Structural and functional multiplicity of the kisspeptin/GPR54 system in goldfish (*Carassius auratus*)

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Abstract

To ascertain the neuroendocrine function of the kisspeptin/GPR54 system in non-mammalian species, full-length cDNAs encoding for Kiss1 and Kiss2 as well as their putative cognate receptors GPR54a and GPR54b, were isolated from goldfish (*Carassius auratus*). The deduced protein sequences between Kiss1 and Kiss2 in goldfish share very low similarity, but their putative mature peptides (kisspeptin-10) are relatively conserved. RT-PCR analysis demonstrated that the goldfish *kiss1* gene (*gfkiss1*) is highly expressed in the optic tectum-thalamus, intestine, kidney, and testis, while the goldfish *kiss2* gene (*gfkiss2*) is mainly detected in the hypothalamus, telencephalon, optic tectum thalamus, adipose tissue, kidney, heart, and gonads. The two receptor genes (*gfgpr54a* and *gfgpr54b*) are highly expressed in the brain regions including telencephalon, optic tectum thalamus, and hypothalamus. Both mature goldfish kisspeptin-10 peptides (gfKiss1–10 and gfKiss2–10) are biologically active as they could functionally interact with the two goldfish receptors expressed in cultured eukaryotic cells to trigger the downstream signaling pathways with different potencies. The actions of gfKiss1–10 and gfKiss2–10 on LH secretion were further investigated *in vitro* and *in vivo*. Intraperitoneal administration of gfKiss1–10 to sexually mature female goldfish could increase the serum LH levels. However, this peptide does not significantly influence LH release from goldfish pituitary cells in primary culture, indicating that the peptide does not exert its actions at the pituitary level. On the other hand, gfKiss2–10 appears to be a much less potent peptide as it exhibits no significant *in vivo* bioactivity and is also inactive on the primary pituitary cells.

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Introduction

Kisspeptin, the product of the *kiss1* gene, was originally identified as a metastasis suppressor in breast cancer and melanoma cell lines (Lee *et al.* 1996, Lee & Welch 1997). In 2003, two independent groups reported that loss-of-function mutations in the G protein-coupled receptor 54 (*gpr54*), the kisspeptin receptor, led to hypogonadotrophic hypogonadism (De Roux *et al.* 2003, Seminara *et al.* 2003). In addition, knockout of either the *kiss1* or *gpr54* gene severely impairs the hypothalamic–pituitary–gonadal axis in mice, suggesting that the kisspeptin/GPR54 system plays an important role in mammalian reproduction (Funes *et al.* 2003, Seminara *et al.* 2003, d' Anglemont de Tassigny *et al.* 2007, Lapatto *et al.* 2007).

In mammals, several mature peptides including kisspeptin-54, -14, -13 and -10 are generated by proteolytic cleavage of the kisspeptin precursor with equal biopotency to activate GPR54 (Kotani *et al.* 2001, Muir *et al.* 2001, Ohtaki *et al.* 2001). Kisspeptin neurons are mainly located in the arcuate nucleus and anteroventral periventricular nucleus (Matsui *et al.* 2004, Irwig *et al.* 2005). These neurons send projections into the preoptic area where *gpr54* is expressed in the GnRH neurons (Matsui *et al.* 2004, Irwig *et al.* 2005). Kisspeptin activates the GnRH neurons to stimulate GnRH release (Gottsch *et al.* 2004, Messager *et al.* 2005, Shahab *et al.* 2005). At very low dose, central administration of kisspeptin markedly elicits LH release in mouse (Gottsch *et al.* 2004, Messager *et al.* 2005), rat (Navarro *et al.* 2004, Irwig *et al.* 2005), sheep (Messager *et al.* 2005) and monkey (Shahab *et al.* 2005, Plant *et al.* 2006). The LH-releasing action of kisspeptin could be eliminated by pretreatment with GnRH antagonists (Gottsch *et al.* 2004, Irwig *et al.* 2005). At the pituitary level, however, the effects of kisspeptin on LH release are controversial. Some studies indicated that kisspeptin could stimulate LH release from the pituitary (Gutierrez-Pascual *et al.* 2007, Suzuki *et al.* 2008), while

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others failed to demonstrate similar effects (Matsui *et al*. 2004, Thompson *et al*. 2004, Smith *et al*. 2008). The discovery of the kisspeptin/GPR54 system has greatly enhanced our understanding of the neuroendocrine regulation of reproduction in various aspects including the molecular timing of puberty onset, the negative and positive feedback control of gonadotropin secretion by sex steroids, the mechanism of sexual dimorphism, the regulation of seasonal reproduction, and the integration of energy homeostasis and reproduction (Kauffman *et al*. 2007, Popa *et al*. 2008).

In lower vertebrates, however, the role and significance of the kisspeptin/GPR54 system in the neuroendocrine regulation of reproduction remains to be established. Recently, two *kiss1* genes, namely *kiss1* and *kiss2*, were identified in zebrafish and medaka (Kitahashi *et al*. 2008) as well as in sea bass (Felip *et al*. 2008). Kiss2 peptide administration could stimulate LH β-subunit and FSH β-subunit mRNA expression in the pituitary of sexually mature female zebrafish (Kitahashi *et al*. 2008). In sea bass, the two kisspeptins were able to induce LH and FSH secretion (Felip *et al*. 2008). In addition, two types of *gpr54s* have been identified in zebrafish (Biran *et al*. 2008). However, a systematic study in a single fish species demonstrating the interplay between the two ligands and the two putative cognate receptors is lacking. Moreover, given that fish consist of evolutionarily divergent species, further studies in different species are highly warranted to reveal other important aspects of kisspeptins on the regulation of fish reproduction. In this study, we have therefore employed goldfish, a recognized model organism for studying the neuroendocrine control of reproduction in lower vertebrates (Popesku *et al*. 2008), to study the role of the kisspeptin/GPR54 system on the neuroendocrine regulation of LH release in fish.

Materials and Methods

Animals and chemicals

Goldfish were obtained from a local fish farm in Guangzhou, China. Tissue samples were collected immediately from decapitated goldfish and snap frozen in liquid nitrogen. All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Sun Yat-Sen University.

Peptides corresponding to goldfish kisspeptins (gfKiss1–10 and gfKiss2–10) and [D-Ala6, Pro9NEt]-LH-releasing hormone (LHRHa) were synthesized by Ningbo Fish Hormone Factory, Zhejiang Province, China. The purity was >95% as determined by analytical HPLC.

Molecular cloning of goldfish kiss1s and GPR54s cDNAs

Total RNA from goldfish brain was prepared using Trizol reagent (Invitrogen). One microgram of isolated RNA was used to synthesize the first-strand cDNA using the ReverTra Ace-α first-strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). Partial cDNA fragments were first obtained by PCR using degenerate primers or gene-specific primers designed according to the predicted sequences. Full-length cDNA sequences were obtained by the RACE using the GeneRacer Kit (Invitrogen). All primers used in the present study are listed in Table 1.

