Available online at www.sciencedirect.com

ScienceDirect

Journal of the Chinese Medical Association 78 (2015) 249–257

www.jcma-online.com

Original Article

Kisspeptin expression in mouse Leydig cells correlates with age

Jyun-Yuan Wang, Meng-Chieh Hsu, Tai-Hsiang Tseng, Leang-Shin Wu, Kuo-Tai Yang, Chih-Hsien Chiu*

Department of Animal Science and Technology, College of Bio-Resources and Agriculture, National Taiwan University, Taipei, Taiwan, ROC

Received June 13, 2014; accepted October 14, 2014

Abstract

Background: Kisspeptin, encoded by the *Kiss1* gene, has many forms including kisspeptin54, kisspeptin14, kisspeptin13, and kisspeptin10, and all these peptides have the same affinity to their receptor KISS1R encoded by the *Kiss1r* gene. The KISS1–KISS1R system was discovered in neurons, and many reports stress on their function in the brain. However, recent studies have shown that *Kiss1* and *Kiss1r* are expressed in the testes. The goal of this study was to demonstrate the roles of *Kiss1* and *Kiss1r* in testicular function, especially their steroidogenic activity.
Methods: Kisspeptin10 and the kisspeptin10 antagonist peptide234 were used to determine their effect on testosterone production. Moreover, expression of steroidogenic genes in mouse testes and their gonadosomatic index (weight of the testes divided by the total body weight) and also serum testosterone level were studied between the ages of 2 weeks and 15 weeks.
Results: Kisspeptin10 and peptide234 did not affect testosterone production in primary Leydig cells from adult mice. *Kiss1* and *Esr1* expression also increased during puberty. The peak gonadosomatic index occurred at 4 weeks of age, and serum testosterone levels plateaued after the age of 4 weeks.
Conclusion: Our results suggest that kisspeptin10 does not affect steroidogenesis in adult Leydig cells, but its pattern of expression follows the stages of testicular development. Future studies should determine if kisspeptin regulates testicular development during puberty.
Copyright © 2015 Elsevier Taiwan LLC and the Chinese Medical Association. All rights reserved.

Keywords: kisspeptin; Leydig cells; puberty; steroidogenesis

1. Introduction

Kisspeptin is the product of the *Kiss1* gene and the ligand for the seven-transmembrane G protein-coupled receptor GPR54, also named KISS1R.[1] The *Kiss1* gene encodes a 145 amino acid protein, which is hydrolyzed into four peptides of different lengths, including kisspeptin54, kisspeptin14, kisspeptin13, and kisspeptin10; all these peptides have the same affinity for KISS1R.[2] Expression of *Kiss1* mRNA is detected in many regions of the mouse brain, including the anteroventral periventricular nucleus, periventricular nucleus, anterodorsal preoptic nucleus, and arcuate nucleus.[3–5] Moreover, kisspeptin is highly expressed in placental syncytiotrophoblasts[6] and other tissues, such as the testes, liver, pancreas, and small intestine.[7] Many studies indicate that kisspeptin stimulates gonadotropin secretion. Injection of kisspeptin into mouse lateral ventricles rapidly induces significant secretion of luteinizing hormone (LH) and follicle-stimulating hormone.[3] Similar effects were observed in rats,[8–10] sheep,[11] monkeys,[12,13] and humans.[14]

However, studies of this system have focused primarily on its regulatory function in the hypothalamic–pituitary–gonadal axis and suppression of tumor cell metastasis, and a few studies have investigated the functions of KISS1 and KISS1R in other tissues, such as the gonads. The goal of the present

Conflicts of interest: The authors declare that there are no conflicts of interest related to the subject matter or materials discussed in this article.

