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Regulation of the two *kiss* promoters in goldfish (*Carassius auratus*) by estrogen via different ERα pathways

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ABSTRACT

Kisspeptin stimulates the synthesis and release of gonadotropin via controlling the secretion of gonadotropin releasing hormone in vertebrates. It also mediates the positive or negative feedback regulation of sex steroids on the hypothalamus–gonadotropic axis. In contrast to mammals, two paralogous genes of kisspeptin (*kiss1* and *kiss2*) have been identified in several teleosts, implying the multiplicity of their physiological functions. In the present study, we cloned the promoters of *kiss1* and *kiss2* genes in goldfish (*Carassius auratus*), and identified the presence of putative binding sites for estrogen receptors, glucocorticoid receptors, Sp1, AP1, C/EBP and Oct-1. We further demonstrated that the goldfish Kiss neurons co-express the estrogen receptors, with *era1* and *erb1* in the habenula Kiss1 neurons and *era1*, *era2* and *erb1* in the preoptic and hypothalamic Kiss2 neurons. Using transient transfection in HEK293T cells of the two goldfish *kiss* gene promoters cloned upstream of a luciferase reporter, estrogen (E_2, 17β-estradiol) treatment was shown to enhance the promoter activities of the two goldfish *kiss* genes in the presence of ERα. Deletion analysis of *kiss1* promoter indicated that the E_2-induced promoter activity was located between position −633 and −317 where no half ERE motifs were found. Point mutation studies on the *kiss2* promoter indicated that the E_2-stimulated promoter activity was mediated by a half ERE site located at position −57. Results of the present study provide evidence that E_2 is capable of exerting positive feedback regulation on the expression of *kiss1* and *kiss2* in goldfish via ERE-independent or ERE-dependent ERα pathway, respectively.

1. Introduction

Kisspeptins, the products of *KISS1*/*Kiss1* gene, regulate the hypothalamus–gonadotropic axis via activating the G protein-coupled receptor 54 (GPR54, or Kiss1R) (Roa et al., 2008; Oakley et al., 2009). Mutation of this receptor has shown to cause absence of puberty and profound hypogonadotropic hypogonadism in human and mouse (Seminara et al., 2003; de Roux et al., 2003). In view of the action of kisspeptins in stimulating gonadotropin release via modulating gonadotropin-releasing hormone (GnRH) secretion (Messager et al., 2005; Navarro et al., 2005a, 2005b), it has been suggested that the Kiss1/GPR54 signaling system plays a central role in the onset of puberty and sexual maturation.

Sex steroids have been identified as important regulators of the hypothalamic Kiss1 system in different developmental stages and species (Garcia-Galiano et al., 2012; Kauffman et al., 2007; Cheng et al., 2010; Homma et al., 2009; Clarkson and Herbison, 2006;

Abbreviations: GPR54 or Kiss1R, G protein-coupled receptor 54; GnRH, gonadotropin-releasing hormone; ER, estrogen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; Sp1, specificity protein-1; Sp3, specificity protein-3; ERE, estrogen response element; RNAP II, RNA polymerase II; AP1, activator protein-1; AP2, activator protein-2; LH, luteinizing hormone; FSH, follicle-stimulating hormone; POA, preoptic area; TESS, Transcription Element Search Software; DIG, digoxigenin; U87MG, human glioblastoma cells; HEK293T, human embryonic kidney cells; FBS, fetal bovine serum; E_2, 17β-estradiol; PR, progesterone receptor; AR, androgen receptor; vHb, ventral part of habenula; nRL, nucleus of the lateral recess; GR, glucocorticoid receptor; C/EBPβ, CCAAT/enhancer binding protein β.

