Molecular Evolution of Kiss2 Genes and Peptides in Vertebrates

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The kiss1 peptide (kisspeptin), a product of the *kiss1* gene, is one of the key neuropeptides regulating vertebrate reproduction. In 2009, we identified a paralogous gene of *kiss1* in the brain of amphibians and named it *kiss2.* Currently, the presence of the *kiss2* gene and the kiss2 peptide is still obscure in amniotes compared with that in other vertebrates. Therefore, we performed genome database analyses in primates and reptiles to investigate the molecular evolution of the *kiss2* gene in vertebrates. Because the mature kiss2 peptide has been identified only in amphibians, we further performed immunoaffinity purification and mass spectrometry to identify the mature endogenous kiss2 peptide in the brains of salmon and turtle that possessed the *kiss2* gene. Here we provide the first evidence for the presence of a *kiss2*-like gene in the genome database of primates including humans. Synthetic amidated human KISS2 peptide activated human GPR54 expressed in COS7 cells, but nonamidated KISS2 peptide was inactive. The endogenous amidated kiss2 peptide may not be produced in primates because of the lack of an amidation signal in the precursor polypeptide. The *kiss2*-like gene may be nonfunctional in crocodilians because of premature stop codons. We identified the mature amidated kiss2 peptide in turtles and fish and analyzed the localization of kiss2 peptide mRNA expression in fish. The present study suggests that the *kiss2* gene may have mutated in primates and crocodilians and been lost in birds during the course of evolution. In contrast, the *kiss2* gene and mature kiss2 peptide are present in turtles and fish. **(*Endocrinology* 154: 4270–4280, 2013)**

The kiss1 peptide (kisspeptin), a product of the *kiss1* gene, belongs to the RFamide or RYamide peptide family that possesses a C-terminal motif of Arg-Phe-NH_2 (RFa) or Arg-Tyr-NH_2 (RYa) (for reviews, see Refs. 1–4). Kiss1 peptide has a stimulatory effect on GnRH neurons via its receptor, G protein–coupled receptor 54 (GPR54), causing up-regulation of the hypothalamo-pituitary-gonadal axis (for reviews, see Refs. 5–13). The *KISS1* gene was originally identified as a human gene that suppresses metastases of human melanomas and breast carcinomas (14). In 2001, the KISS1 peptide was identified as a mature endogenous ligand at various lengths (54-, 14- and 13-amino acid peptides) in human placenta (15, 16). The KISS1 peptide is also called metastin because of its suppressive effects on tumor growth and tumor metastasis (16). In 2003, it was clear for the first time that human KISS1 peptide and GPR54 are involved in the control of reproduction. The loss of function of GPR54 was correlated with the hypogonadotropic hypogonadism in human, and that phenomenon was confirmed by using *Gpr54* gene knockout mice (17, 18). The C-terminal 10 amino acid portion of kiss1 peptide (kisspeptin-10) shows high homology in fish, amphibians, and mammals, which is considered to be the minimal sequence required for the

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Abbreviations: DIG, digoxigenin; GPR54, G protein–coupled receptor; h, human; MALDI TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NRL, nucleus recessus lateralis; POA, preoptic area; RACE, rapid amplification of cDNA ends; SRE, serum response element.

activation of kissr1 (gpr54) in mammals (15, 16). Therefore, kisspeptin-10 is often highlighted and used for functional analyses (19–21).

Recently, we identified a gene encoding a novel RFa peptide and its mature endogenous ligand as a 12-amino acid peptide in the brain of amphibians (22). The C-terminal 10 amino acids of the identified peptide showed a high sequence similarity with that of kisspeptin-10, and thus we named this RFa peptide kiss2 peptide (22). From the synteny analysis, the *kiss2* gene was considered to be a paralogous gene of the *kiss1* gene (22, 23). Genome database analyses suggested that the *kiss2* gene also exists not only in amphibians but also in agnathans, fish, and reptiles (only lizards) (22–25). However, it is still not known whether the *kiss2* gene is present in most reptiles (eg, turtles or crocodiles) and mammals except for platypus, a primitive mammalian species (for a review, see Ref. 26). Therefore, we performed genome database analyses in salmon, reptiles, and primates to investigate the molecular evolution of the *kiss2* gene in vertebrates. The mature endogenous kiss2 peptide has been identified only in the brain of amphibians (22). Therefore, we further sought to identify the mature endogenous kiss2 peptide in the brains of 1 salmon and 1 turtle species that possessed the *kiss2* gene.

Materials and Methods

Animals

Adult masu salmon (*Oncorhynchus masou masou*) and adult kokanee salmon (*Oncorhynchus nerka*; landlocked sockeye salmon) were reared in well water at constant temperature (9–10°C) at the Freshwater Fisheries Research Division, National Research Institute of Fisheries Science (Nikko, Tochigi Prefecture, Japan). Juvenile red-eared slider turtles (*Trachemys scripta elegans*) were purchased from commercial dealers in Japan. All animals were killed by cervical transection. After decapitation, tissues were collected, quickly frozen in liquid nitrogen, and kept at −80°C. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Waseda University and Utsunomiya University.

RNA preparation

Total RNA was extracted from the brains of masu salmon, kokanee salmon, and red-eared slider turtle using Sepasol-RNA I Super (Nacalai Tesque) in accordance with the manufacturer's instructions.

Molecular cloning

All PCR amplifications were performed in a reaction mixture containing Prime STAR HS DNA polymerase (TAKARA BIO Inc) or Bio *Taq* polymerase (Bioline) and 0.2 mM deoxynucleotide triphosphate on a thermal cycler (Program Temp Control System PC-700; Astec). First-strand cDNA was synthesized with the oligo(dT)-anchor primer supplied in the 5′/3′ rapid amplification of cDNA ends (RACE) kit (Roche Diagnostics). All primers used in this study are summarized in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. For 3′ RACE, the first round PCR products were amplified with the anchor primer (Roche Diagnostics) and the first degenerate primers (Kiss2-DG1) corresponding to the conserved amino acid sequence of the kiss2 peptide, Asn^{6}-Pro^{7}-Phe^{8}-Gly^{9}-Leu^{10}-Arg^{11}-Phe^{12}. The first-round PCR products were further amplified with the second degenerate primers (Kiss2-DG2), corresponding to the conserved amino acid sequence of the kiss2 peptide, Lys^{2}-Phe^{3}-Asn^{4}-Phe^{5}-Asn^{6}-Pro^{7}-Phe^{8}-Gly^{9}. All PCRs consisted of 5 cycles for 30 seconds at 94°C, 30 seconds at 45°C, and 1 minute at 72°C and of 30 cycles for 30 seconds at 94°C, 30 seconds at 50°C, and 1 minute at 72°C (10 minutes for the last cycle). For 5′ RACE, template cDNAs were synthesized with the gene-specific primers named MS-AS1 for masu salmon, KS-AS1 for kokanee salmon, and RS-AS1 for red-eared slider turtle, respectively (Supplemental Table 1). These cDNA syntheses were followed by dA-tailing of the cDNA with dATP and terminal transferase (Roche Diagnostics). The poly(A)–tailed cDNAs were amplified with the oligo(dT)-anchor primer (Roche Diagnostics) and gene-specific primers named MS-AS2 for masu salmon, KS-AS2 for kokanee salmon, and RS-AS2 for red-eared slider turtle, respectively (Supplemental Table 1). These were followed by further amplification of the first-round PCR products with the anchor primer and gene-specific primers named MS-AS3 for masu salmon, KS-AS3 for kokanee salmon, and RS-AS3 for red-eared slider turtle, respectively (Supplemental Table 1). Both the first- and second-round PCRs were performed for 30 cycles consisting of 1 minute at 94°C, 30 seconds at 55°C, and 1 minute at 72°C (10 minutes for the last cycle). The second-round PCR products were subcloned into a pGEM-T Easy vector in accordance with the manufacturer's instructions (Promega). The DNA inserts of the positive clones were amplified by PCR with universal M13 primers.

