar (Figure 2B). In summary, no gross morphological difference was observed among the mutants and WT fish.

To examine whether the gametes developed normally, gonad histology was performed for fish from each genotype at 75 dpf (Figure 3). In the testes, haploid spermatids and spermatozoa with condensed spermheads could be observed, indicating that spermatogenesis could be completed in all the genotypes (Figure 3). In the ovaries, follicles of different developmental stages, including full grown follicles could be observed in all the genotypes (Figure 3), indicating that folliculogenesis was not affected.

To determine whether knockout of the *kiss* genes affect the timing of puberty, we have further analyzed gonad histology at 45 and 60 dpf, a period during which zebrafish puberty onset was observed (42). At 45 dpf, the testes contained a large number of spermatids, and the ovaries contained a large number of vitellogenetic follicles (Figure 4), indicating normal puberty onset in all the genotypes. At 60 dpf, spermatozoa and full grown follicles could be observed in all the genotypes (Figure 4). Overall, these data indicate that puberty onset and gamete maturation are not affected in the absence of functional *kiss* genes.

Full reproductive capacity is retained in the zebrafish *kiss1*$^{-/-}$, *kiss2*$^{-/-}$, and *kiss1*$^{-/-}$;*kiss2*$^{-/-}$ mutant lines

We have further assessed the reproductive capacity of the *kiss* mutant lines. Examination of sperm quality showed no significant difference in sperm motility, average curvilinear velocity and average straightness among the different genotypes (Figure 5A). Females from all the mutant lines exhibited regular spawning activity. There was no significant difference in the number of eggs spawned per female and the fertilization rate for each genotype (Figure 5B). Healthy homozygous mutant embryos could be obtained from the intercross of the *kiss1*$^{-/-}$;*kiss2*$^{-/-}$ double mutant line. Collectively, these data indicate that full reproductive capacity is retained in the mutant lines lacking functional *kiss* genes.

Gene expression of *gnrh2*, *gnrh3*, *lhβ* and *fshβ* in the *kiss1*$^{-/-}$, *kiss2*$^{-/-}$, and *kiss1*$^{-/-}$;*kiss2*$^{-/-}$ mutant lines

We have then examined the gene expression of *gnrh2*, *gnrh3*, *lhβ*, and *fshβ* in the matured male and female fish

