Demonstration of a Functional Kisspeptin/Kisspeptin Receptor System in Amphioxus With Implications for Origin of Neuroendocrine Regulation

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Amphioxus belongs to the Cephalochordata, which is the most basal subphylum of the chordates. Despite many studies on the endocrine system of amphioxus, key information about its regulation remains ambiguous. Here we clearly demonstrate the presence of a functional kisspeptin/kisspeptin receptor (Kiss-Kissr) system, which is involved in the regulation of reproduction in amphioxus. Evolutionary analyses revealed large expansion of *Kiss* and *Kissr* (*gpr54*) genes in amphioxus, and they might represent the ancestral type of the *Kiss*/*gpr54* genes in chordates. Amphioxus Kiss was obviously expression at the cerebral vesicle and the Hatschek pit, whereas amphioxus *gpr54* messenger RNA (mRNA) was abundantly present in nerve cord, ovary, and testes. Amphioxus GPR54-Like1 (GPR54L-1) was shown to be located on the cell membrane. The synthetic amphioxus Kiss-like (KissL) peptides were capable of activating the amphioxus GPR54L-1 with different potencies, hinting the interaction between Kiss and GPR54. Moreover, the expression of amphioxus *gpr54* mRNA was significantly decreased during low or high temperature extremes. Importantly, the injection of amphioxus KissL could cause an elevation of zebrafish blood luteinizing hormone level and induce the expression of amphioxus *gpb5*, a gene encoding the ancestral type of vertebrate pituitary glycoprotein hormones. Also, the expression levels of *BjkissL-2* or *Bjgpr54L-1* were downregulated after spermiation or spawning. Collectively, the amphioxus Kiss-Kissr system has a correlation with the regulation of reproduction. Our studies provide insights into the functional roles and evolutionary history of the Kiss-Kissr system, as well as the origin of the vertebrate neuroendocrine axis for controlling reproduction. **(*Endocrinology* 158: 1461–1473, 2017)**

Identification and characterization of the physiologic roles of kisspeptin (Kiss) and its receptor GPR54 (kisspeptin receptor, Kissr) are an important breakthrough in the area of vertebrate reproduction in recent years (1–10). Kiss belongs to the RF-amide family of peptides that possess an Arg-Phe-amide sequence motif at their C-terminus (11, 12). The RF-amide peptide family includes gonadotropin-inhibitory hormone, neuropeptide FF, prolactin-releasing peptide, pyroglutamylated RFamide peptide/26RFamide peptide, and Kiss (12). *Kiss1*, encoding kisspeptin, was first isolated as a tumor metastasis suppressor gene from the human malignant melanoma cell line C8161 (13–15). GPR54, a G protein–coupled receptor, was originally cloned from rat brain and shares 45% sequence identity with galanin receptor (16). Kiss has been recognized as a neuromodulator in vertebrates. The tissue distribution revealed that messenger RNAs (mRNAs) encoding *Kiss* and *gpr54* are strongly expressed in the brain, notably in the hypothalamus (17). In mice, knockout of the *Kiss1* or *Kiss1r* gene leads to the lack of puberty and infertility (18, 19). Furthermore, the Kiss-Kissr system has an important role in initiating secretion of gonadotropin-releasing hormone by acting on the anterior pituitary in mammals (20–24). The system also involves the release of luteinizing hormone (LH), and follicle-stimulating hormone (FSH) (20–24). Besides governing reproduction, kisspeptin

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Abbreviations: cDNA, complementary DNA; FSH, follicle-stimulating hormone; GPR54L-1, GPR54-Like1; HPG, hypothalamic–pituitary–gonadal; Kiss, kisspeptin; KissL, Kiss-like; Kissr, kisspeptin receptor; LH, luteinizing hormone; mRNA, messenger RNA; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time polymerase chain reaction; RACE, rapid amplification of cDNA ends.

signaling is an important and novel regulator of body weight, adiposity, metabolism, and glucose homeostasis as well (25). In addition, regulation of hypothalamic *Kiss1* mRNA levels by key metabolic signals, such as leptin, has been demonstrated in rats and mice (26, 27).

Kiss-Kissr homologous genes have been identified in different vertebrate groups, including mammals, amphibians, fish and Agnatha. In mammals, humans and rodents have only one *Kiss1* and one receptor form *Kiss1r*, but monotremes exhibit two genes of each (17). Three genes for both ligands and receptors were described in amphibians (17). In fish, two *Kiss* and two *Kiss* receptor genes were reported in zebrafish, medaka, sea bass, and goldfish (17, 24, 28–32), but three *Kissr* genes in eel and four *Kissr* genes in both coelacanth and spotted gar were found. Two *Kiss* genes and one *Kissr* gene were identified in Agnatha (lamprey). Pasquier *et al.* (10) suggested that the multiplicity of Kiss and Kissr types in vertebrates probably originated from the two rounds of whole-genome duplication that occurred in early vertebrates. At the same time, independent gene losses throughout vertebrate evolution, led to the presence of various numbers and types of Kiss and Kissr, in the extant vertebrate species (10). However, no clear evidence demonstrates the presence of the functional *Kiss-Kissr* system in cephalochordate.

The cephalochordate amphioxus, which represents the most basal chordate, is of particular importance in understanding the evolutionary origin of vertebrate hypothalamic–pituitary–gonadal (HPG) axis. Developmental gene expression and transmission electron microscopy indicate the presence of a diencephalic forebrain, a possible midbrain, and a hind brain in amphioxus (33, 34). However, unlike those in vertebrates, amphioxus dorsal nerve cords are not protected by bone but by a simpler notochord made up of a cylinder of cells. The nerve cords have only a small anterior swelling (cerebral vesicle), so that amphioxus does not appear to possess a true brain. In addition, amphioxus has the so-called Hatschek pit, which is a deep ciliated fossa on the dorsal midline of the buccal cavity (the region of the gut behind the mouth). The Hatschek pit has been proposed to be a homolog of the vertebrate pituitary gland (35, 36), whereas no pituitary hormone except the growth hormone–like hormone has been reported in amphioxus (37). Therefore, the neuroendocrine regulation in amphioxus is still an enigma. Recent studies showed that several predicted *Kissr-like* gene annotations exist in *Branchiostoma* (cephalochordate), *Saccoglossus* (hemichordate), and sea urchin (echinodermate) (17, 29, 38). Regarding the ligands, kisspeptin-like hormones were recently described in starfish (39) and amphioxus *B. floridae* (38, 40). Because the amino acid sequence homology is low among amphioxus kisspeptin-like peptides and vertebrate kisspeptins, further evidence is needed to clarify the function and evolutionary relationship of these genes. Here, the phylogenetic analysis, tissue expression, interaction, and functional studies on amphioxus putative *Kiss* and *Kissr* genes were performed, indicating presence of a functional Kiss-Kissr system in the basal chordate amphioxus. Our study provides insights into the functional roles and evolutionary history of the Kiss-Kissr system, as well as the origin of the HPG axis of vertebrates for controlling reproduction.

Materials and Methods

Animal

Adult amphioxus *Branchiostoma japonicum* (formerly known as *B. belcheri tsingtauense*) were collected from the sandy bottom of the sea near Shazikou, Qingdao, China and maintained in glass containers with continuous aeration at room temperature. They were fed twice a day with single-celled algae. Zebrafish (*Danio rerio*) used in this study were purchased from a local fish dealer, maintained in well-aerated tap water at 27°C ± 1°C and fed with live bloodworm and TetraMin tropical fish food flakes (Tetra) twice a day and acclimatized for 1 week before the experiments. Animal experiments were approved by the Ethics Committee of the Laboratory Animal Administration of Shandong province (permit number SD2007695).