DNA sequencing

All nucleotide sequences were determined with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and a model 3130 Genetic Analyzer (Applied Biosystems). Universal M13 primers or gene-specific primers were used to sequence both strands. All nucleotide sequences were analyzed by using DNA Sequencing Analysis Software v5.1 (Applied Biosystems). SignalP 3.0 (27, 28) (http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptide sequences.

Peptide extraction and immunoaffinity purification

The brains of adult masu salmon ($n = 100$) were collected, immediately frozen on dry ice, and stored at −80°C until use. Brains were boiled for 7 minutes and homogenized in 5% acetic acid as described previously (29, 30). The homogenate was centrifuged at 10,000 × *g* for 30 minutes at 4°C. The resulting pellet was again homogenized and centrifuged. The pooled supernatants were concentrated using a rotary evaporator at 45°C. The concentrated supernatant mixture was then passed through a disposable C-18 cartridge column (Mega Bond Elut; Varian, Inc). The retained material was eluted with 60% methanol and loaded onto an immunoaffinity column as described previously

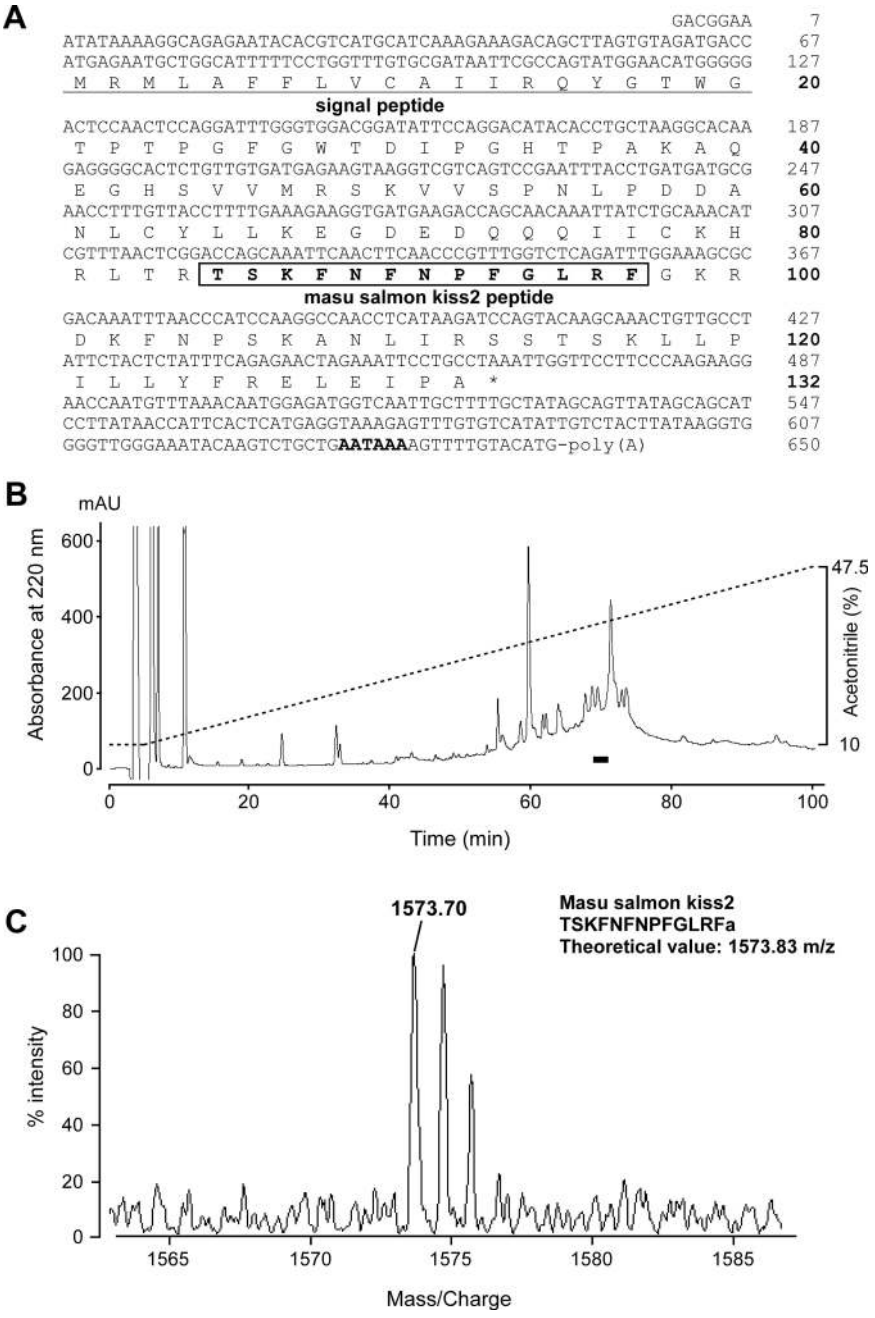

Figure 1. A, Nucleotide sequence and deduced amino acid sequence of the masu salmon kiss2 peptide precursor cDNA. The sequence of masu salmon kiss2 peptide is boxed. The signal peptide (20 amino acids) is underlined. The poly(A) adenylation signal AATAAA is shown in bold. B, Purification of native masu salmon kiss2 peptide. The HPLC profile of the retained material on a reverse-phase HPLC column (ODS-80Tm) is shown. The retained material loaded onto the column was eluted with a linear gradient of 10% to 50% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min for 100 minutes and collected in 50 fractions of 1 mL each. Aliquots (1/100 volume) of each fraction were evaporated to dryness, dissolved in distilled water, and spotted onto a nitrocellulose membrane. Immunoreactive fractions were eluted with 36.4% to 37.2% acetonitrile and are indicated by the horizontal bar. mAU, milli-absorbance units. C, Chromatogram of MALDI-TOF MS of native masu salmon kiss2 peptide. The immunoreactive material in the extract of masu salmon brain using antiserum against kiss2 peptide showed a molecular ion peak of 1573.70 *m/z* ($[M + H]^+$). This value was almost identical to the calculated mass of the predicted masu salmon kiss2 peptide (TSKFNFNPFGLRFa) [1573.83 *m/z* ($[M + H]^+$)].