* Corresponding author. Dr. Chih-Hsien Chiu, Room 209, Laboratory of Animal Physiology, Department of Animal Science and Technology, National Taiwan University, 50, Lane 155, Section 3, Keelung Road, Da'an District, Taipei 106, Taiwan, ROC.
E-mail address: chiuchihhsien@ntu.edu.tw (C.-H. Chiu).

http://dx.doi.org/10.1016/j.jcma.2015.01.004
1726-4901/Copyright © 2015 Elsevier Taiwan LLC and the Chinese Medical Association. All rights reserved.

study was to determine the function of the KISS1–KISS1R system in the testes. The testes are essential for male reproduction through theri functions including spermatogenesis, sperm production, and testosterone secretion. Testosterone is converted from cholesterol in Leydig cells by a process named steroidogenesis, which is regulated by LH. LH binds to its receptors on the cell membrane, activating G proteins, which activate adenylyl cyclase. Activated adenylyl cyclase increases cytoplasmic concentrations of cyclic AMP,[15] triggering phosphorylation of protein kinase A and expression of steroidogenic genes, such as steroidogenic acute regulatory protein (StAR) and cytochrome P450 cholesterol side-chain cleavage (CYP11A1) enzyme. StAR, CYP11A1, and 3-β-hydroxysteroid dehydrogenase (HSD3B1) play critical roles in basal and hormone-regulated steroidogenesis[16] due to the increased activities of multiple transcription factors, which were previously reviewed by Lavoie and King.[17] StAR protein transfers free cholesterol from the cytoplasm to the mitochondrial inner membrane, where cholesterol is converted to pregnenolone by CYP11A1.[18] Pregnenolone is then transported to the smooth endoplasmic reticulum and converted to progesterone by HSD3B1. Additional enzymatic processing converts progesterone to testosterone.

In this study, the regulatory effect of the KISS1–KISS1R system was further investigated in Leydig cells. The function of kisspeptin in testosterone production was evaluated in primary Leydig cells from adult mice. Continuous samples were collected and examined by enzyme immunoassay (EIA), real-time RT-PCR (qPCR), and immunohistochemistry to understand the role of the KISS1–KISS1R system during mouse development. Since there were already studies focusing on the expression of KISS1 and KISSR in brain of different stages, in the present study, we aimed to understand the expression pattern of KISS1 and KISSR in the testes and investigate the effect of KISS1 on the testes.

2. Methods

2.1. Reagents and chemicals

Cell culture Medium 199, trypsin–EDTA (0.25%), penicillin G, streptomycin sulfate, fetal bovine serum, Hank's balanced salt solution, and trypan blue stain were purchased from Life Technologies (Grand Island, NY, USA). Collagenase-type 1 was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Bovine serum albumin, 22-hydroxycholesterol, pregnenolone, and other general chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kisspeptin10 and its antagonist (peptide234) were synthesized by Kelowna International Scientific, Inc. (Taipei, Taiwan), and ovine LH was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases. All nucleotide primers were obtained from Bio Basic, Inc. (Markham, Canada). Reverse-transcription kits were purchased from Bioline (London, UK). SYBR-Green PCR reagent for semiquantitative real-time PCR was also purchased from Life Technologies.

2.2. Animal study

Adult male ICR mice, aged 2–16 weeks, were purchased from the National Taiwan University, Taipei, Taiwan and maintained in a 12-hour light (0800–2000)/12-hour dark (20:00–08:00) cycle at 22 ± 1°C. Mice were provided a chow diet and water *ad libitum* for the duration of the study. The KISS1 and KISS1R expression patterns were examined by semiquantitative real-time PCR and histology in 20 mice. The steroidogenesis function in primary Leydig cells was examined in three male mice. All experimental protocols were approved by the Institutional Animal Care and Use Committee, College of Medicine, National Taiwan University, and all procedures conformed to the National Institutes of Health Guide for the care and use of laboratory animals.