Cloning and sequencing of *Bjgpr54L-1* and *BjkissL-2* complementary DNA

Total RNAs extraction, polyA$^+$ RNA purification and the first-strand complementary DNA (cDNA) synthesis were performed as described by Xu *et al.* (41). The initial fragments of the *Bjgpr54L-1* cDNA were amplified by polymerase chain reaction (PCR) with the primer pairs P1 and P2 (Supplemental Table 1), which were designed by using the Primer Premier 5.0 (Premier Biosoft) program (42) on the basis of the *Bfgpr54L-1* mRNA sequence found in *B. floridae* genome database. To get the full-length cDNA sequence, 5′ ρapid amplification of cDNA ends (RACE) and 3′RACE were performed by using the gene-specific primers P3 and P4 (Supplemental Table 1) and the gene-specific nested primers P5 and P6 (Supplemental Table 1), which were designed according to the sequences obtained above. The 5′- and 3′-RACE-ready cDNAs were synthesized from the total RNAs using the BD SMART RACE cDNA amplification kit (Clontech) according to the instructions. The 5′- and 3′-RACE products were gel-purified, subcloned, sequenced, and assembled. In addition, the fragment of the *BjkissL-2* cDNA was also amplified by PCR with the primer pairs P7 and P8 (Supplemental Table 1) on the basis of the *B. floridae BfkissL-2* mRNA sequence.

Sequence analysis

The cDNAs obtained were analyzed for coding probability with the EditSeq program of the LASERGENE software suit (DNASTAR). The SMART program was used to predict the functional sites and transmembrane domains in the deduced amino acid sequence. Comparison against the GenBank protein database was performed by using the BLASTP network server

at the National Center for Biotechnology Information. The three-dimensional structure was performed by using the iterative threading assembly refinement (I-TASSER) program(Zhang Lab). Phylogenetic trees were constructed by using *p*-distance based on the maximum likelihood method of the MEGA 6.0 software package (43). The reliability of each node was estimated by bootstrapping with 1000 replications (44).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to determine the expression profiles of the *BjkissL-2* and *Bjgpr54L-1* genes in the different tissues of *B. japonicum*. Total RNAs were extracted with Trizol (Invitrogen) from the various tissues of adult female and male *B. japonicum*. The PCR primers specific for *BjkissL-2* (P9 and P10), *Bjgpr54L-1* (P11 and P12), and *ef1-α* (P13 and P14) were designed by using the Primer Premier 5.0 program (Supplemental Table 1). qRT-PCR was performed on an ABI 7500 Real-time PCR system (Applied Biosystems), as described by Wang and Zhang (45). The *ef1-α* (elongation factor 1-α) gene was chosen as the reference for internal standardization. qRT-PCR was also used to determine the expression level of *gpb5* and *β-actin* in *B. japonicum* with the primers P15, P16, P17, and P18 (Supplemental Table 1), as described previously.

Immunohistochemical analysis

Rabbit antikisspeptin 10 polyclonal antibody was purchased from Millipore. Immunohistochemical staining was performed as described by Li *et al.* (37) (Table 1). Each amphioxus *B. japonicum* was severed into three to four pieces and fixed in freshly prepared 4% paraformaldehyde (weight-to-volume ratio) in 100 mM phosphate-buffered saline (PBS) (pH, 7.4) at 4°C for 24 hours. After a series of dehydrations, the samples were embedded in paraffin, sectioned at 8 μm, and dried at 42°C for 24 hours. They were dewaxed in xylene for 10 minutes (two times, 5 minutes each), followed by immersion in absolute ethanol for 10 minutes (two times, 5 minutes each), and then rehydrated in 95%, 90%, 80%, and 70% ethanol (5 minutes each) and brought to 100 mM PBS. After rinsing with distilled water for 5 minutes, the endogenous peroxidase activity in the sections was quenched with incubation in 3% H_2O_2 (volume-to-volume ratio) at room temperature for 15 minutes, which was followed by a 5-minute wash in redistilled water. Subsequently, the sections were preincubated with 5% BSA in 10 mM PBS (pH, 7.4) at room temperature for 30 minutes, washed in 100 mM PBS for 5 minutes, and then incubated overnight in a humidified chamber at 4°C with rabbit antikisspeptin 10 polyclonal antibody (Millipore) diluted 1:800 with 100 mM PBS containing 2% BSA. The control sections were also incubated with primary antibody, which was preadsorbed with BjKissL-2 peptide (Supplemental Table 2). BjKissL-2 with final concentration of 1 mg/mL was mixed with antikisspeptin antibody gently in a 10:1 (peptide: antibody) volume ratio. After incubation at room temperature for 1 hour, preadsorbed antibody was diluted at the same ratio as the untreated antibody (1:800). Similarly, the second control sections were incubated with preimmune rabbit serum. Then the sections were washed four times (5 minutes each) in 100 mM PBS and incubated further at room temperature for 1 hour with peroxidase-conjugated goat antirabbit IgG diluted 1:800 with PBS. The chromogenic reaction was achieved by the addition of 0.015% DAB (weight-to-volume ratio) containing 0.02% H_2O_2 (volume-to-volume ratio) in 50 mM Tris-HCl buffer (pH, 7.6) and maintenance in the dark for 5 minutes. The sections were mounted in Canada balsam, observed, and photographed under a Zeiss microscope (Zeiss).

Eukaryotic expression vector construction

The *mCherry* gene was amplified by PCR using the primer pairs P19 and P20 (Supplemental Table 1), respectively, and the PCR product was digested with *Xba*I and *Apa*I, subcloned into the eukaryotic expression vector pcDNA3.1/V5-His A vector (Invitrogen). The plasmid was designated pcDNA3.1/mCherry. Subsequently, the complete coding region of *Bjgpr54L-1* was amplified by PCR using the primer P21 and P22 (Supplemental Table 1), and the PCR product was digested with *Hin*dIII and *Xho*I and ligated into the pcDNA3.1/mCherry (which was cut with the same restriction enzymes) upstream, to construct the recombinant eukaryotic expression vector, pcDNA3.1/BjGPR54L-1/mCherry.

Cell transfection and fluorescent microscopy

HEK 293T cells were the gifts from Jianfeng Zhou, Laboratory of Molecular Medicine, School of Medicine and Pharmacy, Ocean University of China. Cells were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 10 U/mL penicillin and 100 mg/mL streptomycin. To examine the subcellular localization of BjGPR54L-1, cells were seeded in six-well plates and transfected with the different plasmids (pcDNA3.1/mCherry or pcDNA3.1/BjGPR54L-1/mCherry) using Lipofectamine 2000 Reagent (Invitrogen) according to manufacturer instructions. At 48 hours after transfection, the cells were examined by Leica fluorescence microscopy (Leica DMI300B).

Peptides

The peptides (Supplemental Table 2) corresponding to zebrafish kisspeptins (ZfKiss1-10 and ZfKiss2-10) and amphioxus hypothetical kisspeptins (BfKissL-1, BjKissL-2, BfKissL-4-1, and BfKissL-4-2) were synthesized by Sangon (Shanghai, China). The purity was >95%, as determined by

Table 1. Antibody Table

Peptide/ Protein Target	Antigen Sequence (if Known)	Name of Antibody	Manufacturer, Catalog No., and/or Name of Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used	RRID
Kisspeptin10 (kp10)	Mouse kisspeptin 10	Anti-kisspeptin antibody	Millipore AB9754	Rabbit; polyclonal	1:800	AB_2296529

analytical high-performance liquid chromatography. They were dissolved in PBS and stored at −80°C until use.