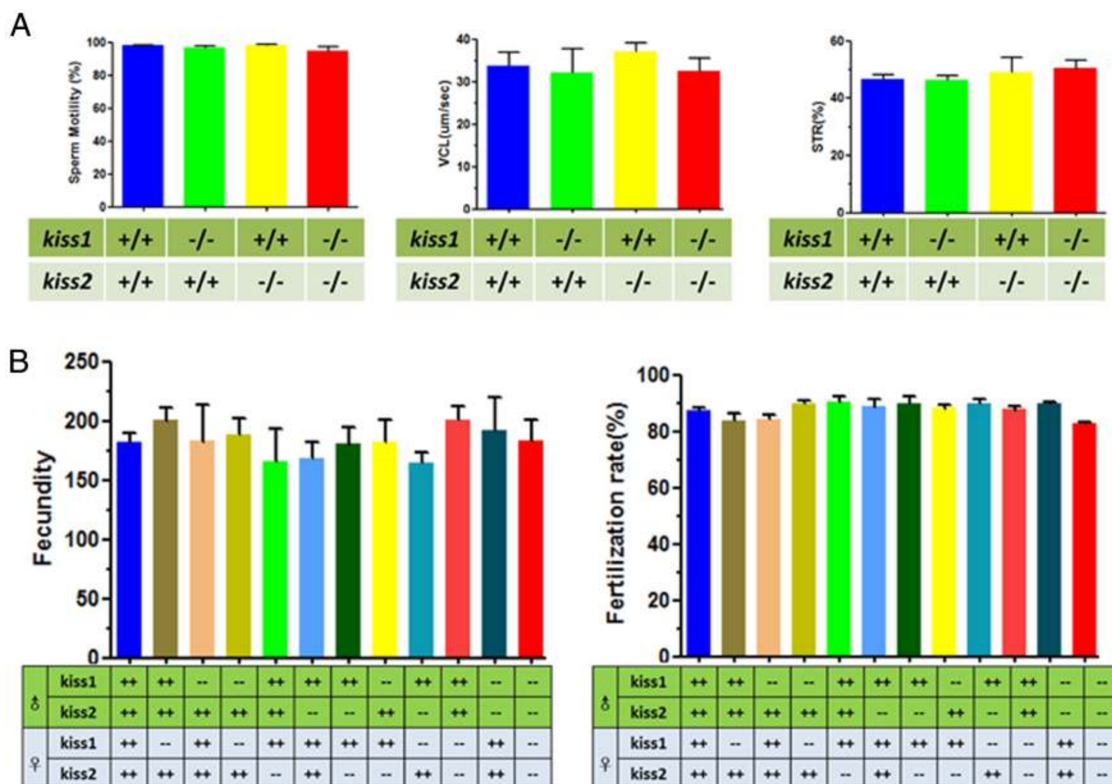

Figure 5. Reproductive capacity of the zebrafish *kiss* mutant lines. A, Sperm quality of male zebrafish *kiss* mutant lines. VCL, curvilinear velocity; STR, straightness. B, Fecundity and fertilization rate of female zebrafish *kiss* mutant lines. Data are expressed as mean values ± SEM from 3 independent replicates (n = 3).

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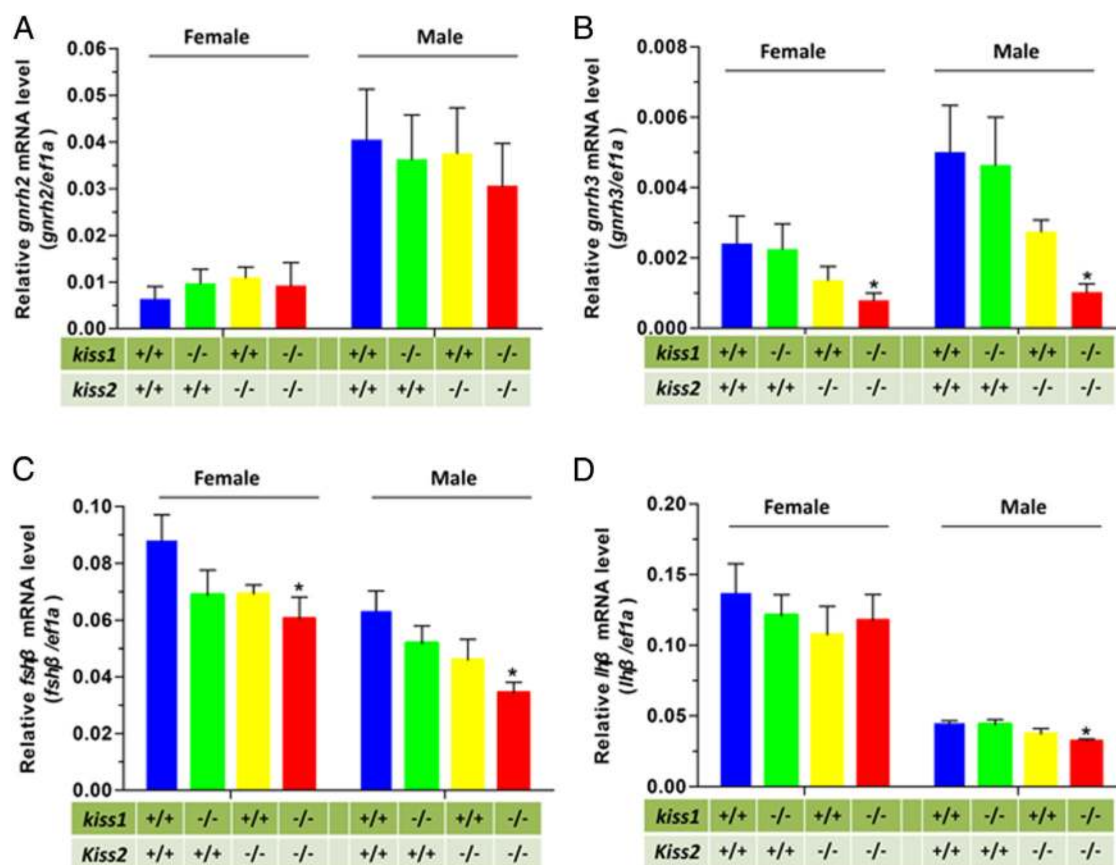