2.3. Primary mouse Leydig cell culture

As previously reported,[19] the testes were removed from sacrificed adult male ICR mice (13–16-week old) and decapsulated in Medium 199 with 10% fetal bovine serum to separate seminiferous tubules. After washing once with isolation buffer (1X Hank's balanced salt solution containing 0.1% bovine serum albumin and 200 U/mL collagenase type 1) and incubating in isolation buffer for an additional 5 minutes at room temperature, seminiferous tubules and cells were filtered with a 250-mesh filter. Leydig cells were collected by centrifugation at 300*g* for 5 minutes. After resuspending the Leydig cells in 10 mL Medium 199 without fetal bovine serum, live cells were counted by trypan blue exclusion and seeded (5×10^4/well) in a flat-bottom 48-well culture plate (Corning Inc., New York, NY, USA). Cells were immediately treated with ovine LH (0 ng/mL or 100 ng/mL), kisspeptin10 (0μM, 1μM, 5μM, or 10μM), or peptide234 (0μM, 1μM, 5μM, or 10μM) following incubation at 37°C with 5% CO_2. Culture media were collected after treatment for 4 hours or 24 hours and stored at −20°C until the EIA was performed.

2.4. EIA for testosterone

The testosterone EIA included an IgM polyclonal antibody against testosterone with specific affinity of 1.1×10^{10}/M. The antibody exhibits cross-reactivity of < 0.01% with bovine serum albumin and other steroids, including pregnenolone, progesterone, estradiol, and estrone. Aliquots (50 μL) of diluted media and horseradish peroxide-linked testosterone conjugate (150 μL) were added to a flat-bottom 96-well plate coated with 200 μL antibody (representing a 1:10,000 dilution). After 30 minutes of incubation at room temperature with gentle shaking and two washes with Tween-20 in 0.01M phosphate buffer (pH 7.0), the color was developed with 3.7mM O-phenylenediamine containing 0.03% H_2O_2 (200 μL each well) for an additional 45 minutes. The reaction was terminated by adding 50 μL of 8N H_2SO_4. The optical density of each sample was determined on a dual-wavelength reader

(BioTek, Winooski, VT, USA) set between 490 nm and 630 nm. Testosterone concentration was determined with a standard curve. Coefficients of variation were 7% (within assays) and 12% (between assays). The sensitivity of the assay was 0.3 pg/mL. All standards and samples were assayed in duplicate.

2.5. Total RNA extraction and real-time PCR

Total RNA was extracted from tissue samples with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. To synthesize cDNA, total RNA (3 μg) was mixed with 2.5μM oligo(dT) and 500μM deoxynucleotide triphosphate, and denatured at 65°C for 5 minutes. After being cooled on ice, the mixture was combined with 40 IU RNase inhibitor and 100 IU SuperScript III RT reverse transcriptase (Life Technologies), and incubated at 45°C for 60 minutes. The reaction was inactivated by heating at 95°C for 5 minutes, and cDNA products were stored at 4°C. Real-time PCR was performed with the ABI StepOne Real-Time PCR System according to the manufacturer's instructions (Life Technologies). Briefly, the 10 μL reaction mixture contained 100 ng cDNA, 0.2μM primer pairs, and 2 × Fast SYBR-Green PCR Master Mix (Life Technologies). PCR amplification was performed at 95°C for 20 seconds, and 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds, followed by melting curve analysis. The primer sequences are shown in Table 1. Primer pairs were designed such that each primer was separated by at least one intron and underwent a pretest to confirm the amplification efficiencies. Gene expression levels were normalized to that of the internal control (18S rRNA) and presented as fold changes, compared to samples from 15-week-old mice.

Table 1
List of primers used in mouse testis.

Gene	Primer sequence (5′–>3′)	Product size (bp)	Accession no.
Lhcgr	F: GCCCGACTATCTCTCACCTATC	111	NM_013582.2
	R: CCTTTCCAGGGAATCACTCTGA		
Star	F: AGCATGTTCCTCGCTACGTT	88	NM_011485.4
	R: GCACAGCTTGGTGCCTTAATC		
Cyp11a1	F: TGGCACACAGAAAATCCATTACC	108	NM_019779.3
	R: TTGGGGTCCACGATGTAAACT		
Hsd3b1	F: TTTGCTCTCTCAGTTGTGACCA	134	NM_008293.3
	R: GCCTGCTTCGTGACCATATTTATT		
Kiss1	F: TGCTGCTTCTCCTCTGTGTCGC	139	NM_178260.3
	R: CAGGCTTGCTCTCTGCATACCGC		
Kiss1r	F: GTGCAAATTCGTCAACTACATCC	103	NM_053244.5
	R: AGCGGGAACACAGTCACATAC		
Esr1	F: TGGACAGGAATCAAGGTAAATG	118	NM_007956.4
	R: TTGAGGCACACAAACTCTTCTC		
18S rRNA	F: CGGACAGGATTGACAGATTA	84	NR_003278.3
	R: CAAATCGCTCCACCAACTAA		

F = forward; R = reverse.