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Clarkson et al., 2009). In rodents, Kiss1 neurons participate in the feedback regulation of estrogen on the hypothalamus–gonadotropic axis via estrogen receptor α (ERα), depending on the area of the brain with positive feedback in the anteroventral periventricular nucleus and negative feedback in the arcuate nucleus of the hypothalamus (Smith et al., 2006; Roa et al., 2009). Estrogens have been shown to differentially regulate *Kiss1* through two distinct ERα signaling mechanisms, namely the classical estrogen response element (ERE) dependent pathway and the nonclassical ERE-independent pathway (Gottsch et al., 2009). A previous study on immortalized GnRH-secreting hypothalamic cells (GT1-7) has revealed that the E_2-induced upregulation of *Kiss1* is mediated by the interaction of ERα/specificity protein-1 (Sp1) complex with GC-rich motifs on *Kiss1* promoter (Li et al., 2007). Further study on a breast cancer cells (MDA-MB-231) has also demonstrated that *Kiss1* is downregulated by E_2 via the nonclassical pathway in association with a reduced number of RNA polymerase II (RNAP II) binding to the proximal region of *Kiss1* promoter and a loss of RNAP II activation along the gene body (Huijbregts and de Roux, 2010). Besides, estrogen can also regulate the expression of genes through interaction of ERα with other genomic pathways such as activator protein-1 (AP1) and nuclear factor-κB, or with non-genomic pathways such as MAPK/PI3K and cAMP/PKA (Safe and Kim, 2008).

Different from human and rodent, two paralogous genes of kisspeptin (*kiss1* and *kiss2*) and their receptors (*kiss1r* and *kiss2r*) have been identified in several teleosts (Servili et al., 2011; Mitani et al., 2010; Li et al., 2009), such as zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), and goldfish (*C. auratus*). In goldfish, the Kiss1 decapeptide could increase the serum luteinizing hormone (LH) levels *in vivo* (Li et al., 2009), and elevate the expression of LH at the pituitary level *in vitro* (Yang et al., 2010). In addition, elevation of LH and follicle-stimulating hormone (FSH) β-subunit gene expression by Kiss2 decapeptide has been reported in several teleost species (Kitahashi et al., 2009; Shi et al., 2010; Felip et al., 2009). These results suggest the critical role of the two kisspeptin systems in the control of the reproductive axis in teleosts. Similar to mammals, estrogen has also been demonstrated to regulate the expression of *kiss* genes in several teleost fish (Servili et al., 2011; Mitani et al., 2010; Kanda et al., 2008, 2012). However, such estrogen responses vary greatly with species. In zebrafish, estrogen treatment increases *kiss1* and *kiss2* expression in the brain (Servili et al., 2011), whereas in medaka, only Kiss1 expressing neurons in the nucleus ventralis tuberis show estrogen response (Mitani et al., 2010). In goldfish, Kiss2 neurons in the preoptic area (POA) are sensitive to estrogen (Kanda et al., 2012). These data suggest the existence of different estrogen signal pathways in regulating the two kisspeptin systems in teleosts. In particular, the exact molecular mechanisms of estrogen regulation on the two *kiss* genes in lower vertebrate species are totally unknown.

In order to better understand how estrogen exerts its action on the expression of *kiss* genes in teleosts, the promoters of the goldfish *kiss1* and *kiss2* genes have been cloned, and the potential estrogen-related regulatory motifs on the two gene promoters examined in the present study. Moreover, the co-expression of estrogen receptors on the *kiss*-expressing neurons was also studied. In addition, the promoter activities of two *kiss* genes in response to estrogen were investigated in HEK293T cells co-transfected with the goldfish ERα. Finally, the regulatory areas and motifs of estrogen on the *kiss1* and *kiss2* gene promoters were characterized by deletion and site-directed mutagenesis analyses.

2. Materials and methods

2.1. Animals

Goldfish (*C. auratus*) were obtained from a local fish farm (Guangzhou, China), weighing between 55 and 65 g. The fish were acclimated for at least 7 days in a recirculating fresh water system at ambient temperature under natural photoperiod (13L:11D) and were fed once daily with commercial pellets until satiety during the experimental period. All animals and experiments were approved by the Animal Research and Ethics Committees of Sun Yat-Sen University and Monash University conducted in accordance with the guidelines of the committees.