Luciferase reporter gene assays

The open reading frame of the *Bjgpr54L-1* was amplified by PCR using the primer P23 and P24 (Supplemental Table 1). The PCR product was digested with *Hin*dIII and *Xho*I and subcloned into the pcDNA3.1/V5-His A vector (Invitrogen) to construct pcDNA3.1/BjGPR54L-1. HEK 293T cells were seeded in 24-well plates. When cells reach a confluency of 90% to 95%, 400 ng of the pCRE-Luc or pSRE-Luc reporter plasmid (Biovector, Beijing, China), 400 ng of pcDNA3.1/BjGPR54L-1, and 40 ng pRL-TK (Promega) were cotransfected into the cells as described above. Twenty-four hours after transfection, cells were serum starved for 18 hours, then stimulated with PBS or various concentrations of ZfKiss1-10, ZfKiss2-10, BfKissL-1, BjKissL-2, BfKissL-4-1, and BfKissL-4-2 for an additional 6 hours. Luciferase activity in cell extracts was determined by using a luciferase assay system (GeneCopoeia) according to the user manual in a luminometer (Promega).

Effects of temperature on *Bjgpr54L-1* gene expression in amphioxus

Four groups of amphioxus (10 each group) were kept in different temperature (15°C, 20°C, 25°C, and 30°C) in separated aquaria for 7 days. Three amphioxus from each group were randomly chosen and the expression of the *Bjgpr54L-1* and *β-actin* (a nonreproductive gene) genes were detected by qRT-PCR as described previously.

Effects of kisspeptin on LH secretion in zebrafish

Sexually mature female zebrafish were injected with various doses of the different kisspeptins twice, separated by a 3-hour interval. The same volume of PBS was used as negative control. Blood samples were collected from the caudal vessels at 3 hours after the second injection. Then the samples were separated by centrifugation at low temperature. LH was measured by using a zebrafish LH enzyme-linked immunosorbent assay kit (MLBIO).

Effects of kisspeptin on *gpb5* expression in amphioxus

Sexually mature female *B. japonicum* were isolated and cultured. They were randomly divided into two groups (three in each group). One group was injected with various doses of the different kisspeptin twice, separated by a 3-hour interval. The second group (control) was administrated with the same volume of PBS only. It is not simple to inject amphioxus because of their size, tough tunic, and continuous twisting when out of sand. The amphioxus was put on a clean gauze pad with a graduated pipette, and the abdomen was moved up by hand with the gauze. Then, 5 μL of various doses of the different kisspeptins was intraperitoneally injected to the amphioxus by using a microsyringe, quickly and gently. The expression of *gpb5* was detected by qRT-PCR as described previously.

Comparison of expression levels of *BjkissL-2* and *Bjgpr54L-1* before and after spermiation (or spawning) in amphioxus

During the natural breeding season (mid-June to mid-July), the sexually mature Qingdao amphioxus *B. japonicum* were collected from the sea near Shazikou in Qingdao. They were separated into a male group and a female group and maintained in glass containers with continuous aeration at room temperature. They were fed twice a day with single-celled algae. No special induction is needed for sexually mature amphioxus to release eggs or sperms in the laboratory. The Qingdao amphioxus starts to spawn at about 7:00 PM. Spawning was observed in he evening. After 48 hours, they were further separated into four groups: male before spermiation, male after spermiation, female before spawning, and female after spawning. Subsequently, three from each group were randomly chosen and the expression levels of the *BjkissL-2* and *Bjgpr54L-1* genes were detected by qRT-PCR as described previously.

Statistical analysis

All the experiments were conducted three times. Statistical analyses were performed using GraphPad Prism 5 software. The significance of differences was determined by two-way analysis of variance. Differences at $P < 0.05$ were considered significant.

Results

Characterization and phylogenetic analyses of amphioxus *Kiss* genes

Four putative kisspeptin-like genes have been reported in amphioxus *B. floridae* (38, 40). One of the genes encoded three putative peptides, whereas other genes encoded one peptide (38). In this study, we performed further sequence analyses on the putative kisspeptin-like peptides in amphioxus. The six putative kisspeptin-like peptides in amphioxus *B. floridae* are named BfKissL-1, BfKissL-2, BfKissL-3, BfKissL-4-1, BfKissL-4-2, and BfKissL-4-3 in this paper (Fig. 1). The signal peptide sequences were identified on the *N*-terminus of the four putative prepro-kisspeptin-like proteins using the SignalP 3.0 Tool (46). Except for BfKissL-4-3 peptide, the putative amidation motifs were also found in other five amphioxus kisspeptin-like peptides (Fig. 1). The conserved C-terminal amidation motif is a common structural characteristic of kisspeptins and is important for their biological activity. Thus, the amphioxus BfKissL-4-3 peptide was excluded from further analysis in this paper.

To understand the evolutionary relationships of these peptides, the phylogenetic tree was constructed with the

BfKissL-1:	------YNPNAWSRFGR------	XP_002608257.1
BfKissL-2:	------PNMNAWGQPWGKR------	XP_002591617.1
BfKissL-3:	------ISPNMFSLHGKR------	XP_002608256.1
BfKissL-4-1:	------YNPNSWSVFGR------	XP_002608492.1
BfKissL-4-2:	------ANLNMWSSFGRR------	XP_002608492.1
BfKissL-4-3:	------VNPAFFLTPFG------	XP_002608492.1

Figure 1. Partial amino acid sequences and GenBank accession numbers of the six putative kisspeptin-like peptides in amphioxus *Branchiostoma floridae*. The putative potential cleavage sites and amidation motifs are marked in gray.

maximum likelihood method (Fig. 2) based on the amino acid sequences of putative mature kisspeptins from the representative species. It was observed that the members of Kiss1 and Kiss2 form two different clusters, respectively. All the vertebrate kisspeptins cluster into Kiss1 group or Kiss2 group, but the five putative kisspeptin-like peptides from amphioxus, together with two kisspeptin-type peptides from starfish, do not cluster into either group in the tree. It is highly possible that the *Kiss1* and *Kiss2* genes in vertebrates may originate from a common ancestral gene, and these putative genes in amphioxus might represent the ancestral type of the *Kiss* genes in chordates. In addition, considering that four genes are present in amphioxus, the amphioxus-specific *Kiss* gene duplication might have occurred in the amphioxus clade during evolution.