For all PCR reactions in this study, amplifications were performed with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 15 s, 52–58 °C for 15 s and 72 °C for 1–1·5 min. The reaction was ended by a further extension of 10 min at 72 °C. The amplification products were purified using the E.Z.N.A. Gel Extraction Kit (Omega BioTek, GA, USA) and ligated into the pTZ57R/T vector (Fermentas, MD, USA). Three different individual positive clones were sequenced to confirm the sequence information on an ABI 3700 sequencer (Applied Biosystems).

Sequence analysis

The signal peptide and the neuropeptide prohormone cleavage sites were predicted using the SignalP3.0 (Bendtsen *et al*. 2004) and Neuropred software (Southey *et al*. 2006) respectively. Multiple sequence alignments were performed using ClustalW (Thompson *et al*. 1994), and the phylogenetic trees were constructed by MEGA 3.1 using the neighbor-joining method (Kumar *et al*. 2004).

RT-PCR analysis for tissue expression of kiss1s and gpr54s in goldfish

To detect the tissue expression profiles of *kiss1s* and *gpr54s* in goldfish, total RNA from different tissues of goldfish was isolated including telencephalon, optic tectum thalamus, hypothalamus, cerebellum, medulla, pituitary, liver, adipose tissue, intestine, gill, heart, kidney, testis, and ovary. One microgram of total RNA from each tissue was digested with DNase I and reverse-transcribed (RT) into cDNA using the ReverTra Ace-first-strand cDNA Synthesis Kit (Toyobo). Mock RT reactions without the reverse transcriptase were used as negative controls.

Cell culture, transfection, and functional assays

The open reading frame (ORF) of the gfGPR54a and gfGPR54b cDNAs were subcloned into the pcDNA3.1 expression vector (Invitrogen). The COS-7 cell line was obtained from ATCC (Manassas, VA, USA). Cells were maintained at 37 °C in DMEM containing 10% fetal bovine serum (FBS). All media were supplemented with antibiotics (10 U/ml penicillin and 100 μg/ml streptomycin).

Twenty hours before transfection, $1{\cdot}5 \times 10^5$ cells/well were seeded into 24-well tissue-culture plates. Five hundred nanograms of the pSRE-Luc or pCRE-luc reporter plasmid

Table 1 The primers used in the present study

	Sequences (from 5′ to 3′)	**Primer**	**Sequences** (from 5′ to 3′)
Primer			
Primers for gfGPR54b partial cDNA			
GPR54bF1	ACCGATATACTTTTCCTGGT	GPR54bR1	GTGTAGCTGCGGCTCACGTCT
Primers for gfGPR54b, gfKiss1 and gfKiss2 3′ end			
GPR54bF2 (first)	TATGTTCTTTGCTGCTTTCGG	GPR54bF3 (neste)	ATCAGCACACAGAGTCTTCGT
Kiss1F1 (first)	CTACAATCTCAACTCCTTCG	Kiss1F2 (neste)	ACTCCTTCGGCCTCCGCTAT
Kiss2F1 (first)	GCACGCGRAAYTTC AAC	Kiss2F2 (neste)	TTCAACTACAARCCGTTTGG
Primers for gfGPR54b, gfKiss1 and gfKiss2 5′ end			
GPR54bR2 (first)	TCTGGTCCGCACTGCTTCAA	GPR54bR3 (neste)	AACAGCAGCACCATTACAACC
Kiss1R1 (first)	TCCTTTTATTCAGATTGTTTG	Kiss1R2 (neste)	CTAAAGTCTGTGAACTATTGC
Kiss2R1 (first)	ATTACGGACTGAAACAAGCG	Kiss2R2 (neste)	TTACCCACAGTTTTCCATTCT
Primers for ORF of gfGPR54a, gfGPR54b, gfKiss1 and gfKiss2			
GPR54aF1	GAAAAACTGCCTCAGTGAC	GPR54aR1	CGACTAGACAGTTGGCATG
GPR54bF4	CTGAAAGAGGTTATTGCTAG	GPR54bR4	AGCCTTTCCAAAACGCCTAG
Kiss1F3	AATGAAGCTACTTACCATC	Kiss1R1	TCCTTTTATTCAGATTGTTTG
Kiss2F3	CAGCTGTGCTCCATAAGCT	Kiss2R1	ATTACGGACTGAAACAAGCG
Primers for tissue distribution			
GPR54aF2	CCGCCACTAACTTTTACATTG	GPR54aR1	TTCAGAGGATACACAGTCACA
GPR54bF5	TATGTTCTTTGCTGCTTTCGG	GPR54bR5	TCTGGTCCGCACTGCTTCAA
Kiss1F4	ACACAAAAAGGAAGCAGAATG	Kiss1R3	CCTCAACGAACAATACACAAG
Kiss2F4	GCAGTTTGACGAGCCCAGTTC	Kiss2R3	AAATCATCATTGGCAGCAGGT
18S F	AGCAACTTTAGTATACGCTATTGGAG	18S R	CCTGAGAAACGGCTACCACATCC

Mixed bases: Y = G + A; R = C + T.

(Stratagene, La Jolla, CA, USA), 100 ng pcDNA-gfGPR54a or pcDNA-gfGPR54b, and 50 ng pRL-CMV (for normalization of transfection efficiency) containing the *Renilla* luciferase reporter gene were co-transfected into the cells in 250 μl serum-free medium using Lipofectamine reagent (Invitrogen). Six hours after transfection, cells were incubated with vehicle or various (from 10^{-10} to 10^{-6} M) concentrations of gfKiss1–10 or gfKiss2–10 for a further 20 h. Luminescence was measured on a Lumat LB 9501 luminometer (EG & G, Berthold, Germany) and the activities of both luciferases were measured sequentially on the same sample. Transfection experiments were performed in triplicate in three independent experiments. A paralleled control transfection experiment was performed with only pcDNA3.1, cAMP response element (CRE) or serum response element (SRE) promoter and an internal control pRL/CMV.

In vitro actions of goldfish kisspeptins on LH secretion from goldfish pituitary cells in primary culture

Sexually mature female goldfish were anesthetized in 0·05% tricaine methanesulfonate before decapitation. Pituitary was removed and washed three times with Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS). Pituitaries were diced into small pieces of 1 mm^3 dimension, and digested with 1 mg/ml trypsin (Invitrogen) at 25 °C for 60 min. The protease digestion was terminated by 1 mg/ml trypsin inhibitor (Sigma–Aldrich). After further digestion with 25 μg/ml DNase I (Invitrogen), the pituitary cells were then washed with calcium-free HBSS solution containing 1 mM EGTA and filtered through a 100 μm nylon membrane. Pituitary cells were harvested by centrifugation (200 ***g*** for 15 min) and were resuspended in Hanks salt medium 199 (M199). The viability of the cells, tested by the trypan-blue method, was >90%. Cells were seeded at a density of $2{\cdot}5 \times 10^5$ cells/well on poly-L-lysine-coated 24-well dishes in 1 ml M199 containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% FBS. After preincubation at 25 °C for 24 h, the medium was aspirated away and replaced with a fresh medium containing the test peptides (goldfish kisspeptins and LHRHa). The culture media were harvested after incubation for 0·5 and 3 h, and were stored at −80 °C until measurement of LH by RIA.