2.2. Cloning of the 5′-flanking regions of the kiss1 and kiss2 genes in goldfish

The 5′-flanking regions of the *kiss1* and *kiss2* genes were isolated using the Universal Genome Walker Kit (BD Biosciences Clontech, USA). Four Genome Walker libraries were respectively constructed by the restriction enzymes *Eco*RV, *Stu*I, *Dra*I and *Pvu*II (Takara, Japan). The primer sequences used are listed in Table 1. The conditions of the first round PCR were: 7 cycles consisting of 94 °C for 30 s and 72 °C for 3 min, followed by 35 cycles at 94 °C for 30 s and 67 °C for 3 min, and then 67 °C for 7 min as the final extension step. Nested PCR was performed under the same conditions except that the cycle numbers were respectively reduced to 5 and 25. The PCR products obtained above were purified using the E.Z.N.A. Gel Extraction Kit (Omega Bio Tek, USA), subcloned into the pTZ57R/T vector (Fermentas, USA), and were verified by sequencing on an ABI 3700 sequencer (Applied Biosystems, USA). To locate the transcriptional start site of the goldfish *kiss1* and *kiss2* genes, 5′-rapid amplification of cDNA ends (RACE) was carried out using SMARTer RACE cDNA Amplification Kit (BD Biosciences Clontech, USA). The cDNAs were tailed, then amplified using a standard PCR protocol with the abridged anchor primers in combination with the gene-specific primers (listed in Table 1) of *kiss1* and *kiss2*. The PCR product was subcloned into the vector pTZ57R/T, and multiple clones were confirmed by sequencing. The transcription initiation (start) site of goldfish *kiss1* and *kiss2* was further confirmed after a comparison with published full-length sequences of the goldfish *kiss1* and *kiss2* cDNA (GenBank accession numbers FJ236327.1 and FJ465138.1) (Yang et al., 2010; Li et al., 2009). The sequences were analyzed using the online Transcription Element Search Software (TESS) (http://www.cbil.upenn.edu/cgi-bin/tess/tess).

2.3. Expression of estrogen receptor (ER) subtypes in laser captured Kiss1 and Kiss2 neurons

The expression of ER subtypes was examined in laser-captured digoxigenin (DIG)-labeled *kiss1* and *kiss2* mRNA expressing neurons by RT-PCR. Brains of sexually mature female goldfish (n = 3–5) were processed for DIG-*in situ* hybridization for the *kiss1* and *kiss2* genes. DIG-*in situ* hybridization for *kiss1* and *kiss2* mRNA was performed as described previously (Kitahashi et al., 2009). Briefly, brain sections (15 μm) fixed with buffered 4% paraformaldehyde were subjected to permeabilization with 0.2 M HCl for 10 min followed by proteinase K (1 μg/ml) treatment for 15 min, and hybridized with the DIG-labeled riboprobes for *kiss1* (256 nt) and *kiss2* (325 nt) at 50 ng/ml at 55 °C overnight in a humidified chamber. DIG was detected with an alkaline phosphatase-conjugated anti-DIG antibody (Roche, at 1:500 dilution) and 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (Roche, USA) was used for chromogenic development. Each population of the laser-microdissected cells were pooled into a sterile 0.2 ml PCR tube containing 50 μl of lysis solution [1 × RT buffer (Applied Biosystems, USA), 1% Nonidet P-40, and 0.05 mg/μl proteinase K] and lysed for 1 h at 50 °C. After DNase I treatment, total RNA was isolated using TRIzol (Life Technologies, USA) and dissolved in 10 μl of DEPC-treated water. The total RNAs were subsequently subjected to cDNA synthesis as above. The cDNA samples were then subjected to RT-PCR analysis for