Characterization and phylogenetic analyses of amphioxus *Kissr* genes

Sixteen putative *Kissr-like* genes were reported in amphioxus *B. floridae* (38, 40). However, we identified

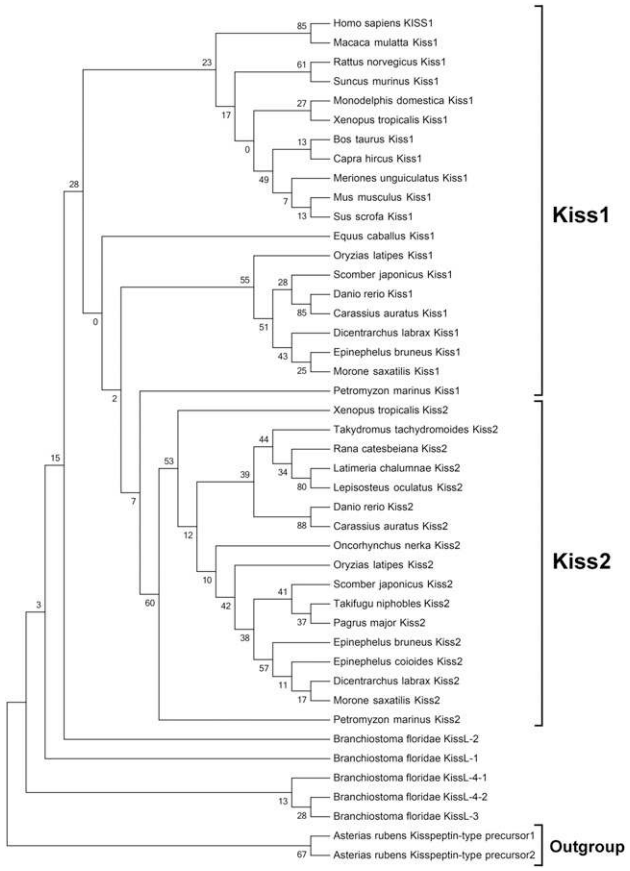

Figure 2. Phylogenetic tree of kisspeptins. The phylogenetic tree was constructed by the MEGA 6.0 software package based on the amino-acid sequences of putative mature kisspeptins using the maximum likelihood method. The reliability of each node was estimated by bootstrapping with 1000 replications. The *Asterias rubens* kisspeptin-type precursor1 and kisspeptin-type precursor2 were used as outgroup. Accession numbers for sequences used are listed in Supplemental Table 3.

27 putative *gpr54-like* genes by extensive BLAST survey. Hydropathy analysis for all 27 putative GPR54-like proteins revealed the presence of seven stretches of hydrophobic amino acid residues, the typical characteristic of G protein–coupled receptor (data not shown). To uncover the origin and evolution of the *gpr54*, 22 putative *gpr54-like* genes were included for the phylogenetic analyses (Fig. 3). The other five putative *gpr54-like* genes were excluded from the analysis because their sequences were obviously longer or shorter than those of the conventional GPR54 proteins. The phylogenetic tree constructed by using the sequences of GPR54 available in the vertebrates and invertebrates show that all the 22 putative amphioxus *gpr54-like* genes, together with *Saccoglossus* and sea urchin *gpr54-like* genes, locate at the basal position of the vertebrate *gpr54* genes. They do not cluster into Kissr1 or Kissr2 group in the tree. Of note, *Kissr* genes were lost in lineage leading to tunicates. It is highly possible that the *Kissr1* and *Kissr2* genes in vertebrates may originate from a common ancestral gene, and these putative genes in amphioxus might represent the ancestral type of the *Kissr* genes in chordates. Also, it is highly possible that the amphioxus-specific *Kissr* gene duplication has occurred in the amphioxus clade during evolution because 27 putative genes are present. The large expansion of *Kiss* and *Kissr* in amphioxus indicates a codiversification of Kiss and Kiss receptor in the *Branchiostoma* genome.

Cloning of *KissL-2* and *gpr54L-1* cDNA in Qingdao amphioxus

Based on the phylogenetic analyses, the genes of *KissL-2* and *gpr54L-1* were selected for cloning and performing further expression and functional study in Qingdao amphioxus *B. japonicum*. They were named *BjkissL-2* and *Bjgpr54L-1*, respectively. The cloned 291-bp *BjkissL-2* cDNA fragment encodes a deduced peptide containing the conserved kisspeptin10 [Supplemental Fig. 1(a)]. The *Bjgpr54l-1* cDNA is 1508 base pairs long, containing an open reading frame of 1221 bp that encodes a deduced protein of 406 amino acids [Supplemental Fig. 1(b)]. Analyses with SMART and InterPro programs showed that BjGPR54L-1 belongs to G protein–coupled receptor, with an extracellular *N*-terminus, a seven-transmembrane domain, and a cytoplasmic C-terminus. I-TASSER prediction showed that BjGPR54L-1 has a three-dimensional structure similar to that of zebrafish GPR54a or GPR54b [Supplemental Fig. 1(c–e)]. They include the same number of transmembrane helices and pleated sheets at the same position.

Tissue distribution of *BjkissL-2* and *Bjgpr54L-1*

The relative expression levels of *BjkissL-2* in different tissues of amphioxus were examined by using real-time

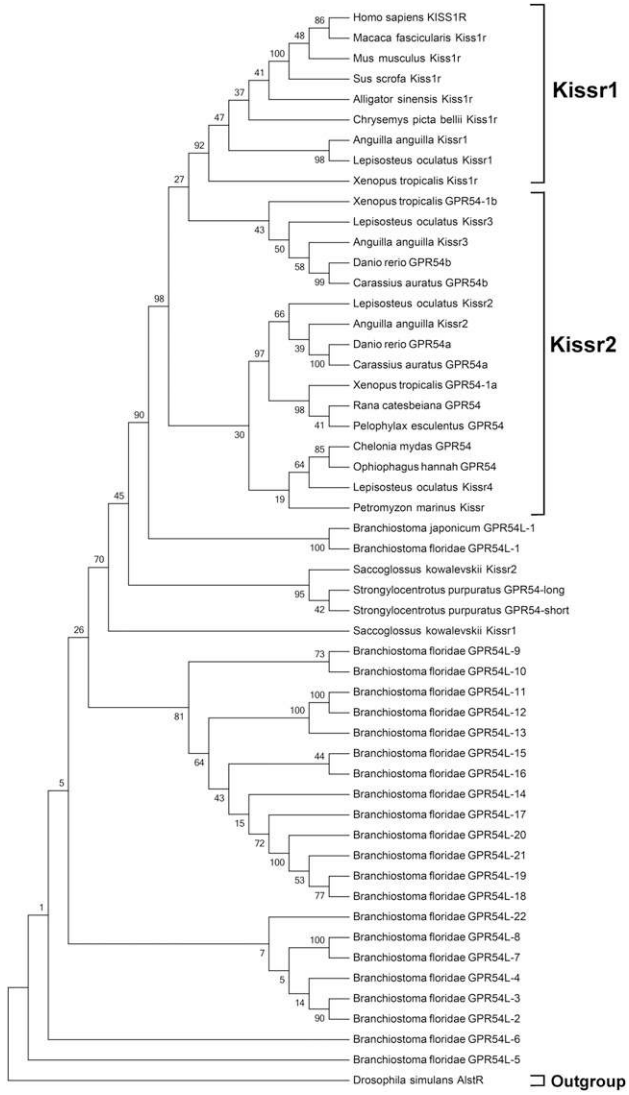

Figure 3. Phylogenetic tree of GPR54s. The phylogenetic tree was constructed by the MEGA 6.0 software package based on the amino-acid sequences of GPR54s using the maximum likelihood method. The reliability of each node was estimated by bootstrapping with 1000 replications. The *Drosophila simulans* AlstR was used as outgroup. Accession numbers for sequences used are listed in Supplemental Table 4.