In vivo effects of goldfish kisspeptins on LH secretion in goldfish

Sexually mature female goldfish, 120–140 g body weight, were kept in indoor tanks supplied with constant water flow. The fish were acclimatized to the environment for 2 weeks and feed on commercially available fish foods without any supplemented hormones. The test peptides were dissolved in a vehicle of 0·7% NaCl. Fish were anesthetized with 0·05% tricaine methanesulfonate and intraperitoneally injected with various doses of the test peptides twice with a 3 h internal. The LHRHa injected group was used as the positive control. Negative control fish were administrated with 0·7% NaCl only. Blood samples were collected from the caudal vessels at 2 and 6 h after the second injection. Serum samples were separated by centrifugation and stored at −80 °C until measurement of LH by RIA.

Hormone measurements

LH levels in the serum samples and culture media were determined using a heterologous RIA as described by Peter *et al.* (1984) and Lin *et al.* (1988) in which the antiserum against the β-subunit of carp LH was used as the antibody and intact carp LH was used as the standard and tracer.

Statistical analysis

All data were expressed as mean values ± S.E.M. Statistical differences were estimated by one-way ANOVA followed by Duncan's multiple range test, and a probability level $<0{\cdot}05$ ($P<0{\cdot}05$) was used to indicate significance. All statistics were performed using SPSS 13.0 (SPSS, Chicago, IL, USA).

Results

Cloning and sequence analysis of kiss1 and kiss2 cDNAs in goldfish

Using the methods described in the Materials and Methods section and the primers described in Table 1, two kiss1 cDNA sequences were cloned from goldfish. They are named gfkiss1 and gfkiss2 (GenBank accession number: FJ465137 and FJ465138 respectively). As shown in Fig. 1, the cDNA of gfkiss1 is 559 bp in length, containing an ORF of 363 bp encoding a precursor protein of 120 amino acids (aa) with a predicted signal peptide of 15 aa. The cDNA of gfkiss2 is 616 bp in length, containing an ORF of 423 bp encoding a precursor protein of 144 aa with a predicted signal peptide of 22 aa.

Sequence comparison among the deduced aa sequences of the vertebrate Kiss1 precursor proteins shows relatively low identity with each other (Fig. 2). However, the mature peptide (Kiss1–10), the putative functional motif, and also the C-terminal cleavage site (GKR or GRR), are well-conserved across vertebrates. The gfKiss1–10 is the same as that of medaka and zebrafish, and differs by only one and two aa with the mouse and human Kiss-10 respectively. Kiss2–10 of goldfish, medaka, and zebrafish are identical as well, and differ only by one aa with that of *Xenopus*, *Takifugu*, *Tetraodon*, and lizard. However, the gfKiss1–10 (YNLNSFGLRY) and gfKiss2–10 (FNYNPFGLRF) differ significantly by four aa with each other. Phylogenetic analysis shows that the Kiss1 sequences are clustered into two separate clades, with Kiss1 in one clade and Kiss2 in the other (Fig. 3A).

Cloning and sequence analysis of GPR54 cDNAs in goldfish

Two full-length GPR54 cDNAs were cloned from goldfish (named gfGPR54a and gfGPR54b; GenBank accession number: FJ465139 and FJ465140). As shown in Fig. 4, both goldfish GPR54 cDNAs consist of an ORF of 1101 bp encoding a protein of 366 aa in length, with an extracellular N-terminus, a seven transmembrane domain

A

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                                                                  AAAA  4
ATGAAGCTACTTACCATCATCTTGATGTTGTCAGTGGCAAATGGTGATCCATACCCTTCAGGCCATTTTCAATATTATTTAGAAGATGAA  94
 M  K  L  L  T  I  I  L  M  L  S  V  A  N  G  D  P  Y  P  S  G  H  F  Q  Y  Y  L  E  D  E  30
ACTCCTAAAGAATCACTCCAGGTTCTCAGGGGAACTGATACTCGTCCCATGGCTGGATCTCCATCTCCCAAGCTCTCAGTGCACTTCTCC 184
 T  P  K  E  S  L  Q  V  L  R  G  T  D  T  R  P  M  A  G  S  P  S  P  K  L  S  V  H  F  S  60
ATGAGTGCAGATCCTCAGCGAAACACACGGTGGTGGGCTCCTGTAAGACCTTACACAAAAAGGAAGCAGAATGTTGCATACTACAATCTC 274
 M  S  A  D  P  Q  R  N  T  R  W  W  A  P  V  R  P  Y  T  K  R  K  Q  N  V  A  Y  Y  N  L  90
AATTCCTTCGGCCTCCGCTATGGAAAGAGAGAGCAGAACATGCTTGCTGAGTTTAAACAGAAGATACCTATGAAGTGAGGACCACTGGGC 364
 N  S  F  G  L  R  Y  G  K  R  E  Q  N  M  L  A  E  F  K  Q  K  I  P  M  K  *              115
CATGCTAAATCTTGTGTATTGTTCGTTGAGGTTTTATATTAATTAGAAATGATTTGTAGATTGATTTCTAGACAAAACATTAGTGCAATA 454
GTTCACAGACTTTAGTAACTGTGAGGACACGTTTTGCTAGTAATAAATACAAACGTTTTGTCAAACAATCTGAATAAAAGGACTTTATTA 544
TAAAAAAAAAAAAAA                                                                            559
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B

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                                                  TTTGTTCACTGAAGAGAGAAAGGCTCAGCTGTGCTCCATAAGCTTTCCAATTCAGCAGC  59
ATGAAAATCAAGGCACTGATTCTCTTCATGTCTGCAATGATCTGTCAGTCCACAGCTTTGAGAGCCTCATTCACTGACATGGATATATCT 149
 M  K  I  K  A  L  I  L  F  M  S  A  M  I  C  Q  S  T  A  L  R  A  S  F  T  D  M  D  I  S  30
GATTCTGAGCCCGTTCCAGACTCCAAGCAGCACTATCTCTCAGTGGAGCGGAGGCAGTTTGACGAGCCCAGTTCTTCAGACGACGCAAGC 239
 D  S  E  P  V  P  D  S  K  Q  H  Y  L  S  V  E  R  R  Q  F  D  E  P  S  S  S  D  D  A  S  60
CTTTGTTTTTTCTTCCAAGAAAAAGACGAATCGACACATATTTCCTGCCAACATCGATTACCACGCGGTAAATTCAACTACAACCCGTTT 329
 L  C  F  F  F  Q  E  K  D  E  S  T  H  I  S  C  Q  H  R  L  P  R  G  K  F  N  Y  N  P  F  90
GGGCTGCGCTTTGGGAAGCGAAATGAAGCGCCAACTGACAGACCCAAACACAAGCACCTGCTGCCAATGATGATTTACCTGAGAAAACAG 419
 G  L  R  F  G  K  R  N  E  A  P  T  D  R  P  K  H  K  H  L  L  P  M  M  I  Y  L  R  K  Q  120
TCAGAAACTACTTGAAAATCACATTTCTGTCAGTCTTCATCAGAATGGAAAACTGTGGGTAATTATTTCAGGCACGCCCTGCCTGTTATT 509
 S  E  T  T  *                                                                              124
ATGCTTTTTTTTTTTATTTCCCAGTCATGGAATTTTATGGCGCGCTTGTTTCAGTCCGTAATTGTATTTGTGCAATAAACGTTTTATTTTG 599
TTTGAAAAAAAAAAAAA                                                                          616
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Figure 1 The nucleotide sequences and the deduced amino acid sequences of goldfish Kiss1 (panel A) and Kiss2 (panel B). The putative signal peptides are underlined. The mature peptides (kisspeptin-10) are boxed.