2.6. Immunohistochemistry

Mouse testes were fixed, embedded in paraffin, sectioned into 5-μm-thick pieces, and mounted on poly-L-lysine-coated slides. Following deparaffinization in xylene, tissues were rehydrated by passage through descending concentrations of ethanol and washing PBS at room temperature. Retrieval of antigen-binding sites was achieved by incubating the slides twice in 10mM sodium citrate buffer [containing 0.05% (v/v) Tween-20; pH 6.0] heated in a microwave oven for 10 minutes. After quenching endogenous peroxidase activity with 1% (v/v) H_2O_2 in methanol for 30 minutes, the sections were rinsed three times (5 minutes each time) in PBS. Slides were incubated in blocking buffer [PBS containing 3% (v/v) normal goat serum and 0.2% (v/v) Triton X-100] for 1 hour and processed with the Avidin/Biotin blocking kit (Vector Laboratories, Burlington, Ontario, Canada) to quench endogenous protein-associated biotin. Slides were incubated in primary antibodies diluted in blocking buffer for 20 hours at 4°C in a moist chamber. The primary antibodies were rabbit anti-kisspeptin145 (1:100, product no. ab19028; Abcam, Inc., Cambridge, UK) and rabbit anti-Kiss1r (1:100, product no. ab12698; Abcam, Inc.). Blocking buffer alone served as a negative control. Slides were washed five times (5 minutes each time) in PBS at room temperature and incubated with biotinylated goat antirabbit secondary antibodies for 60 minutes in a moist chamber. Prior to incubation with an avidin–biotin–horseradish peroxidase complex, the sections were rinsed again in Vectastain Universal ELITE ABC Kit (Vector Laboratories) for 30 minutes according to the manufacturer's protocol. After additional rinsing, slides were incubated for 2–10 minutes at room temperature with diaminobenzidine to visualize immunostaining. Finally, slides were rinsed in distilled water twice (10 minutes each time), counterstained with hematoxylin for 30 seconds, and hydrated with ethanol and xylene prior to adding mounting medium (Hecht-Assistent, Sondheim, Germany). Slides were observed with an Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany) equipped with the digital camera AxioCam ERc 5s (Carl Zeiss).

2.7. Data analysis

Results are expressed as the mean ± standard deviation or standard error of the mean of triplicate samples from three individual experiments. Results of assays were analyzed by one-way ANOVA followed by Duncan's multiple comparison with SigmaStat 3.5 (Aspire Software International, Ashburn, VA, USA). A p value <0.05 was considered significant.

3. Results

3.1. Effect of Kisspeptin10 and peptide234 on ovine LH-stimulated testosterone production in Leydig cells

The main function of Leydig cells is the production of testosterone in response to LH stimulation. In this experiment,