PCR technology [Fig. 4(a) and 4(b)]. It is almost impossible to separate the tissue of notochord from nerve cord, so we examined the mixture of both tissues in this experiment. The dissociation curve of amplification product showed a single peak, indicating that the amplification was specific (data not shown). The expression level of *BjkissL-2* mRNA is obviously higher in the mixed tissues of notochord and nerve cord. In mammals, amphibians, and fish, kisspeptins are highly expressed in the brain, notably in the hypothalamus. To further detect whether the kisspeptin-like proteins in amphioxus are expressed in the homologous regions, the sections of the head part of amphioxus were performed with immunohistochemical analysis with antikisspeptin10 (kp10) antibody. Fortunately, signals were detected in the anterior region of the nerve cord (cerebral vesicle) and the Hatschek pit [Fig. 4 (e–g)], indicating the possible existence of kisspeptin-like protein in amphioxus.

Also, the relative expression levels of *Bjgpr54L-1* in different tissues of amphioxus were examined by using real-time PCR technology [Fig. 4(c) and 4(d)]. It was found that *Bjgpr54L-1* mRNA was predominantly present in the mixed tissues of notochord and nerve cord; middle in testis and ovary; and low in the tissues of gill, muscle, hepatic cecum, and hind-gut of both female and male amphioxus.

Eukaryotic expression of *Bjgpr54L-1*

To investigate the subcellular localization of BjGPR54L-1, we first cloned and sequenced the full-length coding region of *Bjgpr54L-1* cDNA. Then the HEK 293T cell line was transfected with a construct expressing BjGPR54L-1 fused to the fluorescent protein mCherry. The red fluorescence was visualized at the rim of the cells, indicating that BjGPR54L-1-mCherry is localized on the cell membrane [Fig. 5(a)]. In contrast, the fluorescent protein mCherry alone does not localize to the cell membrane but is evenly distributed throughout the cell [Fig. 5(b)], meaning the BjGPR54L-1 molecule is responsible for the redirection of the fluorescent protein to the cell membrane. The subcellular localization of BjGPR54L-1 is in agreement with the localization of the mammalian Kissr molecule as well as the predicted structure of the BjGPR54L-1 molecule with seven transmembrane regions. The localization on the cell membrane of the BjGPR54L-1 protein indicates a possible function as a receptor for the ligand of Kiss.

Ligand selectivity of BjGPR54L-1

To further characterize the ligand–receptor interactions of BjGPR54L-1 with kisspeptins, *CRE* and *SRE* reporter gene assays were performed. As reporter genes, *SRE* and *CRE* follow protein kinase C (PKC) and protein kinase A (PKA) activation, respectively. Graded concentrations of the four synthesized amphioxus kisspeptins (BfKissL-1, BjKissL-2, BfKissL-4-1, and BfKissL-4-2) and two synthesized zebrafish kisspeptins (ZfKiss1-10 and ZfKiss2-10) were applied to HEK 293T cells, which were transfected with BjGPR54L-1. Cells transfected with the empty vector exhibited no response to the kisspeptins treatment (data not shown). In the CRE-Luc reporter system [Fig. 6(a)], treatment with high concentrations of ZfKiss1-10, ZfKiss2-10, or BjKissL-2 could obviously trigger the receptor signaling pathway. However, no obvious receptor signaling could be detected when treatment with the kisspeptin of BfKissL-1, BfKissL-4-1, or BfKissL-4-2. In the SRE-Luc reporter system [Fig. 6(b)], BjKissL-2 clearly induced a concentration-dependent increase in SRE-luc activity. ZfKiss1-10, ZfKiss2-10, or BfKissL-4-2 was

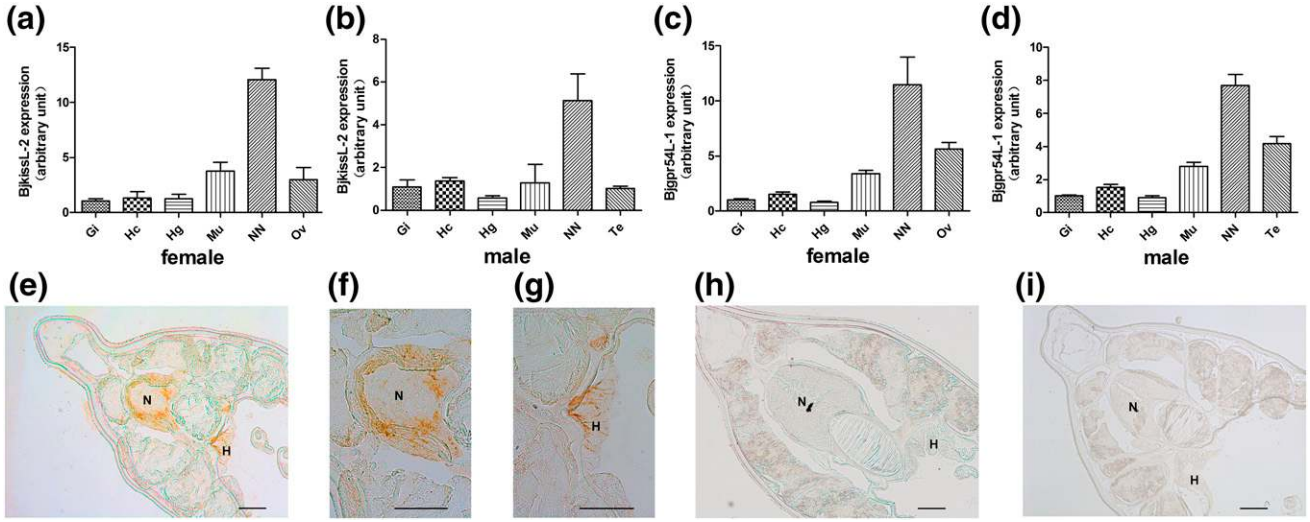

Figure 4. Expression of BjkissL-2 and BjGPR54L-1. (a–d) Relative expression of *BjkissL-2* and *Bjgpr54L-1* mRNAs in the different tissues in both female and male amphioxus *B. japonicum*. The *ef1-α* gene was chosen as internal control for normalization. Gi, gill; Hc, hepatic cecum; Hg, hindgut; Mu, muscle; NN, notochord and nerve cord; Ov, ovary; Te, testis. (e–h) The clear reactivity in the cerebral vesicle (N) and Hatschek pit (H) with antikisspeptin 10 (kp10) antibody were shown (e–g). Control experiments performed by preadsorbed primary antibody with BjKissL-2 peptide (h) and preimmune rabbit serum instead of primary antibody (i). Scale bar, 50 μm.

also able to activate BjGPR54L-1 but exhibited relatively low potency. However, BfKissL-1 or BfKissL-4-1 had no obvious effect. In addition, SRE-Luc reporter system exhibited higher potency in comparison with the CRE-Luc reporter system. The data revealed that BjKissL-2 and BfKissL-4-2 were able to activate their putative receptors BjGPR54L-1, suggesting the presence of a functional Kiss-Kissr system in amphioxus. Here it should also be noted that the luciferase activity was detected only at very high concentrations relative to physiologic levels, and this may not reflect a true indication of what is happening *in vivo*.

Effects of temperature on *Bjgpr54L-1* mRNA expression

Temperature is a critical factor in regulating reproduction, especially in ectothermic animals (47–50). Normally, the temperature range experienced by Qingdao amphioxus in its natural environment is from 2.2°C in winter to 26.5°C in summer (the breeding season) (51). We examined the effects of temperature on the expression of *Bjgpr54L-1* mRNA. The amphioxus was reared in the laboratory at a temperature of 20°C ± 1°C. We found that a low (15°C) or a high (30°C) temperature obviously suppressed the expression of *Bjgpr54L-1* mRNA [Fig. 7(a)], but no significant differences in the expression of β-actin were observed among different temperature groups [Fig. 7(b)], indicating that the regulation of reproduction by temperature might be mediated through the Kiss-Kissr system.