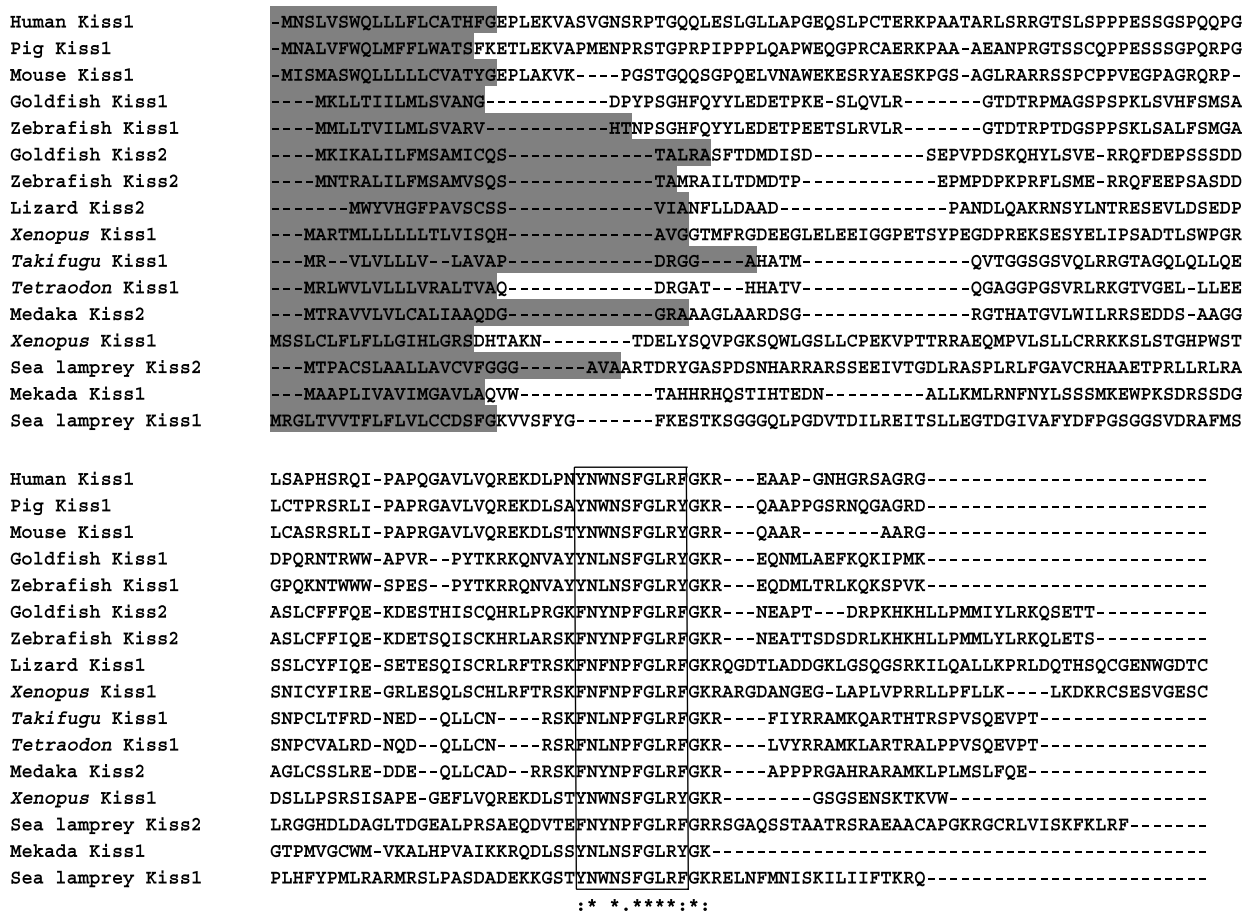

Figure 2 Comparison of amino acid sequences of kisspeptin precursors from different species. The signal peptides are shaded in grey and the mature peptides are boxed. Sequences were aligned by the ClustalW program. Identical sequences are indicated by asterisks. Gaps (indicated by hyphens) are introduced in some sequences to maximize alignment.

and a cytoplasmic C-terminus. The two gfGPR54s share only 55·6% identity in aa sequence with each other. In the phylogenetic analysis (Fig. 3B), it can be seen that gfGPR54a and gfGPR54b are clustered into two clearly separate groups.

Tissue distribution of two kiss1s and two gpr54s in goldfish

Using gene-specific primers (Table 1) designed from the cloned sequences, RT-PCR analysis was performed to examine the tissue distribution patterns of the two *gfkiss1* and *gfgpr54* genes. As shown in Fig. 5, *gfkiss1* and *gfkiss2* exhibit rather different expression patterns in the central and peripheral tissues. The *gfkiss1* is highly expressed in optic tectum thalamus, intestine, kidney, and testis, with slightly lower levels in hypothalamus and liver. The *gfkiss2* is observed in almost all tissues examined except the cerebellum, with significant expression in the hypothalamus, telencephalon, optic tectum thalamus, adipose tissue, kidney, heart, and gonads.

In the brain regions, high expression of both *gfgpr54a* and *gfgpr54b* could be detected. In peripheral tissues, *gfgpr54a* is expressed exclusively in the gonads and adipose tissue, whereas *gfgpr54b* is expressed in all peripheral tissues examined.

Goldfish kisspeptins functionally interact with goldfish GPR54s expressed on cultured eukaryotic cells

To further characterize the ligand–receptor interactions of the two kisspeptins and the two GPR54s cloned from goldfish, CRE, and SRE reporter gene assays were performed. Cells transfected with the empty vector exhibited no response to the kisspeptins treatment (data no shown). For the CRE promoter, gfKiss1–10 could trigger similar potencies via activating both receptors. For the CRE promoter activity induced by gfKiss2–10, no significant post-receptor signaling could be detected in cells transfected with gfGPR54a but a dose-dependent increase was clearly observed in the gfGPR54b-expressing cells (Fig. 6A and B).

For the SRE promoter, gfKiss1–10 could trigger the post-receptor signaling pathway in a clearly dose-dependent manner in cells expressing gfGPR54a but possesses no significant effect at all on cells expressing gfGPR54b. On the other hand, gfKiss2–10 exhibits low potency in activating the SRE promoter in cells transfected with gfGPR54a, but causes a marked increase in the SRE-driven promoter activity in cells expressing gfGPR54b (Fig. 6C and D).