(29, 30). Antiserum against the C-terminal sequence of the *Xenopus laevis* kiss2 peptide (SKFNFNPFGLRFa) was conjugated to cyanogen bromide–activated Sepharose 4B (GE Healthcare Bio-Sciences Corp) as an affinity ligand. The brain extract was applied to the column at 4°C, and the adsorbed materials were eluted with 0.3 M acetic acid containing 0.1% 2-mercaptoethanol. An aliquot of each fraction (1 mL) was analyzed by a dot immunoblot assay with the antiserum against kiss2 peptide. The peptide extraction and immunoaffinity purification using the brains of juvenile red-eared slider turtle ($n = 200$) were also conducted by the same method as described above.

HPLC and structure determination

Immunoreactive fractions were loaded onto an HPLC column (ODS-80Tm; Tosoh Corp) and eluted with a linear gradient of 10% to 50% acetonitrile containing 0.1% trifluoroacetic acid over 100 minutes at a flow rate of 0.5 mL/min. The eluted fractions were collected every 2 minutes and assayed by immunoblotting. For masu salmon, fractions corresponding to the elution time of 69 to 71 minutes showed intense immunoreactivity. For red-eared slider turtle, fractions corresponding to the elution time of 58 to 62 minutes showed intense immunoreactivities. These immunoreactive fractions were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (AXIMA-CFR plus; Shimadzu Corp).

In situ hybridization

The expression of masu salmon and red-eared slider turtle kiss2 peptide precursor mRNA in the brain was localized by in situ hybridization. Salmon were anesthetized in 0.06% 2-phenoxyethanol and perfused by 4% paraformaldehyde in 0.1 M phosphate buffer. The dissected brains were immersed in refrigerated 4% paraformaldehyde in 0.1 M phosphate buffer for about 24 hours. Turtles were killed by decapitation after being anesthetized by injection of ethyl *m*-aminobenzoate methanesulfonate (MS222). Brains were rapidly collected and immersed in refrigerated 4% paraformaldehyde in 0.1 M phosphate buffer for about 24 hours. Subsequently, the brains of turtles and salmons were soaked in a refrigerated sucrose solution (30% sucrose in PBS) until they sank. They were embedded in OCT compound (Miles Inc) and sectioned frontally at

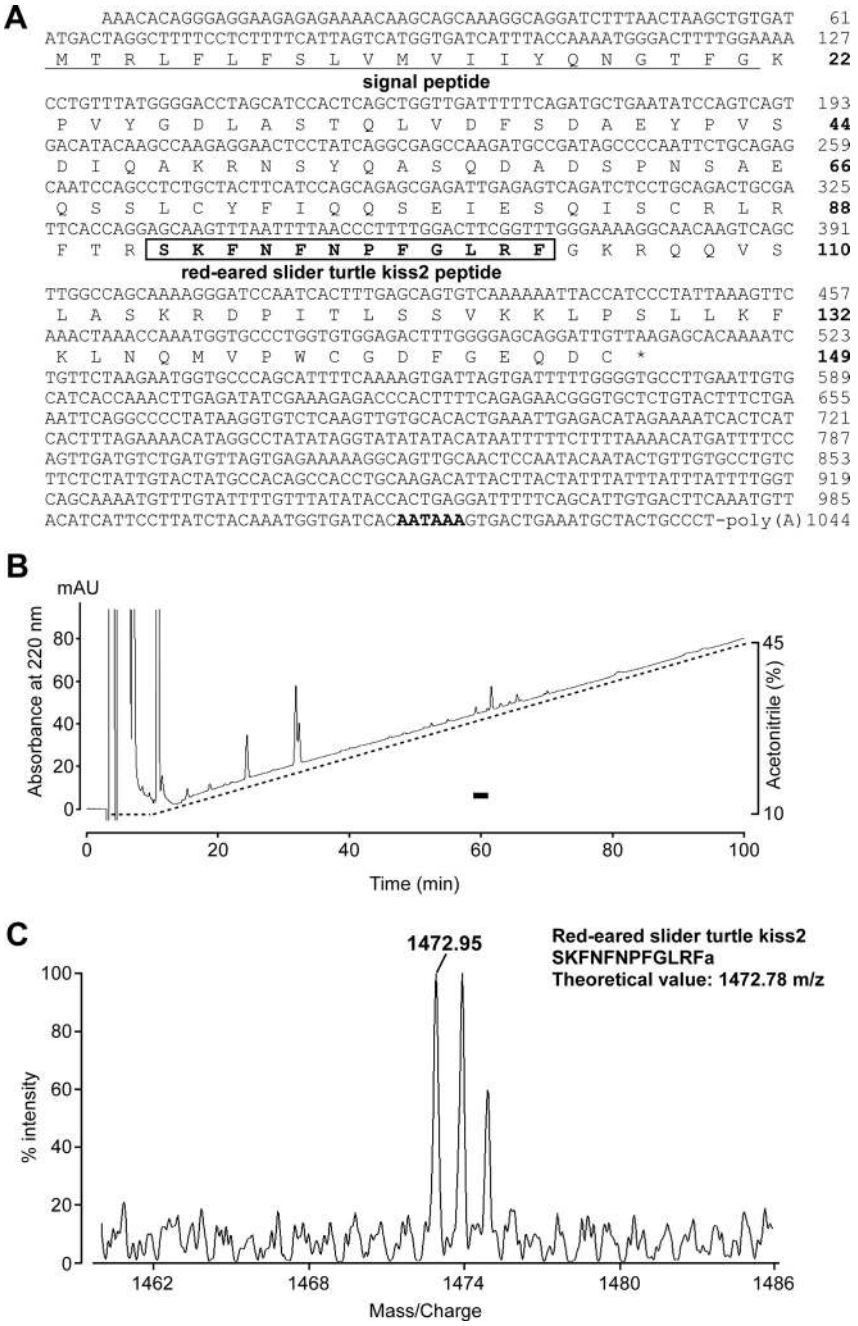

Figure 2. A, Nucleotide sequence and deduced amino acid sequence of the red-eared slider turtle kiss2 peptide precursor cDNA. The sequence of red-eared slider turtle kiss2 peptide is boxed. The signal peptide (21 amino acids) is underlined. The poly(A) adenylation signal AATAAA is shown in bold. B, Purification of native red-eared slider turtle kiss2 peptide. The HPLC profile of the retained material on a reverse-phase HPLC column (ODS-80Tm) is shown. The retained material loaded onto the column was eluted with a linear gradient of 10% to 50% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min for 100 minutes and collected in 50 fractions of 1 mL each. Aliquots (1/100 volume) of each fraction were evaporated to dryness, dissolved in distilled water, and spotted onto a nitrocellulose membrane. Immunoreactive fractions were eluted with 29.2% to 30.8% acetonitrile and are indicated by the horizontal bar. mAU, milli-absorbance units. C, Chromatogram of MALDI-TOF MS of native red-eared slider turtle kiss2 peptide. The immunoreactive material in the extract of turtle brain using antiserum against kiss2 peptide showed a molecular ion peak of 1472.95 *m/z* ($[M + H]^+$). This value was almost identical to the calculated mass of the predicted red-eared slider turtle kiss2 peptide (SKFNFNPFGLRFa) [1472.78 *m/z* ($[M + H]^+$)].