Figure 6. The expression of *gnrh2*, *gnrh3*, *lhβ*, and *fshβ* in the zebrafish *kiss* mutant lines. A and B, The relative expression of *gnrh2* and *gnrh3* in the brain of WT and mutant lines. C and D, The relative expression of *fshβ* and *lhβ* in the pituitary of WT and kiss mutant lines. Data are expressed as mean values ± SEM (n = 5).

of different genotypes. The *gnrh2* expression was not significantly changed among different genotypes (Figure 6A). In both male and female, the *gnrh3* expression was significantly decreased in the double mutant line (Figure 6B). The *fshβ* expression was also significantly down-regulated in the double mutant line of both sexes (Figure 6C). The *lhβ* expression was significantly down-regulated in the double mutant line of male fish but not female fish (Figure 6D). These results suggested that kisspeptin signaling could stimulate *gnrh3* and gonadotropin gene expression. However, the decreased expressions of these genes were not sufficient to cause pronounced phenotypic consequences given the normal reproductive performance of the *kiss* mutant lines.

Establishment of the zebrafish *kissr1*$^{-/-}$, *kissr2*$^{-/-}$, and *kissr1*$^{-/-}$;*kissr2*$^{-/-}$ mutant lines

To further confirm the *kiss/kissr* signaling is not required for zebrafish reproduction, we have designed TALENs for *kissr* genes (Figure 7, A and D). The TALEN target sites are located at the first and the second exon for the *kissr1* and *kissr2* genes, respectively. High frequencies of somatic mutations were induced by TALENs (Supplemental Figure 6), and germline mutations were obtained in F1 generation for both genes. The *kissr1* mutant with an 8-bp deletion and the *kissr2* mutant with a 10-bp deletion were further analyzed (Figure 7, B and E). These mutations could induce ORF shifts and thus generating truncated proteins with no functional 7 transmembrane domains (Figure 6, C and F). Using a similar strategy to establish the *kiss* mutant lines, we have obtained the *kissr* mutant lines (*kissr1*$^{+/+}$;*kissr2*$^{+/+}$, *kissr1*$^{-/-}$;*kissr2*$^{+/+}$, *kissr1*$^{+/+}$;*kissr2*$^{-/-}$, and *kissr1*$^{-/-}$;*kissr2*$^{-/-}$) in F3 generation. The genotype of each fish is confirmed by sequencing. The transcript levels of both receptors were significantly decreased in the mutants (Supplemental Figure 4), suggesting decay of the mutated mRNA transcripts.

Reproductive functions are not impaired in the zebrafish *kissr1*$^{-/-}$, *kissr2*$^{-/-}$, and *kissr1*$^{-/-}$;*kissr2*$^{-/-}$ mutant lines

All the zebrafish *kissr* mutant lines were viable and developed normally. No gross morphological difference could be observed among the mutant lines and WT fish (Supplemental Figure 7). At 75 dpf, the ovaries and testes from each group were fully developed (Supplemental Fig-