A

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                GAAAAACTGCCTCAGTGACTAGCATGAATTTTATGAAAAAACACCAGATTCTAAAGGCGTATTCTTTAAATTAATTG 77
GATTGGTGGGCATGGGGACTTTGTCACTGCCAGAAAGAAGAAACTTGACTTTTTTCATCCTGTTTCAAGGTCTCTGCCAAGTCTAAAAAT 167
ATGTTTCCAAGTGAAGATTGGAACTCAAGTGAGCTGCTTAACAGCTCCATTGGAAACTCCTCTATGGAGGACACGGAGGACGAGGAGCAC 257
 M  F  P  S  E  D  W  N  S  S  E  L  L  N  S  S  I  G  N  S  S  M  E  D  T  E  D  E  E  H  30
CCCTTCCTGACGGATGCTTGGCTGGTGCCCCTTTTCTTCTCCCTCATCATGCTAGTGGGGCTTATCGGAAACTCACTGGTGATCTATGTC 347
 P  F  L  T  D  A  W  L  V  P  L  F  F  S  L  I  M  L  V  G  L  I  G  N  S  L  V  I  Y  V  60
ATCTCCAAGCACAGACAGATGAGGACCGCCACTAACTTTTACATTGCCAACTTGGCTGCCACTGACATCATTTTCCTGCTGTGCTGCGTG 437
 I  S  K  H  R  Q  M  R  T  A  T  N  F  Y  I  A  N  L  A  A  T  D  I  I  F  L  L  C  C  V  90
CCGTTCACTGCTACCCTCTACCCTCTGCCTGGCTGGATATTTGGGGACTTCATGTGCAAATTTGTTGCTTTTCTCCAACAGGTGACCGTA 527
 P  F  T  A  T  L  Y  P  L  P  G  W  I  F  G  D  F  M  C  K  F  V  A  F  L  Q  Q  V  T  V  120
CAGGCGACGTGCATCACTCTTACGGCAATGAGTGGAGACCGTTGCTATGTGACTGTGTATCCTCTGAAATCCCTGCACCACCGAACCCCT 617
 Q  A  T  C  I  T  L  T  A  M  S  G  D  R  C  Y  V  T  V  Y  P  L  K  S  L  H  H  R  T  P  150
CGTGTCGCAATGATTGTTAGCATCTGTATCTGGATCGGTTCCTTCATCCTTTCCATACCAATCTTCCTGTACCAGAGGCTTGAGGATGGC 707
 R  V  A  M  I  V  S  I  C  I  W  I  G  S  F  I  L  S  I  P  I  F  L  Y  Q  R  L  E  D  G  180
TTTTGGTATGGACCAAGAAAATACTGCATGGAGAGGTTTCCATCAAAGACCCACGAGAAAGCTTTCATCCTCTATCAGTTCATAGCCGTA 797
 F  W  Y  G  P  R  K  Y  C  M  E  R  F  P  S  K  T  H  E  K  A  F  I  L  Y  Q  F  I  A  V  210
TATCTACTGCCTGTCATTACCATCTCCTTCTGTTATTCCTTCATGCTGAAGAGAGTGGGACAAGCCTCTGTGGAACCAGTGGATAACAAC 887
 Y  L  P  V  I  T  I  S  F  C  Y  S  F  M  L  K  R  V  G  Q  A  S  V  E  P  V  D  N  N  240
CATCAGGTCCACCTGCTCTCAGAGAGAACTATCTCCATTAGGAGTAAGATTTCCAAAATGGTAGTGGTCATTGTTGTTCTCTTCACCATC 977
 H  Q  V  H  L  L  S  E  R  T  I  S  I  R  S  K  I  S  K  M  V  V  V  I  V  V  L  F  T  I  270
TGCTGGGGTCCCATTCAGATCTTTGTCCTGTTCCAGTCTTTCTATCCCAGCTTCAAAGCCAACTACACAACATATAAGATCAAGACATGG 1067
 C  W  G  P  I  Q  I  F  V  L  F  Q  S  F  Y  P  S  F  K  A  N  Y  T  T  Y  K  I  K  T  W  300
GCCAACTGCATGTCCTATGCCAACTCCTCTATCAACCCTATTGTCTACGGTTTTATGGGCGCCAGCTTCCGCAAGTCCTTCAGGAAGACC 1157
 A  N  C  M  S  Y  A  N  S  S  I  N  P  I  V  Y  G  F  M  G  A  S  F  R  K  S  F  R  K  T  330
TTTCCCTTCCTCTTCAGACACAAAGTGAGAGACAGCAGTGTCGCCTCCCGCACGGCCAATGCAGAAATAAAGTTCGTAGCAACGGAGGAG 1247
 F  P  F  L  F  R  H  K  V  R  D  S  S  V  A  S  R  T  A  N  A  E  I  K  F  V  A  T  E  E  360
AGCAACACTGAGAGGAAATGAGAGCGGATGACACCTCAACCATGAATCCATGCCAACTGTCTAGTCG                        1317
 S  N  T  E  R  K  *                                                                       366
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B

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 M  A  E  S  N  R  T  T  E  V  A  E  L  I  L  C  N  N  E  A  N  I  Y  D  C  N  Q  S  D  P  30
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 M  G  S  Q  S  P  V  P  L  T  D  A  W  L  V  P  V  F  F  I  L  I  L  F  V  G  L  V  G  N  60
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 F  L  S  S  G  P  *                                                                       366
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Actions of goldfish kisspeptins on LH release

The *in vitro* action of the two goldfish kisspeptins on LH secretion were examined in primary culture of pituitary cells prepared from mature female goldfish. As shown in Fig. 7, neither gfKiss1–10 nor gfKiss2–10 could significantly stimulate LH release at any dose after 0·5 or 3 h of incubation with the peptides, while the positive control LHRHa significantly increased LH release at 3 h of incubation.

The *in vivo* action of the two goldfish kisspeptins on LH release was also investigated. As shown in Fig. 8, peripheral administration of gfKiss1–10 significantly increased serum LH levels in a dose-dependent manner. At 2 h after the second injection, all tested doses of gfKiss1–10 significantly increased serum LH levels in the sexually mature female goldfish (Fig. 8A). At 6 h post-injection, the stimulatory effect was persistent in fish injected with high doses (0·1 and 1·0 μg/g body weight) of gfKiss1–10. On the other hand, there was no obvious effect of gfKiss2–10 on serum LH levels at all doses of the peptide used and at all sampling times (Fig. 8B). The positive control of LHRHa treatment elicited a significant increase in serum LH levels at all time points tested.

Discussion

In the present study, two kiss1 and two GPR54 sequences were cloned and functionally evaluated in goldfish. Based on synteny analysis, it is proposed that the two *kiss1* genes in fish originated from the same ancestor gene (Felip *et al.* 2008). Interestingly, deletion of either the *kiss1* or *gpr54* gene in mouse or mutation of the *gpr54* gene in human resulted in uncompensated impairment of the reproductive axis (Funes *et al.* 2003, Seminara *et al.* 2003, d' Anglemont de Tassigny *et al.* 2007, Lapatto *et al.* 2007), indicating that there is no functional redundancy in the kisspeptin/GPR54 system in mammals. A genome-wide scan revealed that only *kiss2* and one type of *gpr54* are present in the *Takifugu rubripes* (Fugu), *Tetraodon nigroviridis* (pufferfish), and *Anolis carolinensis* (green anole lizard) genomes, and it seems that chicken has lost both *kiss1s* and *gpr54s*. These observations might be interpreted as the co-evolution of ligand/receptor pairs (Moyle *et al.* 1994). The loss of either the ligand or the receptor would lead to functional redundancy of its partner, thus both the ligand and the receptor would be lost at last.