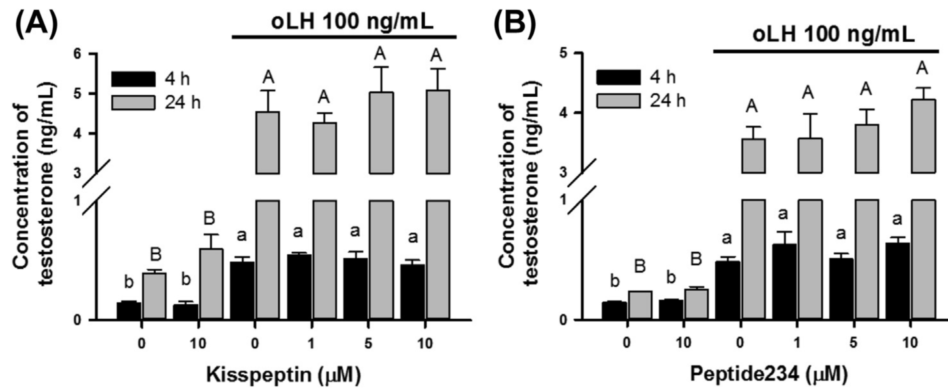

Fig. 1. Effects of kisspeptin and peptide234 on ovine LH-enhanced testosterone production in primary mouse Leydig cells. Cells were treated with (A) kisspeptin or (B) an antagonist of kisspeptin, peptide234, alone or in combination with 100 ng/mL ovine LH for 4 hours (black bars) and 24 hours (gray bars). Data are presented as mean ± standard error of the mean, $N = 3$. Results of assays were analyzed by one-way ANOVA, followed by Duncan's multiple comparison. Different lowercase letters indicate significant differences between black bars; different capital letters indicate significant differences between gray bars. ANOVA = analysis of variance; LH = luteinizing hormone.

primary mouse Leydig cells were obtained and treated with kisspeptin10 to determine the effects on testosterone production. As shown in Fig. 1A, kisspeptin10 did not alter the production of testosterone in Leydig cells after 4 hours (black bars) or 24 hours (gray bars) of treatment. Although Leydig cells responded to ovine LH (100 ng/mL) by increasing testosterone production, cotreatment with ovine LH and kisspeptin10 did not further enhance ovine LH-stimulated production of testosterone. Similar results were discovered in Leydig cells treated with peptide234 (Fig. 1B). Peptide234 alone or treated with ovine LH did not affect basal or ovine LH-stimulated testosterone production, respectively.

3.2. Effects of Kisspeptin10 and peptide-234 on 22-hydroxycholesterol- or pregnenolone-stimulated production of testosterone in Leydig cells

To investigate whether kisspeptin affects CYP11A1 or HSD3B1 activity, primary Leydig cells were treated with a substrate of CYP11A1, 22-hydroxycholesterol (Fig. 2A), or a substrate of HSD3B1, pregnenolone (Fig. 2B). Treatment with these steroid substrates for 4 hours or 24 hours dramatically increased testosterone production in Leydig cells. However, cotreatment with kissppetin10 did not modulate testosterone production.

3.3. Testis growth and serum testosterone levels during development

Changes in the gonadosomatic index (weight of the testes/whole body weight) and serum testosterone concentrations during ICR mouse development from 2 weeks to 15 weeks are shown in Fig. 3A. The gonadosomatic index increased significantly during the age of 2−4 weeks but gradually decreased to a plateau during 5−15 weeks. Serum testosterone concentrations were initially elevated at 3 weeks, gradually increased after 6 weeks, and reached their highest levels at 15 weeks. These results suggested that the testes were growing