a 10-μm thickness with a cryostat at −20°C. The sections were placed onto MAS-coated slides (Matsunami Glass Ind., Ltd). In situ hybridization was performed according to our previous method (29, 30) using a digoxigenin (DIG)-labeled antisense RNA probe. The DIG-labeled antisense RNA probe was produced with an RNA labeling kit (Roche Diagnostics) from the part of the peptide precursor cDNA (complementary to nucleotides 419–644 for masu salmon and nucleotides 352–643 for red-eared slider turtle). Control for specificity of the in situ hybridization of kiss2 peptide precursor mRNA was performed by using the DIG-labeled sense RNA probe, which was complementary to a common sequence of the antisense probe sequence.

Transient transfection and luciferase assay

The human (h) KISS1 peptide (hKISS1; YNWNSFGLRFa), C-terminal amidated KISS2 peptide (hKISS2-NH_2; AFKFNFGLRFa), and C-terminal free KISS2 peptide (hKISS2-free; AFKFNFGLRFS) used for luciferase assays were synthesized with a peptide synthesizer (PSSM-8; Shimadzu). COS7 cells were maintained in DMEM (GIBCO/Invitrogen) supplemented with high glucose (4.5 g/L) containing 10% fetal bovine serum (GIBCO/Invitrogen) and 1% penicillin/streptomycin antibiotics (GIBCO/Invitrogen) in a humidified 5% CO_2 atmosphere at 37°C. For luciferase assays, COS7 cells were plated in 24-well plates and grown to 70% to 80% confluence for 24 hours before transfection. Cells were then cotransfected with 200 ng of hGPR54 cDNA (OriGene Technologies Inc), 100 ng of the pGL4.33[luc2P/SRE/Hygro] (firefly luciferase reporter construct; Promega) and 5 ng of pRL-null (*Renilla* luciferase reporter construct; Promega) using X-treme GENE 9 DNA Transfection Reagent (Roche) according to the manufacturer's instructions. Cells were starved overnight in serum-free DMEM and then challenged by kisspeptins (hKISS1, hKISS2-free, and hKISS2-NH_2) for 6 hours. Cell extracts were prepared, and luciferase activity was measured using the dual-luciferase reporter assay system (Promega). The ratio of firefly luciferase activity to *Renilla* luciferase activity was used in the results to coordinate the dif-

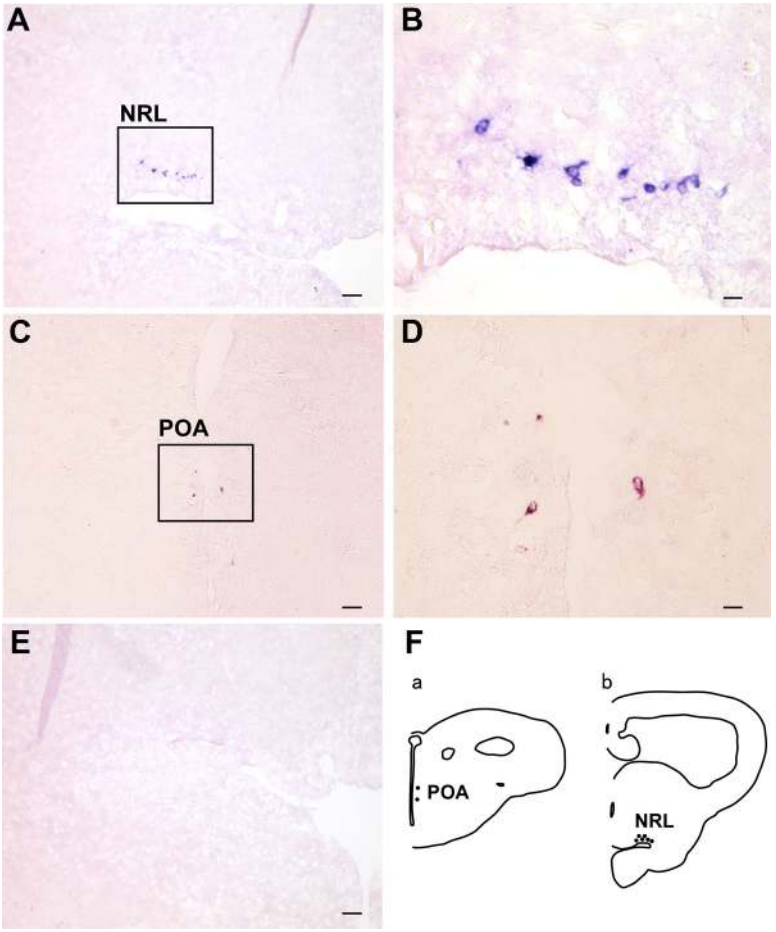

Figure 3. Cellular localization of masu salmon kiss2 precursor mRNA in the transverse brain sections. Masu salmon kiss2 peptide precursor mRNA was expressed in the POA (A) and NRL (C). The rectangular area in A or C is magnified in B or D. E, The sense RNA probe produced no hybridization signal. F, Schematic representation of the distribution of masu salmon kiss2 mRNA. Masu salmon kiss2-expressing cell bodies in the POA (a) and NRL (b) are shown by closed circles. Scale bars correspond to 60 μm (A, C, and E) and 15 μm (B and D).

ferences in transfection efficiency among samples. All assays were performed in duplicate and repeated 3 times. Statistical significance was assessed by Prism statistical software (GraphPad Software Inc).

Bioinformatics analyses

The Ensembl genome database (http://www.ensembl.org/index.html) was used to search the *kiss2* gene in primates, mammals, birds, and reptiles by using the TBLASTN program. BLAST+ (version 2.2.26+) (31) was used on the local computer to search the *kiss2* gene in the genome sequences of American alligator (*Alligator mississippiensis*) and salt-water crocodile (*Crocodylus porosus*) that were provided by the crocodilian genomics consortium (32; http://crocgenomes.org/index.html). GENSCAN (33) (http://genes.mit.edu/GENSCAN.html) was used to predict exons of *kiss2* gene. We also performed the synteny analysis by comparing the gene loci of the *kiss2* gene in primates, birds, reptiles, and fish with the Ensembl genome database and crocodilian genome data. Multiple sequence alignments of the precursors of the kiss2 peptide were performed by using ClustalW 1.83 (European Molecular Biology Laboratory). The data for multiple sequence alignments were displayed by the GeneDoc program (34). SignalP 3.0 (27, 28; http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptide sequences.