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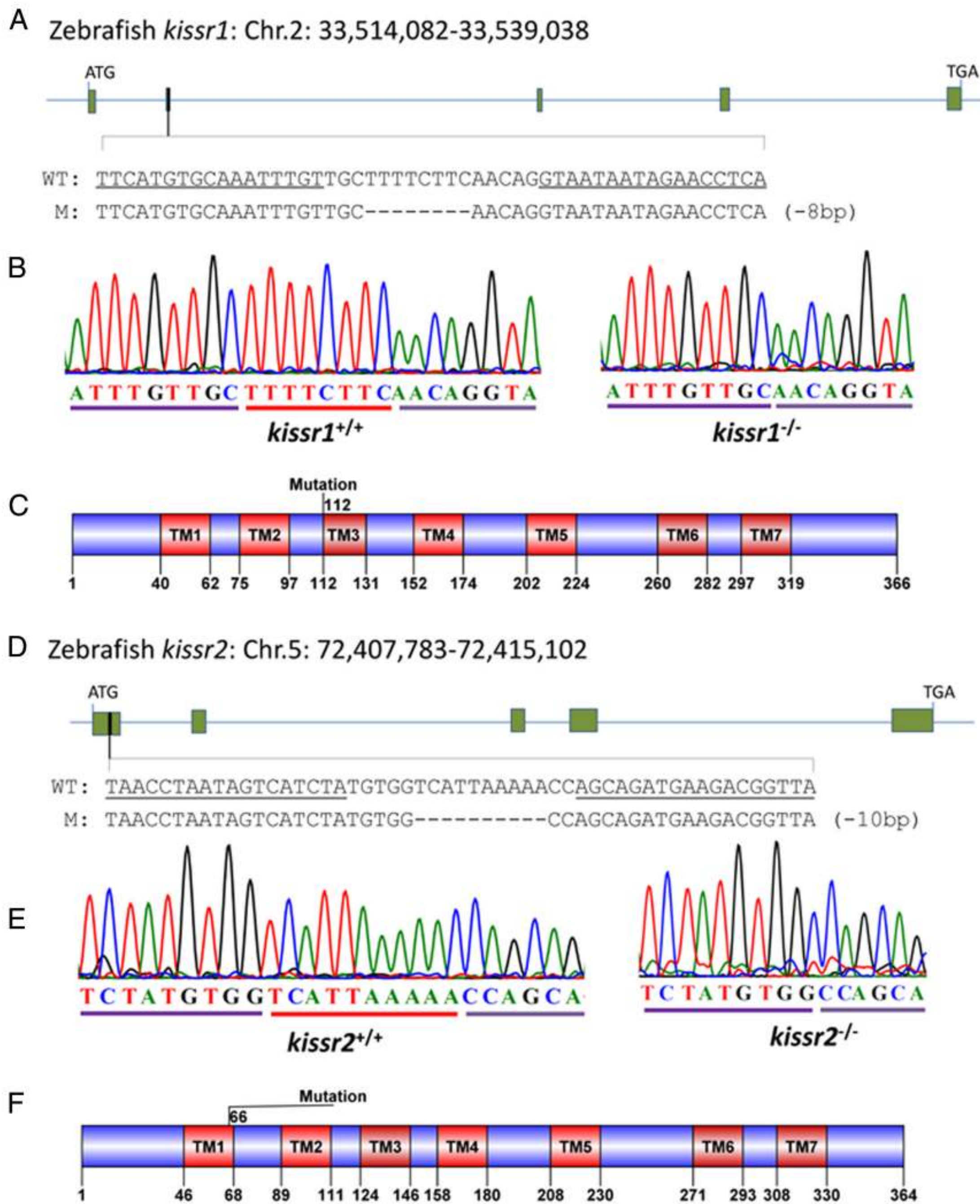

Figure 7. Targeted disruption of zebrafish *kissr1* and *kissr2* genes. A, The location of the TALEN binding sites on the zebrafish *kissr1* gene and the mutated genotype analyzed in this study. The TALEN binding sites are underlined. The deleted nucleotides are indicated by dash lines. B, Sequencing results of *kissr1* locus from the WT (*kissr1*$^{+/+}$) and homozygous mutant (*kissr1*$^{-/-}$). C, The position of the mutation in the Kissr1 protein. D, The location of the TALEN binding sites on the zebrafish *kissr2* gene and the mutated genotype analyzed in this study. The TALEN binding sites are underlined. The deleted nucleotides are indicated by dash lines. E, Sequencing results of *kissr2* locus from the WT (*kissr2*$^{+/+}$) and homozygous mutant (*kissr2*$^{-/-}$). F, The position of the mutation in the Kissr2 protein.

ure 7). Histological analyses indicate that matured gametes were present in the gonad of all the mutated genotypes (Figure 8). Healthy embryos could be obtained from self-crossing of each mutant line. Collectively, these data demonstrate that reproductive functions were not impaired in zebrafish lacking the functional *kissr* genes.