Although the two goldfish kisspeptins possess low similarity in primary structure, they still share some features in common such as the relatively well-conserved functional mature peptide regions (Kiss1–10) and C-terminal amide motif. Phylogenetic analysis revealed that gfKiss1 and gfKiss2 are clustered into two separate branches. Both gfGPR54s contain the NPxxY in the TMH7 and the DRY motif, suggesting that they belong to the rhodposin-like GPCR family (Schwartz *et al.* 2006). Two goldfish GPR54s share high aa sequence identities in the transmembrane regions, but showed rather low similarities in the extracellular N-terminus and the C-terminal tail.

The expression of the goldfish *kiss1*s and *gpr54*s were observed in the neuronal and reproductive related tissues, including the brain, pituitary, and gonads. Nevertheless, the two *gfkiss1* showed rather different expression patterns in the reproduction related tissues, suggesting that the two peptides might play different roles in the fish reproductive system. On the other hand, both *gfgpr54a* and *gfgpr54b* are widely expressed in the brain regions, indicating the potential involvement of goldfish kisspeptins in neural functions. In addition, the low expression level of *gfkiss1*s and *gfgpr54*s in the pituitary is consistent with the lack of *in vitro* actions of the peptides on the pituitary cells. Moreover, the goldfish *kiss1*s and *gpr54*s mRNA transcripts were also detected in other peripheral tissues, but their physiological significance remains unclear.

Several studies have demonstrated the functional region of kisspeptin, Kiss1–10, transduces its activity via the protein kinase C and protein kinase A pathways (Stafford *et al.* 2002, Biran *et al.* 2008, Moon *et al.* 2009). In the present study, we have examined the ligand specificity of the two goldfish kisspeptins on the two goldfish GPR54s functionally expressed on cultured eukaryotic cells. It is interesting to note that there are distinct differences in the ligand selectivity exhibited by the two gfGPR54s. The gfGPR54a shows high potency to gfKiss1–10 activation compared with the gfGPR54b at high dose, while the gfGPR54b exhibits higher preference for gfKiss2–10. Distinct differences in the post-receptor signaling events evoked by the ligand-receptor interactions can be observed. It appears that gfKiss1–10

Figure 3 (A) Phylogenetic analysis of kisspeptin precursors in vertebrates. The phylogenetic tree was constructed by MEGA 3.1 using the neighbor-joining method with 1000 bootstrap replicates. The number shown at each branch indicates the bootstrap value (%). GenBank accession numbers for the sequence are: human Kiss1 (NP_002247); pig Kiss1 (ACH68409.1); mouse Kiss1 (AAI17047); zebrafish Kiss1 (ABV03802), *Xenopus* Kiss2 (BX850386), zebrafish Kiss2 (AB439561), medaka Kiss2 (AB439562). Sequences predicted from Ensemble: sea lamprey Kiss2 (Contig Contig37453.1 at location 1700–6241); *Xenopus* Kiss1 (on scaffold_608 at location 39 460–69 716); lizard Kiss2 (on scaffold_15 at location 4 601 534–4 601 935); sea lamprey Kiss1, *Takifugu*, and *Tetraodon* Kiss2 sequences were previously predicted by van Aerle *et al.* (2008). (B) Phylogenetic analysis of GPR54 sequences in vertebrates. Phylogenetic tree was performed using MEGA 3.1 by performing using the neighbor-joining method with 1000 bootstrap replicates. The number shown at each branch indicates the bootstrap value (%). GenBank accession numbers for the sequences are: human GPR54 (AAK83235); rhesus monkey GPR54 (AAV70982); pig GPR54 (ABE73452); dog GPR54 (XP_855198); platypus duckbill GPR54 (XP_001515272); mouse GPR54 (AAK83236); rat GPR54 (AAD19664); grey short-tailed opossum GPR54 (XP_001374752); bull frog GPR54 (ACD44939); tilapia GPR54 (BAD34454); zebrafish GPR54a (ABV44612); zebrafish GPR54b (ABV44613); cobia GPR54 (ABG82165); Atlantic croaker GPR54 (ABC75101); grey mullet GPR54 (ABG76790); senegalese sole (ABW96362). GPR54 predicted by Ensemble: *Takifugu* GPR54 (ENSTRUG00000013755); medaka GPR54a (ENSORLG00000017731); medaka GPR54b (ENSORLG00000 001694); lizard GPR54 (on scaffold_10 at location 6 382 042–6 393 152).