Results

Characterization of the cDNA encoding kiss2 peptide precursor and its mature peptide in the brain of fish

The cDNA sequence and deduced amino acid sequence of the kiss2 peptide precursor of masu salmon are shown in Figure 1A. This precursor may be translated from Met^1, because hydropathy plot analysis of the precursor demonstrated that the most hydrophobic moiety, which is typical in a signal peptide region, followed Met^1 (27, 28). The cleavage site of the signal peptide in the precursor may be the Gly^{20}-Thr^{21} bond according to the −3, −1 rule (Figure 1A) (35). The predicted signal peptide sequence was also supported by SignalP 3.0 (27, 28). In addition, We identified a *kiss2* cDNA in kokanee salmon that is identical to that in masu salmon except that Phe^6 is changed to Val^6 (Supplemental Figure 1). Immunoaffinity purification and HPLC purification were conducted to purify the mature endogenous kiss2 peptide in masu salmon. Fractions corresponding to an elution time of 69 to 71 minutes for masu salmon showed intense immunoreactivities (Figure 1B). These fractions were examined by MALDI-TOF MS. A molecular ion peak in the spectrum of the substance on the MALDI-TOF MS was observed at 1573.70 *m/z* ($[M + H]^+$) in masu salmon (Figure 1C). This value was close to the theoretical mass number at 1573.83 *m/z* ($[M + H]^+$) in masu salmon calculated for the deduced amidated peptide sequence. These analyses indicated that the mature masu salmon kiss2 peptide is a 13-amino acid sequence (TSKFNFNPFGLRFa) with amidated C termini.

Characterization of the cDNA encoding kiss2 peptide precursor and its mature peptide in the brain of reptiles

The cDNA sequence and deduced amino acid sequence of kiss2 peptide precursor of red-eared slider turtle are

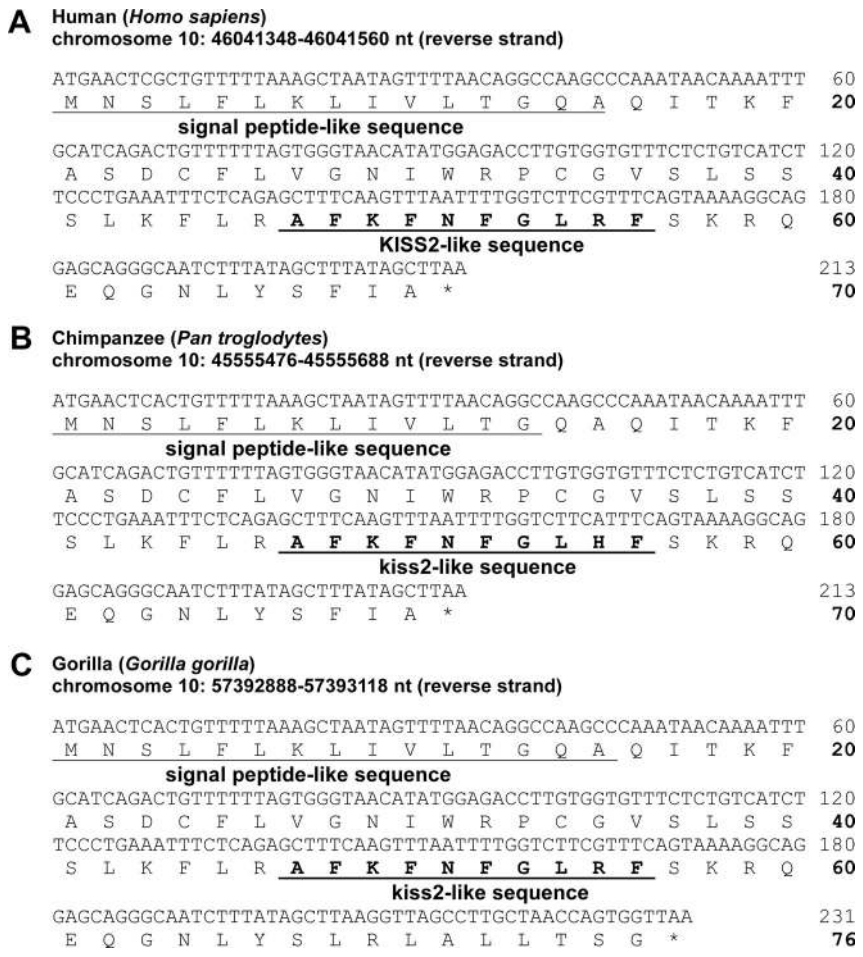

Figure 4. Nucleotide sequences and deduced amino acid sequences of human (A), chimpanzee (B), and gorilla (C) encoding a kiss2 peptide-like sequence obtained from the Ensembl genome database. Each kiss2 peptide-like sequence is underlined. Each signal peptide-like sequence is also underlined.

shown in Figure 2A. An initiating methionine was predicted as described above. The cleavage site of the signal peptide in the precursor may be the Gly^{19}-Lys^{20} bond according to the −3, −1 rule (Figure 2A) (35). The predicted signal peptide sequence was also supported by SignalP 3.0 (27, 28). The mature endogenous kiss2 peptide was purified as described above. Fractions corresponding to an elution time of 58 to 62 minutes for red-eared slider turtle showed intense immunoreactivities (Figure 2B). These fractions were examined by MALDI-TOF MS. A molecular ion peak in the spectrum of the substance on the MALDI-TOF MS was observed at 1472.95 *m/z* ($[M + H]^+$) in red-eared slider turtle (Figure 2C). This value was close to the theoretical mass number at 1472.78 *m/z* ($[M + H]^+$) in red-eared slider turtle calculated for the deduced amidated peptide sequence. These analyses indicated that the mature red-eared slider turtle kiss2 peptide is a 12-amino acid sequence (SKFNFNPFGLRFa) with amidated C termini.

Cellular localization of the kiss2 peptide precursor mRNA in the brain

The expression of masu salmon kiss2 peptide precursor mRNA was detected mainly in the nucleus recessus lateralis (NRL) in the hypothalamus (Figure 3, A and B). Small numbers of neurons expressing kiss2 peptide precursor mRNA were also found in the preoptic area (POA) (Figure 3, C and D). The control study using the sense RNA probe resulted in a complete absence of staining (Figure 3E). The distribution of neurons expressing masu salmon kiss2 mRNA is summarized in Figure 3F.