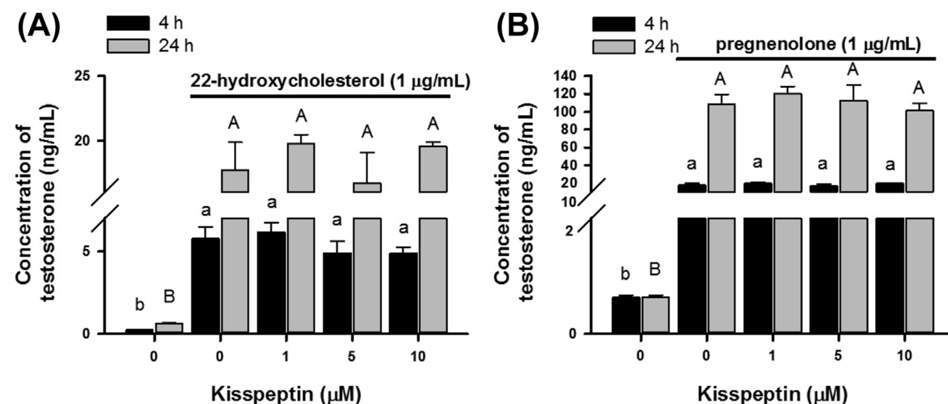

Fig. 2. Effect of kisspeptin on 22-hydroxycholesterol- and pregnenolone-enhanced testosterone production in primary mouse Leydig cells. Cell were treated with kisspeptin alone or cotreated with 1 μg/mL (A) 22-hydroxycholesterol or (B) pregnenolone for 4 hours (black bars) or 24 hours (gray bars). Data are presented as mean ± standard error of the mean, $N = 3$. Results of assays were analyzed by one-way ANOVA, followed by Duncan's multiple comparison. Different lowercase letters indicate significant differences between black bars; different capital letters indicate significant differences between gray bars. ANOVA = analysis of variance.

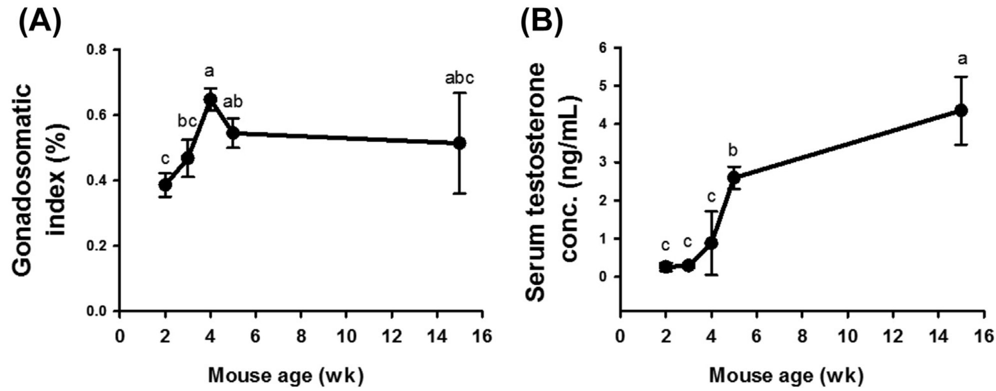

Fig. 3. (A) Gonadosomatic index and (B) serum testosterone concentration during mouse development. The gonadosomatic index was calculated with the following formula: testis weight (g)/body weight (g) × 100. Serum was sampled from mice orbital sinus, and testosterone concentration was assayed with EIA. The mean ± standard deviation of three to five animals in each group is shown. Results of assays were analyzed by one-way ANOVA, followed by Duncan's multiple comparison. Groups with different superscript letters are significantly different ($p < 0.05$). ANOVA = analysis of variance; EIA = enzyme immunoassay.

rapidly during 2−5 weeks, which is referred to as the pubertal period of mice.

3.4. *Lhcgr, Star, Cyp11a1, and Hsd3b1* mRNA expression during development

Steroidogenesis and testicular development require LH signaling through the LH receptor in the testes. We examined *Lhcgr* expression in the testes of growing and adult mice (Fig. 4A). *Lhcgr* levels increased steadily during pubertal development. Further, expression levels of genes required for steroidogenesis were examined in whole testicular tissues (Fig. 4B−D). *Star* and *Cyp11a1* levels increased significantly during 2−4 weeks, with the highest levels being observed in adult mice. By contrast, expression of *Hsd3b1* was not significantly different between pubertal and adult mice.