Discussion

Although the functional roles of the *kiss1/gpr54* system in controlling the reproductive axis have been well established in mammals, studies in nonmammalian vertebrates lag behind. Some data suggest that the *kiss/kissr* systems also participate in regulating the reproductive axis in non-

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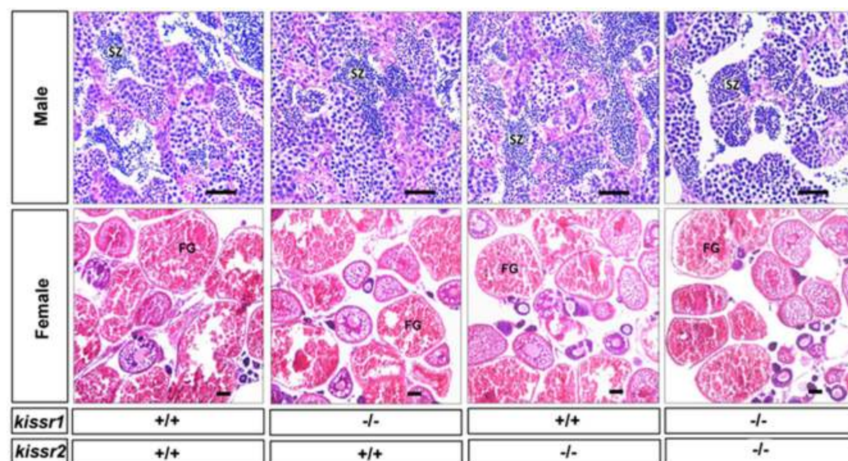

Figure 8. Gonad histology of the zebrafish *kissr* mutant lines. Male and female zebrafish are killed at 75 dpf. SZ, spermatozoa; FG, full grown follicle. The scale bars in the testicular sections represent 20 μm and in the ovarian sections represent 100 μm.

mammalian vertebrates, but whether this system is essential for reproduction is unknown. Because multiple *kiss*/*kissr* paralogs are present in a single nonmammalian vertebrate, functional redundancy among the *kiss* or *kissr* genes may exist. In this study, we have addressed these issues using the zebrafish gene knockout model. Our data indicate that the *kiss*/*kissr* systems are dispensable for zebrafish reproduction.

Using our optimized TALENs system, we have systematically generated the *kiss*/*kissr* mutant lines. For *kiss1* and *kiss2*, the mutations were created at the first exon where the signal peptide is encoded. The mutations were confirmed at both the genomic and transcript levels. Using primers to amplify both the mutant and WT mRNA transcripts, we have demonstrated that the transcript levels of *kiss*/*kissr* genes are significantly decreased. cDNA sequencing confirmed that all mRNA transcripts are actually derived from the mutated genome sequences. Because these mutations can cause ORF shifts, thus no functional mature kisspeptins could be produced. Moreover, the signal peptide is essential for the cellular transportation of the neuropeptide precursor, thus the lack of a functional signal peptide could also disrupt the protein transport. We have further demonstrated that Kiss1 protein is absent in the *kiss1*;*kiss2* double mutant line. The Kiss2-immunoreactivity was dramatically reduced in the brain of kiss mutants, although we still detected some Kiss2-immunoreactive fibers in the brain. However, because our Kiss2 antiserum was generated against the C-terminal region of preopro-Kiss2, these signals detected are not necessarily from the functional peptide. In fact, the presence of Kiss2 peptide in the mutant was ruled out from our gDNA and cDNA sequencing results. To further confirm that the *kiss*/*kissr* systems are not required for zebrafish reproduction, we have mutated the *kissr* genes as well. Both *kissr* genes encode G protein-coupled receptors with 7 transmembrane domains. The mutations were introduced at the functional transmembrane domain, thus disrupting the receptor functions. Consistent with the phenotypes of the ligand gene knockouts, the reproductive functions were not impaired in the receptor mutant lines. However, because of the lack of Kissr antibody, the Kissr protein level has not been examined. Further study confirming the absence of Kissr protein in the mutant is needed to fully ascertain that the *kissr* are not required for zebrafish fertility.