Database search for *kiss2* gene from fish to primates

We found that a signal peptide-like motif and a kiss2-like amino acid sequence are encoded in the genome sequence of primates, such as human, chimpanzee, and gorilla (Figure 4). In confirmation of other reports (23, 25, 26), we could not find kiss2-like amino acid sequences from other mammalian species except for platypus. We also found that a kiss2-like amino acid sequence is encoded in the genome sequence of American alligator and salt-water crocodile (Supplemental Figure 2). However, clear exon composition was not obtained in American alligator and salt-water crocodile by using GENSCAN analysis. Multiple sequence alignment of kiss2 precursor polypeptides showed that the amino acid sequence homology was high in the peptide coding region in fish, amphibians, and reptiles (Figure 5). Newly discovered primate kiss2 precursors encoded a kiss2-like amino acid sequence; however, glycine next to the C-terminal phenylalanine that is essential for the C-terminal amidation of the mature kiss2 peptide was not conserved (Figures 4 and 5). In addition, the second amino acid from the C-terminal of the chimpanzee kiss2 peptide was histidine (Figures 4 and 5). The signal peptide sequence of the primate *kiss2*-like gene is not in a complete form (Figure 4). The *kiss2*-like gene of primates has no introns in contrast to *kiss2* genes of other vertebrates (Figure 4). In confirmation of other reports (23, 25, 26), we could not find

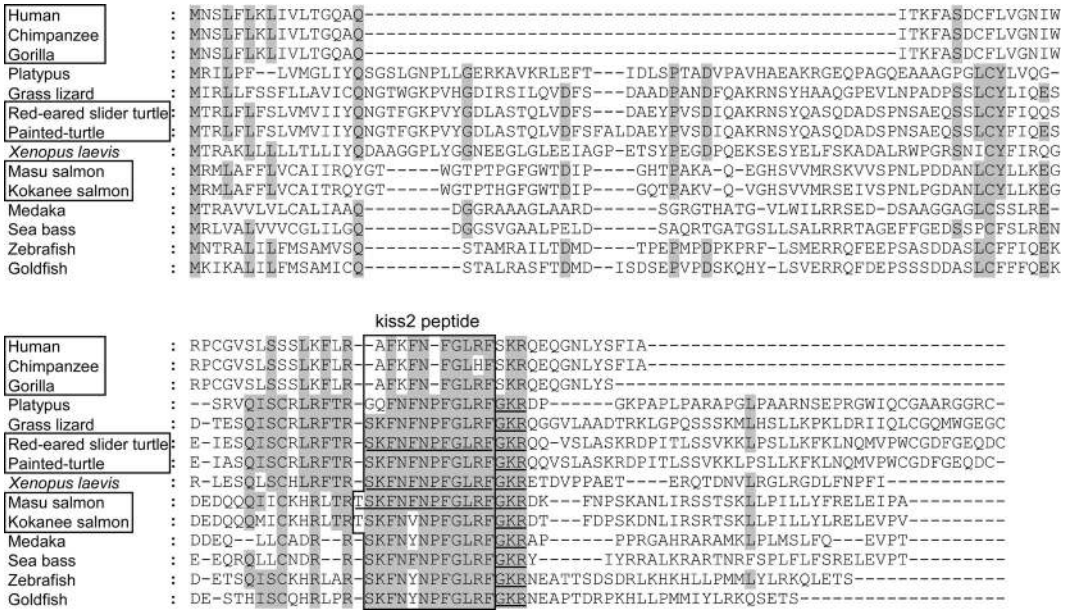

Figure 5. Multiple amino acid sequence alignments of the precursors of kiss2 peptide. The conserved amino acids are shaded. The kiss2 peptide coding region is boxed. Kiss2 peptide sequences that were identified as mature endogenous ligands are underlined. The C-terminal cleavage sites and amidation motifs (GKR) are also underlined. Gaps marked by hyphens were inserted to optimize homology. The names of animals that are boxed indicate that the kiss2 peptide precursor sequences of these animals are identified in the present study.

a *kiss2*-like gene in the genome database of chicken, zebra finch, and budgerigar.

Bioactivity of the human KISS2 peptide

Because humans lack GPR54–2 (25, 26), we used GPR54 expressed in COS7 cells to analyze the bioactivity of the human KISS2 peptide. The serum response element (SRE)-luciferase reporter system was used to examine the effect of the human KISS2 peptide on the phospholipase C/protein kinase C pathway because human GPR54 couples with $G\alpha_{q/11}$ (36). The C-terminal amidated human KISS2 peptide induced an approximately 3.5-fold increase in luciferase activity over basal at 10^{-6} M, which is probably not physiological (Figure 6). On the other hand, C-terminal free human KISS2 peptide did not induce luciferase activity (Figure 6). Human KISS1 that is used for a positive control induced an approximately 9-fold increase in luciferase activity over basal at both 10^{-7} and 10^{-6} M (Figure 6).

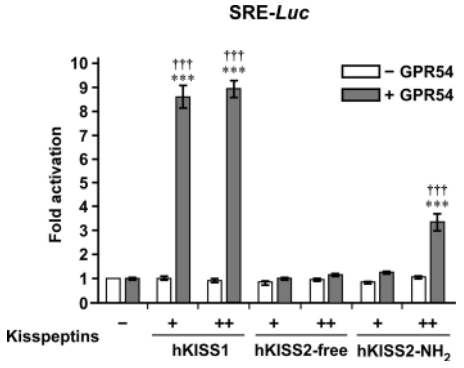

Figure 6. COS7 cells were transiently transfected with a SRE-luciferase (SRE-Luc) vector in the presence (+GPR54, gray bars) or absence (−GPR54, white bars) of human GPR54 cDNA expression vector. Cells were then treated with hKISS1, hKISS2-free, and hKISS2-NH_2 at the concentrations of 10^{-7} (+) and 10^{-6} (++) M for 6 hours. The luciferase activity was measured from cell lysates and is expressed as fold activation over its respective basal level. Data shown are means ± SEM of 3 independent experiments performed in duplicate. ***, $P < .001$ vs vehicle (−), 1-way ANOVA followed by Tukey posttests; †††, $P < .001$ vs in the absence of hGPR54 (−GPR54), 2-way ANOVA followed by Bonferroni posttests.

Synteny analysis of the *kiss2* gene from fish to primates

Synteny analysis of the *kiss2* gene loci showed that the primate *kiss2*-like genes and the American alligator *kiss2*-like gene were in a different chromosome or scaffold compared with those of other vertebrates (Figure 7). In contrast, the putative painted turtle and Chinese softshell turtle *kiss2* genes as well as the anole lizard *kiss2* gene were in the conserved synteny region (Figure 7). In confirmation of other reports (23, 25, 26), we could not find a *kiss2*-like gene in the conserved synteny region of chicken (Figure 7).