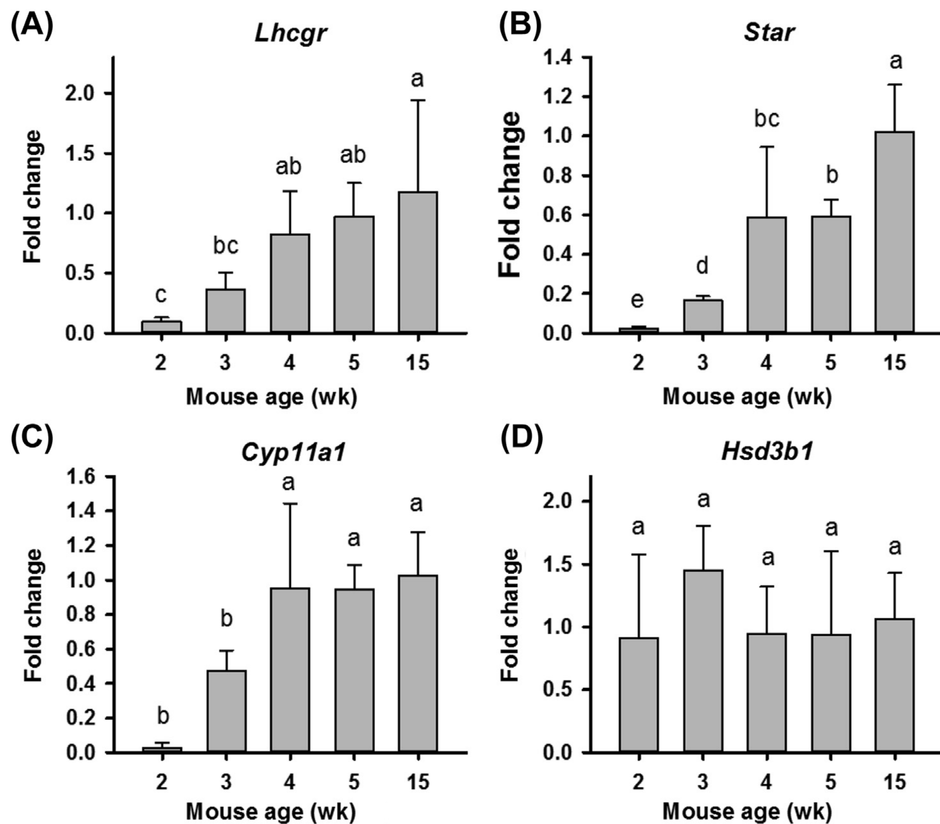

Fig. 4. Expression of *Lhcgr, Star, Cyp11a1*, and *Hsd3b1* mRNA levels during development in the whole mouse testis. Data are shown for (A) *Lhcgr*, (B) *Star*, (C) *Cyp11a1*, and (D) *Hsd3b1* as fold changes compared to the expression levels in 15-week-old mice. The mean ± standard deviation of three to six animals per group is shown. Results of assays were analyzed by one-way ANOVA, followed by Duncan's multiple comparison. Groups with different superscript letters are significantly different ($p < 0.05$). ANOVA = analysis of variance.

3.5. *Kiss1, Kiss1r, and Esr1 expression during development*

Similar to the expression patterns of *Lhcgr*, *Star*, and *Cyp11a1*, expression levels of *Kiss1* and *Esr1* increased during 2–4 weeks, with the highest levels being observed after 4 weeks, while the expression of *Kiss1r* was not altered during 2–15 weeks (Fig. 5). Finally, we examined the expression pattern of KISS1 and KISS1R in the testicular tissue of mice with immunohistochemical staining. As shown in the middle panel of Fig. 6, the signal of KISS1 was detected in the testes of 5- and 15-week-old mice but not in those of 2-week-old mice, while signal of KISS1R was detected after 2 weeks of age.

4. Discussion

The *Kiss1* gene was originally identified as a metastasis suppressor gene.[7,20,21] Kisspeptin and its receptor, KISS1R, which is also known as G-protein-coupled receptor GPR54, regulate the secretion of GnRH from the hypothalamus and affect mammalian reproduction.[22,23] Mutations in *Kiss1* or *Kiss1r* cause hypogonadotropic hypogonadism in mice and result in the reduced production of gonadotropins and sex steroids, which further prevents complete sexual maturation.[24,25] *Kiss1* and *Kiss1r* are expressed in many tissues.[7,26] Some studies suggest that *Kiss1* is expressed in the hypothalamus, kidneys, and testes,[3,7,27] whereas other reports reveal that *Kiss1* or *Kiss1r* are expressed in the ovary or luteum.[28,29] Chronic implantation of a single high dose of kisspeptin causes the seminiferous tubules to shrink and also decreases the volume of the testes. This phenomenon can be reversed by treating with GnRH antagonist, indicating that the effects are due to hyperstimulation of the hypothalamic–pituitary–gonadal axis.[27,30] In addition, intraperitoneal injection of kisspeptin10 (1 ng or 1 μg) twice per day for 12 consecutive days into a 35-day-old rat reduced LH and testosterone levels.[31] Moreover, a recent study showed that the peripheral injection of 50 μg kisspeptin caused a LH surge in rhesus monkeys, followed by an increase of testosterone level. Kisspeptin treatment also enhanced hCG-supported testosterone production in rhesus monkeys pretreated with GnRH antagonist acyline.[32] The aforementioned reports revealed that the kisspeptin function may vary depending on the dose and duration of treatment. These reports also focused on the effect of kisspeptin on the hypothalamic–pituitary–gonadal axis, but not on the direct effect of kisspeptin on the testes and testosterone-secreting regulation.