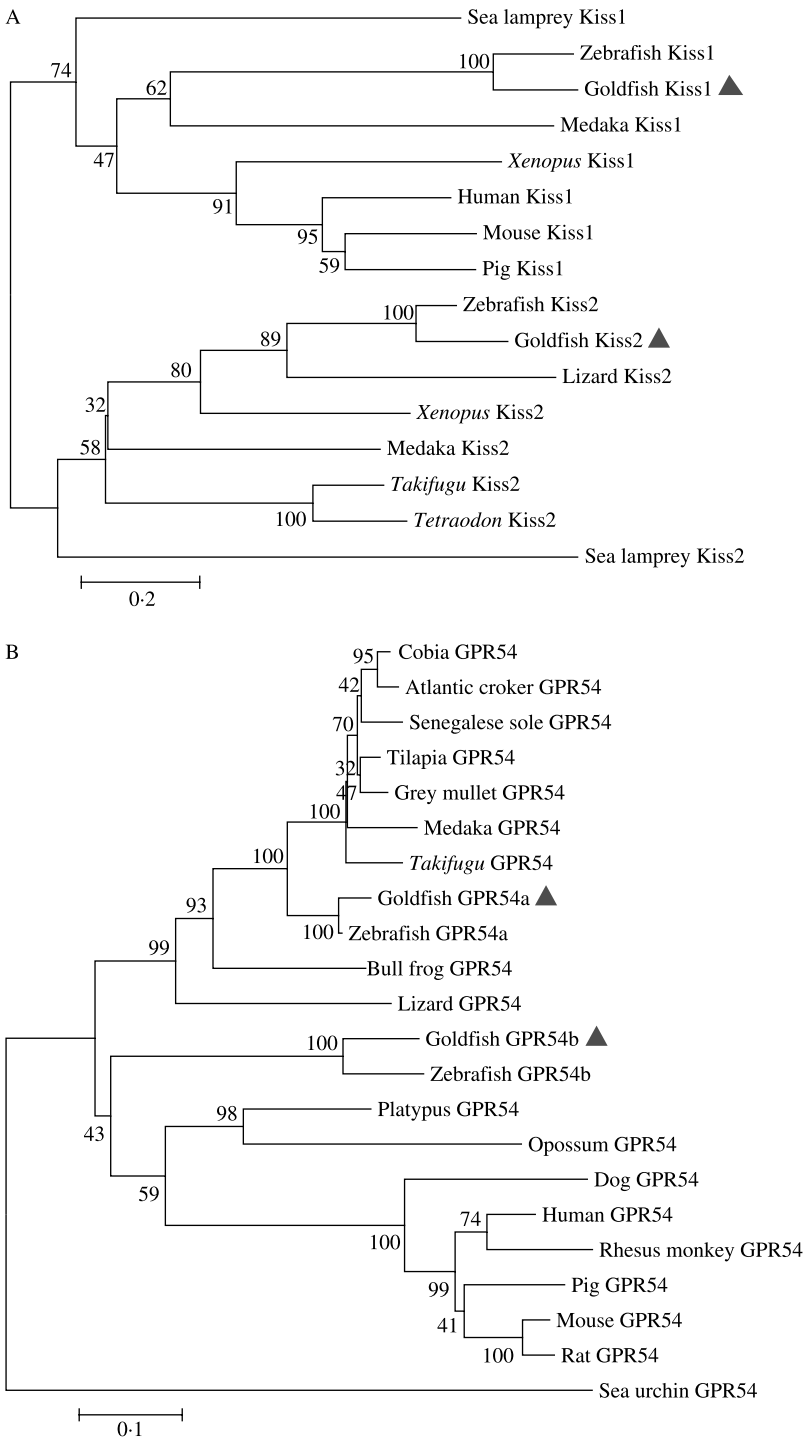

Figure 4 The nucleotide sequences and the deduced amino acid sequences of goldfish GPR54a (A) and GPR54b (B). The transmembrane regions are underlined.

cannot differentiate the two receptors by CRE signaling, whereas the gfKiss2–10 can activate CRE signaling of the gfGPR54b only, but not the gfGPR54a. On the other hand, a completely different picture is observed for the SRE signaling. The gfKiss1–10 can only activate the SRE signaling pathway of gfGPR54a but not gfGPR54b, while the preference for the two receptors is reversed for gfKiss2–10. The multiplicity of the ligands and the cognate receptors of

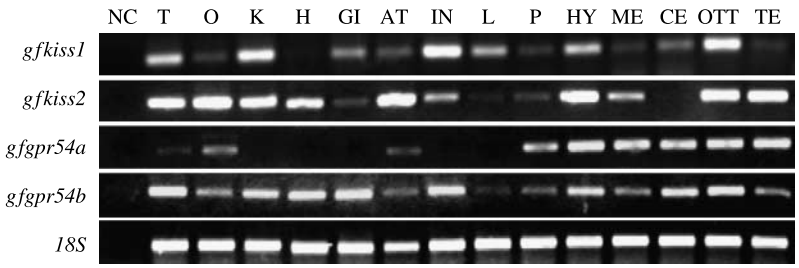

Figure 5 RT-PCR analysis of tissue expression patterns of *kiss1*, *kiss2*, *gpr54a*, *gpr54b* in goldfish. Amplification of *18S* was used as the house-keeping gene control. TE, telencephalon; OTT, optic tectum thalamus; CE, cerebellum; ME, medulla; HY, hypothalamus; P, pituitary; L, liver; IN, intestine; AT, adipose tissue; GI, gill; H, heart; K, kidney; O, ovary; T, testis; NC, negative control.

the kisspeptin/GPR54 system in goldfish do appear to provide additional levels of subtleties in mediating the actions of these peptides through the receptors.

In mammals, kisspeptin plays a central role in controlling reproductive activities by stimulating gonadotropin release which is mediated by increasing hypothalamic GnRH release (Kauffman *et al.* 2007, Popa *et al.* 2008). However, it is not known whether kisspeptin in fish serves the same role as their mammalian counterparts on the reproductive axis. In the present study, *in vivo* administration of synthetic gfKiss1–10 potently stimulated LH release in mature female goldfish, indicating that the stimulatory action of kisspeptin on LH release is conserved from mammals to fish. On the other hand, there is no stimulatory effect on LH secretion in cultured pituitary cells from mature female goldfish, suggesting that Kiss1–10 does not act at the pituitary level to exert its physiological effect. In tilapia, *gpr54* was expressed in GnRH neurons (Parhar *et al.* 2004), affording the important proof for the direct regulation of GnRH secretion by fish kisspeptin. In our studies, it was observed that both the goldfish *kiss1*s and *gpr54*s are abundantly expressed in the central nervous system, especially in the hypothalamus. Taken together, these results suggest that the increase of serum LH levels observed *in vivo* after gfKiss1–10 administration is probably caused by increase of GnRH release in the hypothalamus. The *in vivo* potencies of the two goldfish kisspeptins are very different with no significant LH-release activities observed for the gfKiss2–10. The differences in the *in vivo* effect of goldfish kisspeptins on LH release might be caused by their sequence variations in the functional region. The gfKiss1–10 and gfKiss2–10 differ with each other by four aa. Thus, the two *kiss1* genes in goldfish might acquire different functions during the long time of evolution. Despite that Kiss2–10 might lose its ability to elicit LH release in goldfish, several observations indicated that it might play other important functions, e.g., the preservation of the gene as well as the functional motif of Kiss2–10 during the long evolutionary history; the ability of gfKiss2–10 to activate the gfGPR54 functionally expressed on cultured eukaryotic cells with a clear preference towards gfGPR54a; and the existence of only *kiss2* in some species (Felip *et al.* 2008). During the preparation of this manuscript, Kitahashi *et al.* (2008) reported that zebrafish Kiss1–10 had no effect on the gene expression of gonadotropins (*lhb* and *fshb*), but zebrafish Kiss2 peptide significantly increased the *lhb* and *fshb* mRNA levels in the pituitary of sexually mature zebrafish after peripheral administration. And more recently, Alicia Felip *et al.* (2008) reported that Kiss-2 peptide is more potent than Kiss-1 in inducing gonadotropin secretion in sea bass. Taken together,

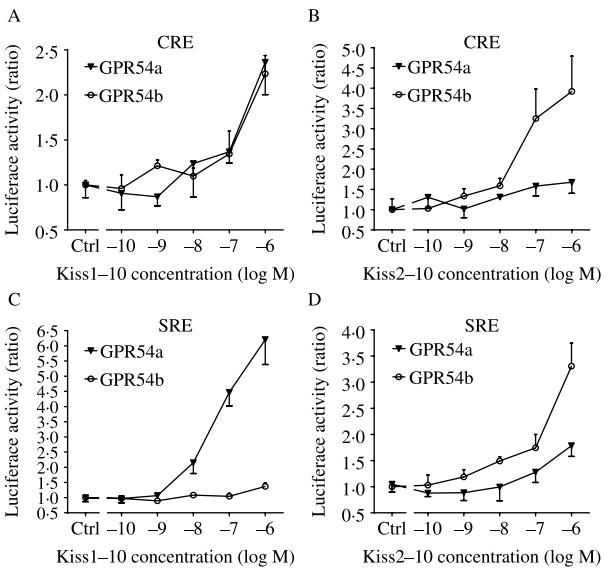

Figure 6 Functional interaction between goldfish kisspeptins and goldfish GPR54s. Induction of CRE-driven luciferase activities in COS-7 cells transfected with GPR54a or GPR54b by Kiss1–10 (A) and Kiss2–10 (B). Induction of SRE-driven luciferase activities in COS-7 cells transfected with GPR54a or GPR54b by Kiss1–10 (C) and Kiss2–10 (D). Results are mean values ± S.E.M. from three independent experiments each conducted in triplicates, and expressed as the ratio of increase in luciferase activity above the control (in the absence of any stimulating peptide).