Effects of administration of kisspeptins on LH release

The Kiss-Kissr system plays an important role in reproduction in mammals. To investigate whether the amphioxus kisspeptins have correlation with the regulation of reproduction, the actions of four synthesized amphioxus kisspeptins and two synthesized zebrafish kisspeptins on zebrafish blood LH level was investigated *in vivo*. As shown in Fig. 8, ZfKiss2-10 administration significantly increased blood LH levels in a dose-dependent manner, whereas ZfKiss1-10 increased blood LH levels at a high concentration. For amphioxus kisspeptins, the high concentration of BfKissL-4-2 significantly increased LH levels, whereas the peptides of BfKissL-1, BjKissL-2, and BfKissL-4-1 showed no effect. The survival rate after injection was 100%. These data demonstrated that amphioxus kisspeptins could influence fish gonadotropin release. Note that the response was shown only at very high concentrations relative to physiologic levels, and this may not reflect a true indication of what is happening *in vivo*.

Effects of administration of kisspeptins on the expression of *gpb5* in amphioxus

The above data suggest that amphioxus peptide BfKissL-4-2 has a correlation with the regulation of reproduction in zebrafish. We then wanted to know its role in the amphioxus. However, the pituitary glycoprotein hormones of LH, FSH, and thyroid-stimulating hormone are not yet found in amphioxus. Recently, thyrostimulin, a heterodimeric glycoprotein hormone composed of an α2 (GPA2) and β5 (GPB5) subunit, has been reported in amphioxus. Phylogenetic analyses suggested that thyrostimulin in amphioxus is an ancestral type of the glycoprotein hormones in chordates. Therefore, we examined the expression changes of *gpb5* mRNA with administration of kisspeptins in amphioxus.

First, the different expression patterns of *gpb5* mRNA between female and male amphioxus were

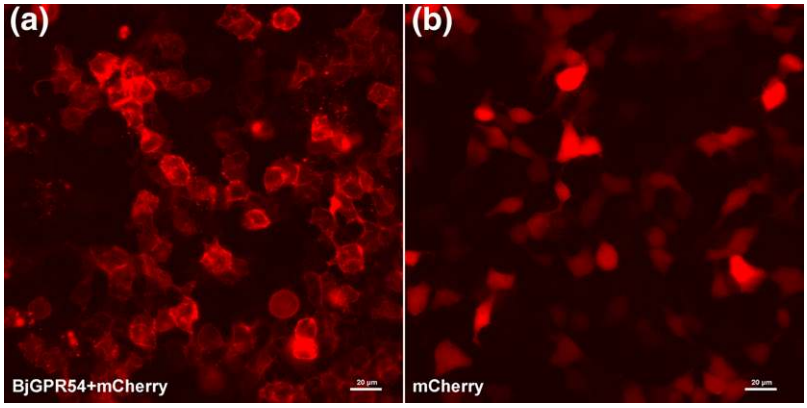

Figure 5. Eukaryotic expression of BjGPR54L-1. The HEK293T cells were transiently transfected with (a) pcDNA3.1/BjGPR54L-1/mCherry or (b) pcDNA3.1/mCherry. After 48 hours, the cells were imaged by fluorescence microscopy.

checked. As shown in Fig. 9(a), the expression of *gpb5* mRNA in females was about twofold higher than that in males. Then, the female amphioxus was used in the following experiment. The zebrafish kisspeptin (ZfKiss1-10 or ZfKiss2-10) injection provoked an increase in *gpb5* expression, with ZfKiss1-10 more potent than ZfKiss2-10. In the case of amphioxus kisspeptins, BjKissL-2 significantly increased the expression of *gpb5* mRNA, whereas BfKissL-1, BfKissL-4-1, and BfKissL-4-2 had no effect on the expression [Fig. 9(b)]. The survival rate after injection was 100%. Collectively, the functional kisspeptin may exist in amphioxus.

Expression levels of *BjkissL-2* and *Bjgpr54L-1* are related to reproduction in amphioxus

To further detect the correlation of the Kiss-Kissr system with the regulation of reproduction in amphioxus, we compared the expression levels of *BjkissL-2* or *Bjgpr54L-1* mRNA before and after spermiation (or spawning). Obviously, the expression levels of both *BjkissL-2* and *Bjgpr54L-1* genes were downregulated 48 hours after spermiation [Fig. 9(d) and 9(f)]. Forty-eight hours after spawning, the expression of *Bjgpr54L-1* was also decreased [Fig. 9(e)], but no significant difference was detected for the expression of *BjkissL-2* [Fig. 9(c))].

Discussion

Ancestral and functional *Kiss* genes exist in amphioxus

Cephalochordate amphioxus represents the most basal chordate. The lack of whole-genome duplications in amphioxus and the slow rate of evolution support the use of amphioxus as a proxy for the ancestral chordate (34). In amphioxus, four putative *Kiss-like* genes encoding six kisspeptin-like peptides were reported by Mirabeau and Joly (38). However, the amino acid sequences of kisspeptins are highly divergent between amphioxus and vertebrate, so further evidence is needed to clarify the evolutionary relationship of these kisspeptins, as well as the functions of the *Kiss-like* genes in amphioxus. Here we showed that amphioxus kisspeptin-like peptides can activate Kissr, induce zebrafish LH release, and increase the expression of amphioxus *gpb5* mRNA, suggesting the presence of the functional kisspeptin-like peptides in amphioxus. Notably, only two kisspeptin-like peptides, BjKissL-2 and BfKissL-4-2, among the four peptides tested in this study displayed correlation with the regulation of reproduction. Considering that kisspeptins in vertebrates have shown diverse functions, such as adiposity, metabolism, and glucose homeostasis (25), it would be interesting to investigate additional functions of the other two kisspeptin-like peptides (BfKissL-1 and BfKissL-3) in the future.

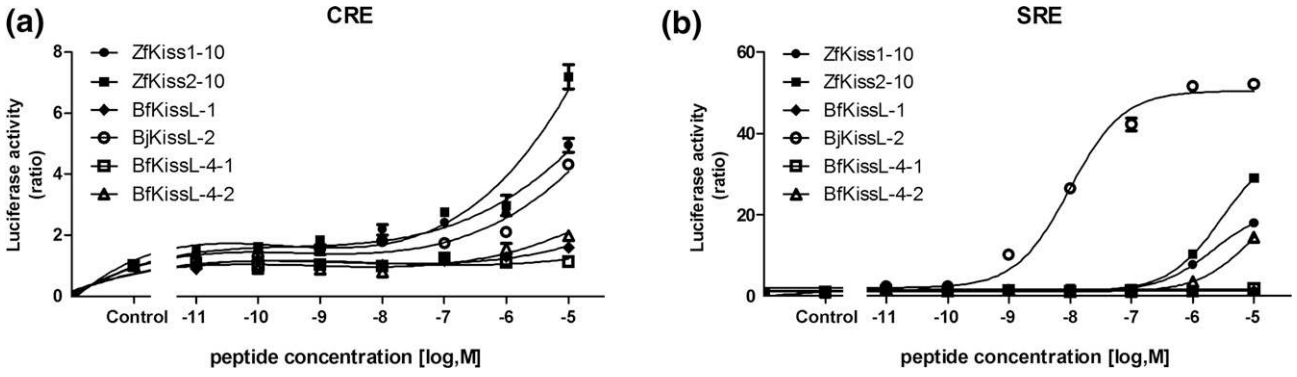

Figure 6. Ligand selectivity of BjGPR54L-1. Induction of (a) CRE-driven and (b) SRE-driven luciferase activities in HEK293T cells transfected with BjGPR54L-1 by ZfKiss1-10, ZfKiss2-10, BfKissL-1, BjKissL-2, BfKissL-4-1, and BfKissL-4-2, respectively. The data are expressed as the change in luciferase activity over basal activity. Vertical bars represent the mean ± standard deviation (n = 3). The amino acid sequences of kisspeptins are shown in Supplemental Table 2.