In humans and mice, mutations in either *Kiss1* or *Gpr54* lead to failure of puberty onset (3, 4), suggesting that no compensation mechanism exists for *Kiss1*/*Gpr54* signaling in controlling the reproductive axis in mammals. Given the presence of multiple forms of *kiss* and *kissr* and crossactivity between different kisspeptins and Kissr isoforms in nonmammalian vertebrates (9, 10), functional redundancy may exist among the *kiss*/*kissr* systems in the regulation of reproduction. We have therefore generated double mutant lines. Both *kiss1*$^{-/-}$;*kiss2*$^{-/-}$ and *kissr1*$^{-/-}$;*kissr2*$^{-/-}$ double mutant lines are fertile, excluding the possibility that the 2 ligand genes or the 2 receptor genes could compensate for the loss of each other's functions in the regulation of reproduction.

Although there was no effect of the loss of *kiss*/*kissr* systems on reproductive phenotypes, we noted the significant reduction of mRNA levels of *gnrh3*, *lh*β, and *fsh*β levels in the *kiss* mutant lines, indicating the involvement of the *kiss*/*kissr* systems in the regulation of the reproductive axis in zebrafish. These data also correspond with previous studies demonstrating the expression of *kissr* gene in GnRH neurons in some species (43, 44). However, there are also several studies that failed to demonstrate the coexpression of *kissr* in GnRH neurons in zebrafish (18), medaka (45), and sea bass (46). These contradictory observations could be due to low expression levels of *kissr* in GnRH neurons or the different physiological status of the animals. These data suggest that the *kiss*/*kissr* systems play modulatory but nonessential roles in the regulation of reproduction in fish.

Different mechanisms have evolved for the neuroendocrine control of reproduction between mammals and fish. In mammals, it is well established that GnRH release is the final gateway for the control of gonadotropin release. The *Gpr54* gene is expressed in GnRH neurons (47, 48), and targeted deletion of *Gpr54* in the GnRH neurons could

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cause reproductive defects as shown in the *gpr54*-null mouse (48). Accumulating evidence indicated that *Kiss1/Gpr54* signaling plays a critical role in controlling hypothalamic pulsatile GnRH release in mammals (49). Therefore, the loss of *Kiss1/Gpr54* system in human and mouse generates profound reproductive defects. In fish, however, the reproductive axis is likely controlled by multiple independent neuroendocrine factors (29, 31). Besides GnRH, numerous other neuropeptides and neurotransmitters directly innervating the gonadotrophs could stimulate gonadotropin release (29, 31). Although previous studies reported that kisspeptin treatment could stimulate gonadotropin release and advance gonad development in pubertal and mature fish (9, 10, 25–28), our gene knockout studies demonstrate that the lack of *kiss/kissr* signaling does not impair zebrafish reproductive functions. One possibility is that the action of kisspeptin signaling in stimulating the reproductive axis could be compensated by other neuropeptides with stimulatory actions, such as neurokinin B (50), neuropeptide Y (51), and secretoneurin (52). It appears that teleost have evolved a multifactorial strategy for the neuroendocrine control of reproduction to ensure their reproductive success.

In summary, we have systematically generated zebrafish mutant lines lacking either the *kiss* or *kissr* genes. We have found that spermatogenesis and follicugenesis as well as reproductive capacity are not impaired in all of these mutant lines. Our data demonstrate that the *kiss/kissr* systems are not essential for zebrafish reproduction, indicating that the *kiss/kissr* systems are dispensable for reproduction in certain nonmammalian vertebrates. These new data also provide genetic evidence supporting a multifactorial strategy for the neuroendocrine control of reproduction in fish. The action of kisspeptin signaling in stimulating the reproductive axis might be compensated by other neuropeptides or neurotransmitters. Moreover, the mutant fish lines generated in this study would be valuable resources for investigating kisspeptin signaling in the regulation of other processes such as development (53), animal behavior (40), and disease processes (54).

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