Discussion

The kiss2 peptide and the kiss1 peptide are considered to be involved in the control of reproduction (for reviews, see Refs. 3–13). Because the mature endogenous kiss2 peptide has only been identified in the brain of amphibians, we sought to identify the mature endogenous kiss2 peptide in

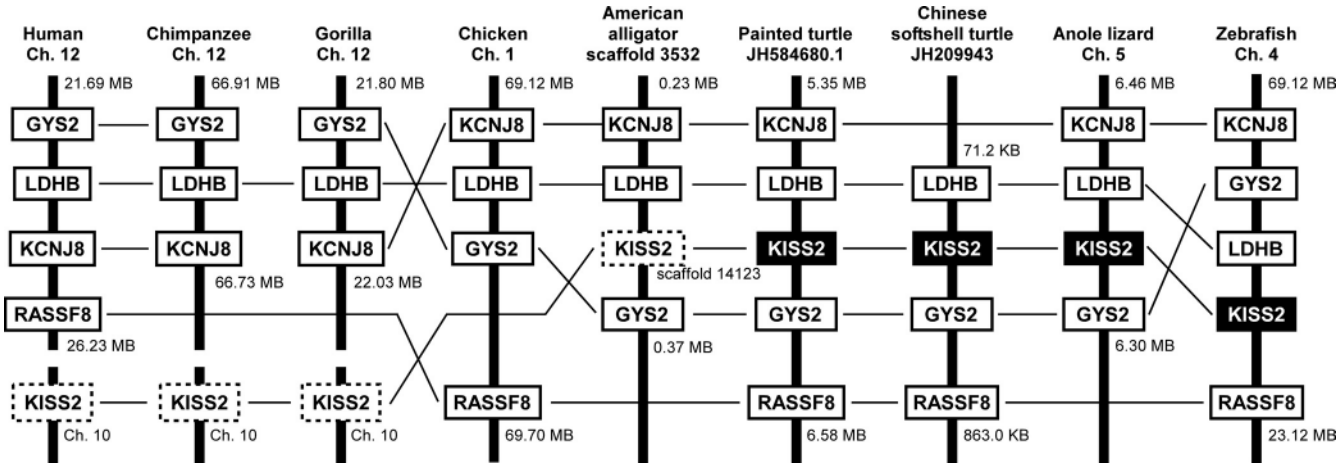

Figure 7. Synteny analysis of *kiss2* gene loci in vertebrates. Orthologous genes are linked by horizontal lines. The *kiss2* genes are shown as white type in black boxes. The *kiss2* genes in human, chimpanzee, gorilla, and American alligator that are considered to be nonfunctional are boxed by a broken line. Ch., chromosome.

the brains of masu salmon and turtle that possessed the *kiss2* gene. In addition, it is still not clear whether the *kiss2* gene is present in mammals and reptiles although the genomes of many species have been examined for the *kiss2* gene (for reviews, see Refs. 25, 26, and 37). In this study, we therefore performed genome database analyses in primates and reptiles to investigate the molecular evolution of the *kiss2* gene in vertebrates.

The *kiss2* gene has been identified in many fish species and some reptile species (for reviews, see Refs. 25, 26, and 37). However, the mature endogenous kiss2 peptide has not been identified in these animals. As a result of cDNA cloning, immunoaffinity purification, and mass spectrometry, mature endogenous kiss2 peptides as well as their cDNAs were identified in the brains of masu salmon and red-eared slider turtle. On the basis of MALDI-TOF MS analysis, the isolated kiss2 peptides were found to have the following structures: TSKFNFNPFGLRFa (masu salmon kiss2 peptide) and SKFNFNPFGLRFa (red-eared slider turtle kiss2 peptide). The red-eared slider turtle kiss2 peptide was a 12-amino acid peptide whose sequence was identical to that of the *X. laevis* kiss2 peptide that was characterized previously (22) (Figure 5). Putative kiss2 peptides in other vertebrates, such as grass lizard, painted turtle, and sea bass were also identical to those of red-eared slider turtle and *X. laevis* (Figure 5). Interestingly, the identified masu salmon kiss2 peptide and putative kokanee salmon kiss2 peptide were 1 amino acid longer than other kiss2 peptides at the N terminus (Figure 5). The 13-amino acid form of kiss2 peptides in masu salmon and kokanee salmon may reflect the phylogenetic position of salmonid fish that belong to *Protacanthopterygii*. However, other fish that belong to *Acanthopterygii* (medaka and sea bass), or *Ostariophysi* (zebrafish and goldfish) generally had the 12-amino acid form of putative kiss2 peptides (38) (Figure 5).

In situ hybridization showed that the masu salmon *kiss2* mRNA was expressed in the NRL (Figure 3). We also found small numbers of neurons expressing *kiss2* mRNA in the POA. The distribution of neurons expressing masu salmon *kiss2* mRNA is similar to that of zebrafish (39), seabream (40), and goldfish (41). Servili et al (39) showed that estradiol affected *kiss1*, *kiss2*, and *kissr2* (*gpr54–2*) mRNA expression in the hypothalamus of juvenile zebrafish. Kanda et al (41) also showed that the kiss2 neurons in the POA are controlled by estrogen feedback and *kiss2* mRNA expression in the POA is lower that that in the nonbreeding fish than breeding fish. Because we used nonbreeding masu salmon, *kiss2* mRNA expression was low in the POA in a way similar to that of the nonbreeding goldfish (41). We could not detect a positive signal of the *kiss2* mRNA in the red-eared slider turtle. Because we used juvenile turtles, the *kiss2* mRNA expression level may have been quite low. We also need to clarify the sites of expression of the *kiss2* gene in the brain of adult turtles.

Recent studies (42, 43) showed that kiss1 and kiss2 peptides do not necessarily act as a stimulator of the hypothalamo-pituitary-gonadal axis in fish. In European eel, kiss1 and kiss2 peptides inhibited LHβ expression (41). According to Zmora et al (43), the function of a putative endogenous 12-amino acid peptide of the striped bass kiss2 peptide changes, depending on their reproductive stages. This putative endogenous form stimulated the plasma LH level and *gnrh1* and *kissr2* mRNA expressions at the prepubertal stage but down-regulated *gnrh1* and *kissr2* mRNA expressions at gonadal recrudescence in striped bass (43). Such complicated effects of the kiss2 peptide may become clear when we use endogenous kiss2

peptides. In our previous study (22) and the present study, we showed that mature kiss2 peptides are present in the brain not only in amphibians but also in reptiles and fish. This information for mature endogenous kiss2 peptides in vertebrates is important for future functional analyses using species-specific kiss2 peptides.