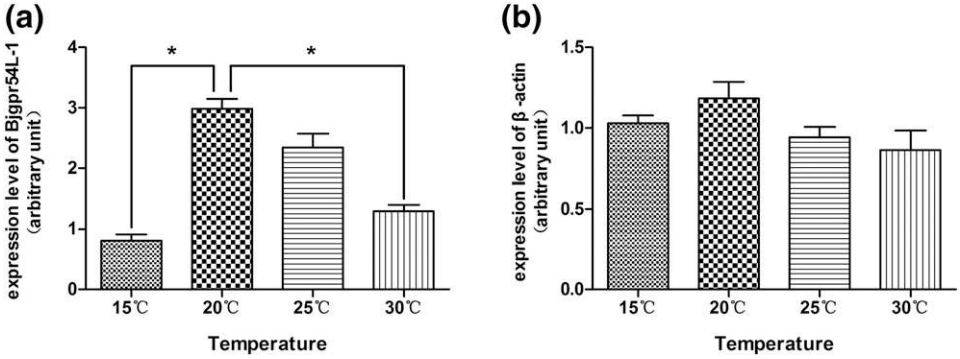

Figure 7. Effects of temperature on the expression of *Bjgpr54L-1* mRNA. The expression of (a) *Bjgpr54L-1* and (b) *β-actin* mRNA was detected by qRT-PCR. Vertical bars represent the mean ± standard deviation (n = 3). Statistically significant differences across control are indicated by asterisk (*$P < 0.05$ vs 20°C).

Interaction of amphioxus Kissr with Kiss

We identified 27 putative GPR54-like in amphioxus *B. floridae* by extensive BLAST survey. To investigate whether they can bind to the putative Kiss ligands, we selected one (*Bjgpr54L-1*) from them to clone from Qingdao amphioxus and performed *CRE* and *SRE* reporter gene assays. It is interesting to note that BjGPR54L-1 exhibited higher preference for two zebrafish Kiss peptides. It has been also reported that human kisspeptin was capable of activating fish GPR54 (52). Our data further confirm the conservation of GPR54 from amphioxus to human. In the case of amphioxus kisspeptin-like peptides, two peptides, BjKissL-2 and BfKissL-4-2, showed functionally interaction with BjGPR54L-1 with different potency, indicating the multiplicity of the ligand and the receptor in amphioxus.

Signaling pathway activated by amphioxus Kissr

In mammals, Kiss1r conveyed its signal *via* the PKC pathway instead of the cyclic adenosine monophosphate/PKA pathway (53, 54), but this is not true for fish Kiss receptors. The ability of fish Kiss to activate the PKA pathway has been identified by several groups (24, 29, 55). In the current study, we found that BjGPR54L-1 could activate both PKC and PKA pathways, supporting the conclusion in fish. In addition, a distinct difference in the post-receptor signaling events evoked by the ligand–receptor interaction was observed. It seems that BjGPR54L-1 is better able to activate the PKC pathway than the PKA pathway because the SRE-driven luciferase activity showed a much higher potency in comparison with CRE-driven luciferase activity.

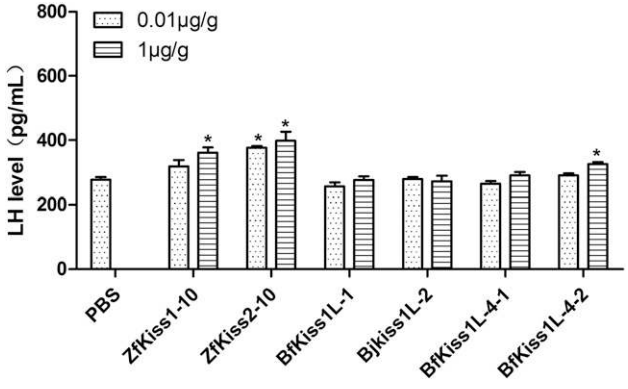

Figure 8. Effects of administration of kisspeptins on LH release. Sexually mature zebrafish was injected with different amounts of kisspeptins. PBS-injected group served as control. Blood samples were collected and serum LH levels were determined. Vertical bars represent the mean ± standard deviation (n = 3). *$P < 0.05$.

Presence of functional Kiss-Kissr system in amphioxus

The functional roles of the Kiss-Kissr system have been well established in mammals and nonmammalian vertebrates (56). In amphioxus *B. floridae*, four putative *Kiss-like* genes encoding 6 kisspeptin-like peptides and 16 putative *Kissr-like* genes (27 were identified in this paper) were reported (38). In this study, we further evaluated the existence of a functional Kiss-Kissr system in amphioxus. First, amphioxus *Kiss* and *gpr54* were mainly expressed in tissues of nerve cord, testes, and ovary. Second, amphioxus Kiss could induce zebrafish LH release and increased the expression of amphioxus *gpb5* mRNA. Third, the expression levels of *BjkissL-2* or *Bjgpr54L-1* genes were downregulated after spermiation or spawning. Therefore, amphioxus Kiss-Kissr system has a correlation with the regulation of reproduction. This is in line with the role of Kiss-Kissr system in vertebrates that kisspeptins influence gonadotropin release. In female zebrafish, Kiss1/2 could significantly induce the expression of FSHβ and LHβ subunits (31). In sea bass, Kiss1/2 could induce LH and FSH release in prepubertal fish (28). In rats, administration of kisspeptin-10 through the tail vein promoted a significant increase of oxytocin (53).

In addition, kisspeptin is a peptide with a diverse and multifunctional nature, involving varied whole body