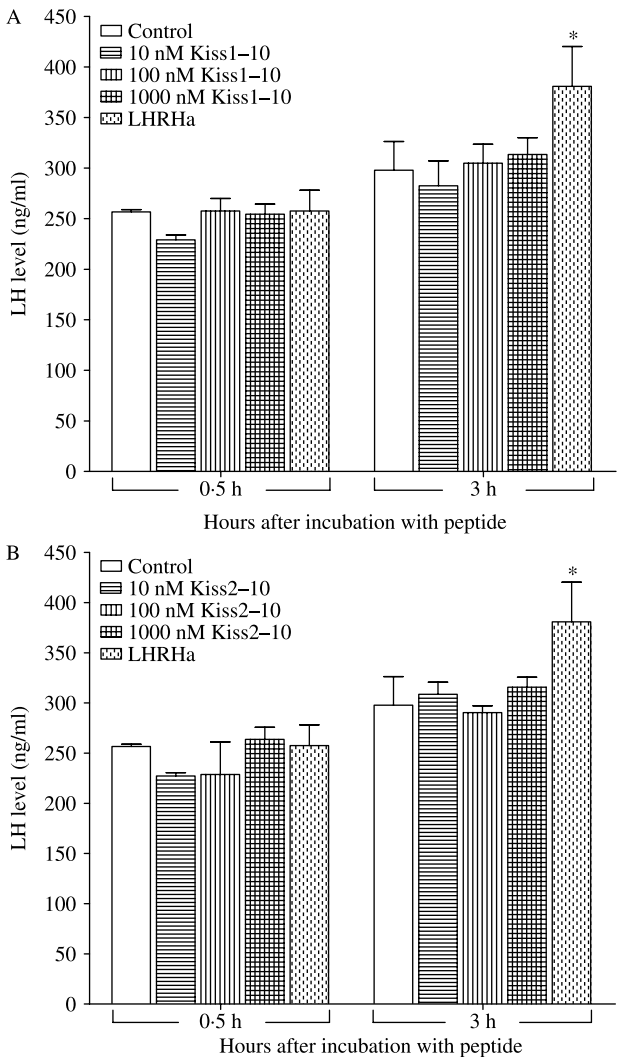

Figure 7 *In vitro* action of goldfish Kiss1–10 (A) and Kiss2–10 (B) on LH release in goldfish pituitary cells. Primary culture of goldfish pituitary cells were challenged with different concentrations of the goldfish kisspeptins and 10^{-8} M of LHRHa was used as the positive control. The cell culture media were harvested at 30 and 180 min, and LH levels were determined. Hormone values are expressed as mean values ± S.E.M. ($n=4$). *$P<0{\cdot}05$ versus the corresponding control.

these results suggest that the two kisspeptins in teleost fish have evolved differentially and the relative importance of the two peptides in regulating gonadotropin secretion varies greatly in different species.

In conclusion, we have cloned two kiss1 and two gpr54 cDNA sequences from goldfish, and demonstrated that the two goldfish kisspeptins possess different features in terms of their primary structure, expression profile, receptor subtype preference, and signaling pathway specificity. And for the first time, we have demonstrated that gfKiss1–10 stimulates LH release *in vivo* in goldfish. These results provide strong evidence for the structural and functional conservation of the kisspeptin/GPR54 system in the regulation of the reproductive axis across vertebrates. The multiplicity of the peptides and the receptors in telesot fish provide the basis for the subtle differences of this system in various fish species. The elucidation of the kisspeptin/GPR54 system in a fish species where the neuroendocrine control of reproduction is well studied paves the way for further evaluating the significance and the detailed mechanisms of how this system controls reproduction.

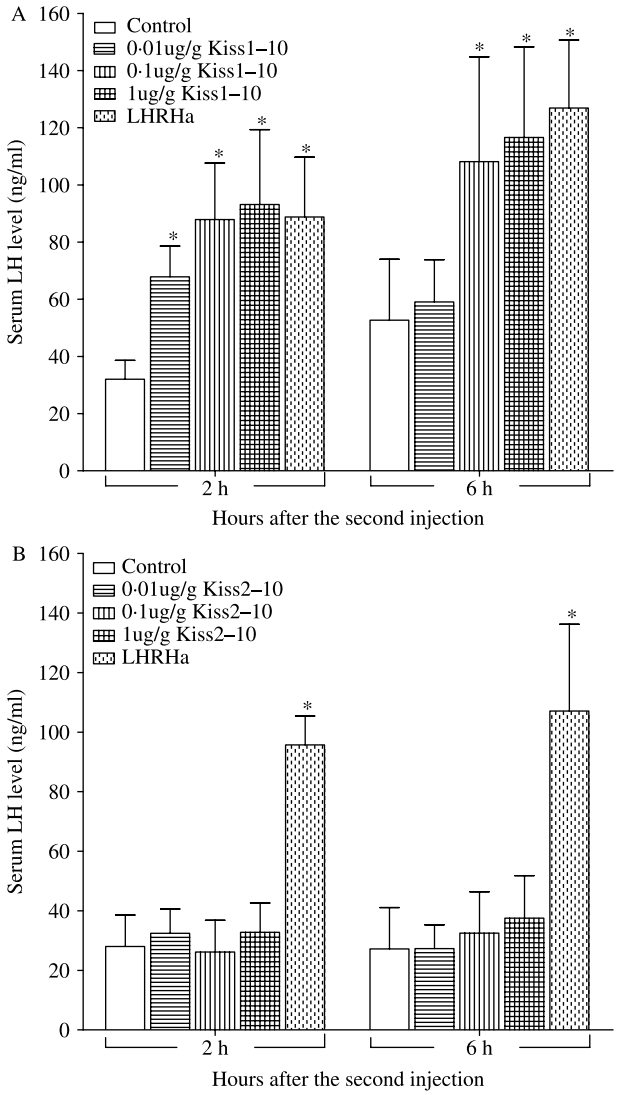

Figure 8 *In vivo* action of goldfish Kiss1–10 (A) and Kiss2–10 (B) on LH release in goldfish. Goldfish were injected i.p. with different amounts of goldfish kisspeptins and 10^{-8} ng/g body weight of LHRHa was used as the positive control. Blood samples were collected and serum LH levels were determined 2 and 6 h post injection. Hormone values are expressed as mean values ± S.E.M. ($n=7$–9). *$P<0{\cdot}05$ versus the corresponding control.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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