In birds and in mammals, whether the *kiss2* gene is present except in platypus is not clear because the *kiss2* gene or *kiss2*-like gene has not been found even in the genome of these animals (for reviews, see Refs. 25, 26, and 37). By using bioinformatics, we found a new putative *kiss2*-like gene in the genome database of primates including humans. This is the first evidence of the presence of the *kiss2*-like gene in primates. However, the kiss2 peptide precursor polypeptide of primates had a serine residue at the C-terminal amidation site of kiss2 peptide, whereas the precursors of other vertebrates have a glycine that is essential for amidation of the kiss2 peptide (Figure 5). The difference in the amino acid at the C-terminal amidation site suggests that the amidated kiss2 peptide is not produced as an endogenous ligand in primates. The codon AGT (encoding serine) at the C-terminal amidation site in primates can change into GGT (encoding glycine) by a single nucleotide substitution (Figure 4), although there is no report about the polymorphism in this locus in the human genome to the best of our knowledge. We conducted in vitro assays to analyze whether the human KISS2 peptide has biological activity when it is amidated. Interestingly, the synthetic amidated human KISS2 peptide activated human GPR54 expressed in COS7 cells, whereas nonamidated human KISS2 peptide did not (Figure 6). Because the C-terminal motif is conserved in human KISS2 peptide, the amidated human KISS2 activated human GPR54, although its potency was less than that of human KISS1 peptide (Figure 6). The precursor of the human KISS2 peptide suggests that the amidated human KISS2 peptide is not produced as the endogenous ligand (Figure 4). Therefore, human KISS2 peptide may be nonfunctional. Synteny analyses showed that the locus of the primate *kiss2*-like genes was different from that of other vertebrates (Figure 7). In addition, the primate *kiss2*-like genes have no introns (Figure 4). The processed pseudogene is a kind of pseudogene that arises by retrotransposition of the mRNA transcript, and it does not have introns (44). Therefore, the primate *kiss2*-like gene may be a processed pseudogene. Although the orthology between *kiss2* genes and primate *kiss2*-like genes could not be confirmed because of the incomplete synteny data in primates, these results suggest that the human *KISS2*-like gene is related to *kiss2* genes in other vertebrates. The original *kiss2* gene may have been lost during the evolution of primates, whereas the *kiss2*-like gene may have remained as a processed pseudogene in the primate lineage. We confirmed that a *kiss2* gene is not present in the genome database of birds, such as chicken, zebra finch, and budgerigar (Figure 7). Furthermore, the *kiss1* gene has not been found in birds in previous studies (3, 23, 25, 45). Therefore, both the *kiss1* and *kiss2* genes may have been lost in birds.

For reptiles, the *kiss2* gene was only described in lizard in the previous study (22). In this study, we identified the *kiss2* gene and mature kiss2 peptide in turtles as described above. We further analyzed the genome database of crocodilians to search for the *kiss2* gene. We found a *kiss2*-like gene in crocodilians, including American alligator and salt-water crocodile (Supplemental Figure 2), which have diverged from the same ancestor as birds (32). However, we could not find introns and exons of the crocodilian *kiss2*-like gene. In addition, many stop codons were

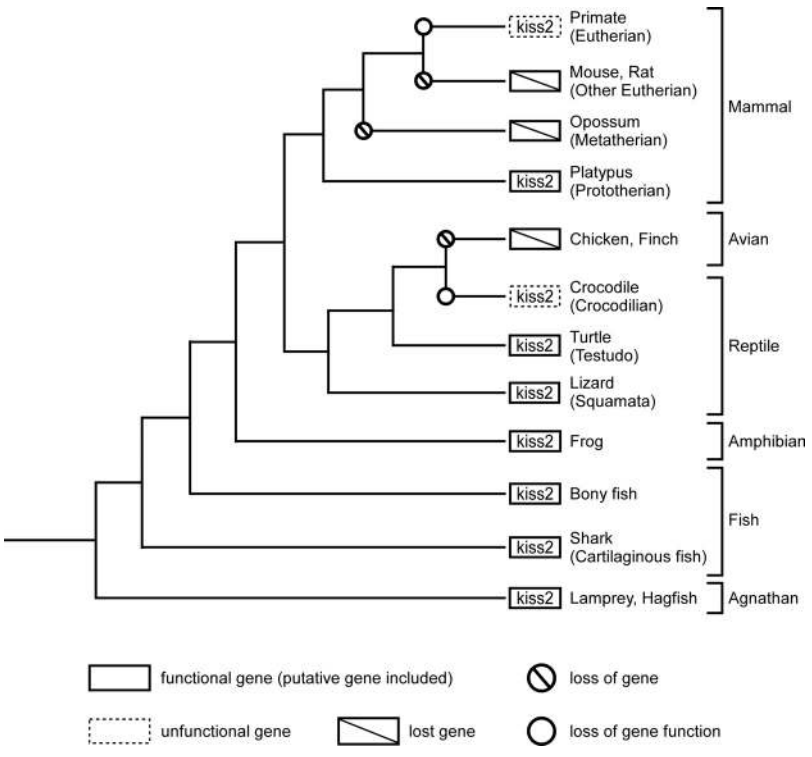

Figure 8. Proposed model of the molecular evolution of the *kiss2* gene in vertebrates. Functional genes including putative genes are boxed by solid lines. Nonfunctional genes are boxed by broken lines. Lost genes are indicated as boxes with diagonal lines. The loss of gene or loss of function is indicated by a circle with diagonal line or open circle, respectively.

found before the peptide-coding regions in the genome sequence of crocodilians. Translocation of the *kiss2*-like gene was also observed in American alligator by synteny analysis (Figure 7). These results suggest that nucleotide mutations and translocations may have led to the loss of function of the *kiss2*-like gene in crocodilians.

Taken together, these results have added to the knowledge of the evolutionary history of the *kiss2* gene (Figure 8). The *kiss2* gene has been conserved in agnathans, fish, and amphibians. In the reptilian and avian lineages, their common ancestor may have retained the *kiss2* gene. However, the function of *kiss2* gene may have been lost because of nucleotide mutations and translocation in the crocodilian lineage after the divergence from the common ancestor of crocodilians and other reptiles. The complete loss of the *kiss2* gene may have occurred at the emergence of birds. In mammals, it was predicted that loss of the *kiss2* gene occurred before the divergence of metatherians and eutherians because opossum lacks a *kiss2* gene in its genome database (23, 46). In a recent review of the evolutionary history of kisspeptin genes, it was suggested that the 2 rounds of whole genome duplication resulted in the 4 kisspeptin genes in the early stage of vertebrate evolution (25). As a result of the following loss of genes, only the *kiss1* gene has been remained in eutherian mammals and only the *kiss2* gene in lizards (25). In agreement with previous studies, we could not find a *kiss2* gene by a genome database search in metatherians, such as opossum and wallaby. However, the present study showed the presence of a *kiss2*-like gene in primates, suggesting that the *kiss2*-like gene was conserved until the divergence of primates and other eutherians. In reptilians, we found that *kiss2* gene may be functional in turtles, whereas it may be a pseudogene in crocodilians, suggesting that the *kiss2* gene may have lost its function after the divergence of turtles and crocodilians. The presence of the *kiss2* gene in platypus, a representative species of prototherians, suggests that the selection pressure has conserved the *kiss2* gene during the course of evolution. Because prototherians are oviparous unlike other mammals, the *kiss2* gene may be needed in the regulation of egg-laying of these animals. Searching for a *kiss2* gene in echidnas, the other representative species of prototherians, may provide an answer to this question.

In summary, we showed that the *kiss2* gene or "its remnant" is present from fish to humans. We further showed that mature endogenous kiss2 peptides are present in a fish and turtle species. The *kiss2* gene may have mutated in crocodilians and primates including humans and been lost in birds during the course of evolution. These results provide novel insights into the molecular evolution of *kiss2* genes and peptides in vertebrates.

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The sequences reported in this article have been deposited in the DNA DataBank of Japan (DDBJ), The European Molecular Biology Laboratory (EMBL), and GenBank database (accession numbers AB753099, AB435387, and AB753100 for cDNA sequences of kiss2 precursors).

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