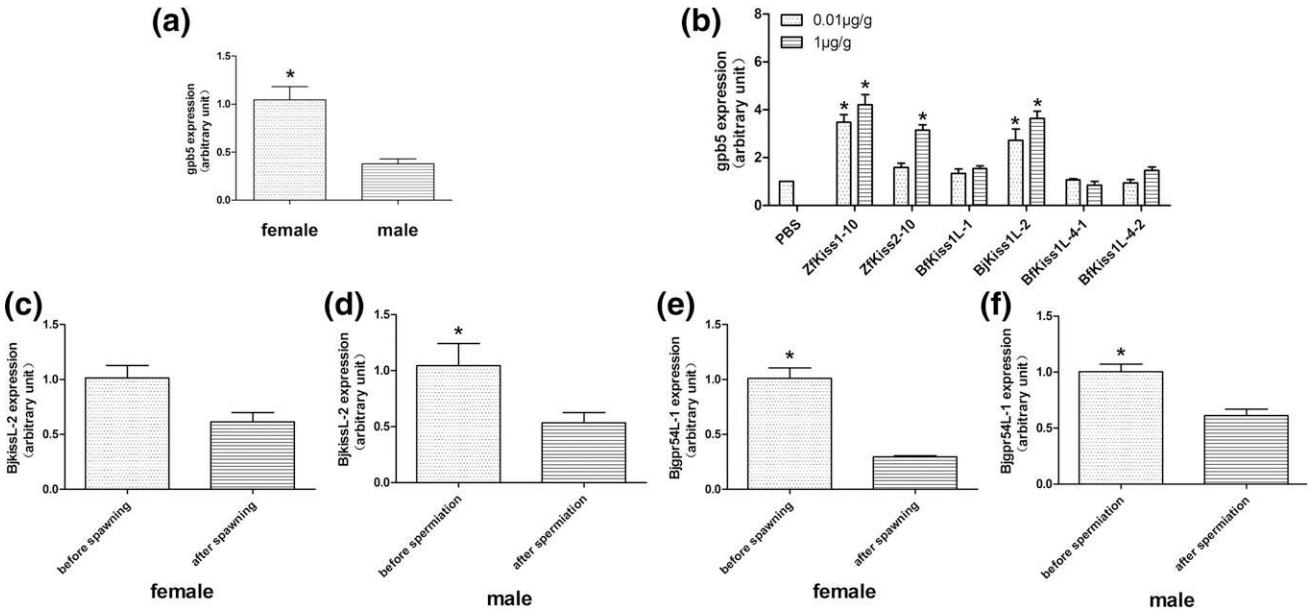

Figure 9. The effects of amphioxus Kiss-Kissr system on the reproduction. (a, b) Effects of administration of kisspeptins on the expression of *gpb5*. (a) The expression of *gpb5* in female and male amphioxus *B. japonicum* detected by qRT-PCR. (b) Sexually mature female *B. japonicum* were injected with different amounts of kisspeptins. PBS-injected group served as control. The expression of *gpb5* was detected by qRT-PCR. (c–f) The comparison of the expression levels of *BjkissL-2* and *Bjgpr54L-1* before and after spermiation (or spawning) in amphioxus *B. japonicum* detected by qRT-PCR. Vertical bars represent the mean ± standard deviation (n = 3). $*P < 0.05$.

physiologic systems and acting at all levels of the reproductive axis: brain, pituitary, gonad, and accessory organs (57). In amphioxus, the Kiss-Kissr system is distributed in many tissues, such as the nerve cord, the Hatschek pit, testis, ovary, gill, and muscle, suggesting that amphioxus Kiss may also act at all levels of the reproductive axis. Other roles unrelated to reproduction would be expected for the amphioxus Kiss-Kissr system as well.

Temperature regulates the *Kissr* expression

In this study, we showed that a low (15°C) or a high (30°C) temperature obviously suppressed the expression of *Bjgpr54L-1* mRNA, which is in line with the regulation of the Kiss2/Kissr2 system during low or high temperature extremes in zebrafish (48). Temperature is a critical factor in regulating reproduction. An increase or decrease in water temperature inhibits or harms reproduction. Our studies support the proposal that kisspeptin is a part of the environmental regulatory factors of reproduction. It is possible that the inhibition on reproduction due to low or high temperature is mediated through the inactivation of the Kiss-Kissr system. This has been substantiated in several models of metabolic stress, known to inhibit reproductive function, where the expression of Kiss1 was also suppressed (58).

This newly discovered role for the kisspeptin system extends our understanding of the relationship between reproduction and energy balance. It may also provide insight into various metabolic diseases. In fact, Tolson *et al.* (25) showed that kisspeptin signaling was an important and novel regulator of body weight, adiposity, metabolism, and glucose homeostasis recently.

Origin and evolution of the HPG axis of vertebrates for controlling reproduction

It is well known that the HPG axis is important in the regulation of reproduction in vertebrates. Kisspeptin plays a major role in the regulation of the reproductive axis by directly stimulating gonadotropin-releasing hormone neurons *via* binding Kissr (59). Qingdao amphioxus has two distinct sexes and breeds once a year. Comparisons of developmental gene expression together with three-dimensional reconstructions from serial transmission electron microscopy have shown that the amphioxus cerebral vesicle was the homolog to the vertebrate brain (34). Our immunohistochemical analysis with antikisspeptin 10 antibody revealed clear reactivity in the cerebral vesicle, supporting the hypothesis. In addition, the Hatschek pit in amphioxus has been considered to be homologous to the vertebrate pituitary gland (35, 36), but Kubokawa *et al.* (60) suggested that the Hatschek pit has different roles from the vertebrate pituitary gland. Recently, a functional GH-like hormone has been demonstrated in amphioxus. It is predominantly distributed in the Hatschek pit and appears to be the member of the vertebrate pituitary hormone family (37). Thus, further studies are needed to decipher the functional homology between amphioxus Hatschek pit and vertebrate pituitary gland. In this paper, we showed that the

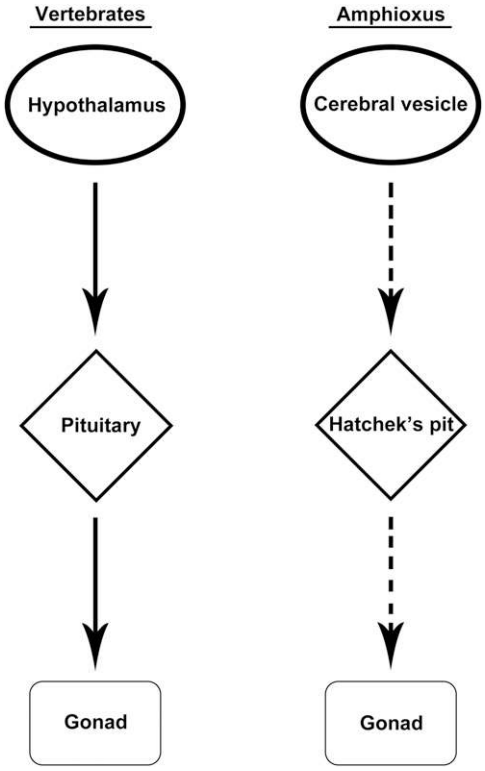

Figure 10. Diagram showing the hypothetical endocrine control system of reproduction in amphioxus and vertebrates. Based on the hypothesis that the amphioxus cerebral vesicle has homologs of most of the features of the vertebrate brain and that amphioxus Hatschek pit is homologous to vertebrate pituitary gland, the system in amphioxus is composed of the cerebral vesicle, Hatschek pit, and the gonad. In contrast, vertebrates have the hypothalamus–pituitary–gonadal axis that controls reproductive functions.

amphioxus Kiss could induce zebrafish LH release. Also, Kiss treatment increased the expression of amphioxus *gpb5*, the β subunit of thyrostimulin. Kubokawa *et al.* (60) have suggested that thyrostimulin is a candidate for the ancestral pituitary hormone, which has diverged into FSH, LH, and thyroid-stimulating hormone in gnathostomes. Therefore, it seems that a vertebrate-like neuroendocrine axis setting has already emerged in amphioxus (Fig. 10).

Conclusions

In summary, this study highlights the existence of a functional Kiss-Kissr system in the basal chordate amphioxus. Kiss-Kissr system has a correlation with the regulation of reproduction in amphioxus, based on the data that amphioxus Kiss can interact with Kissr, induce zebrafish LH release, and increase the expression of amphioxus *gpb5* gene. In addition, we found that the amphioxus-specific *Kiss/Kissr* gene duplication had occurred in the amphioxus clade during evolution and that these genes might represent the ancestral type of the *Kiss/Kissr* genes in chordates. It is highly likely that a vertebrate-like neuroendocrine axis for controlling reproduction has already emerged in amphioxus.

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