Thus, to investigate the direct effect of kisspeptin on testosterone secretion, our first aim was to determine the

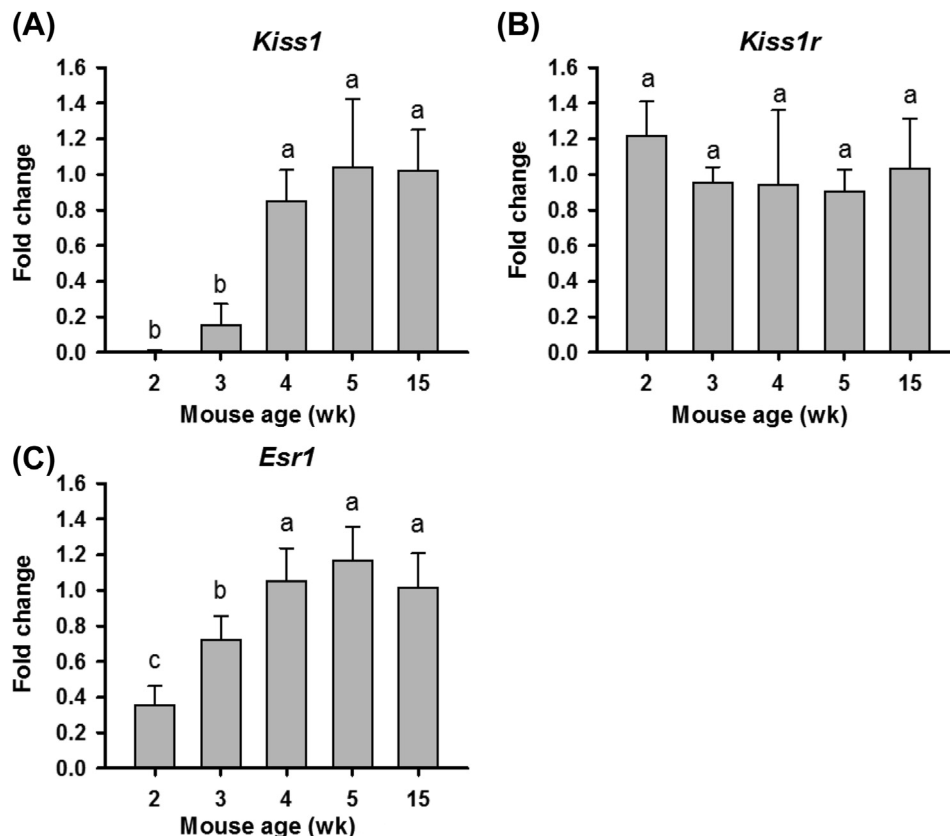

Fig. 5. Expression of *Kiss1*, *Kiss1r*, and *Esr1* mRNA levels during development in the whole mouse testis. Data for (A) *Kiss1*, (B) *Kiss1R*, and (C) *Esr1* are shown as fold changes compared to the expression levels in 15-week-old mice. The mean ± standard deviation of three to six animals per group is shown. Results of assays were analyzed by one-way ANOVA, followed by Duncan's multiple comparison. Groups with different superscript letters are significantly different ($p < 0.05$). ANOVA = analysis of variance.

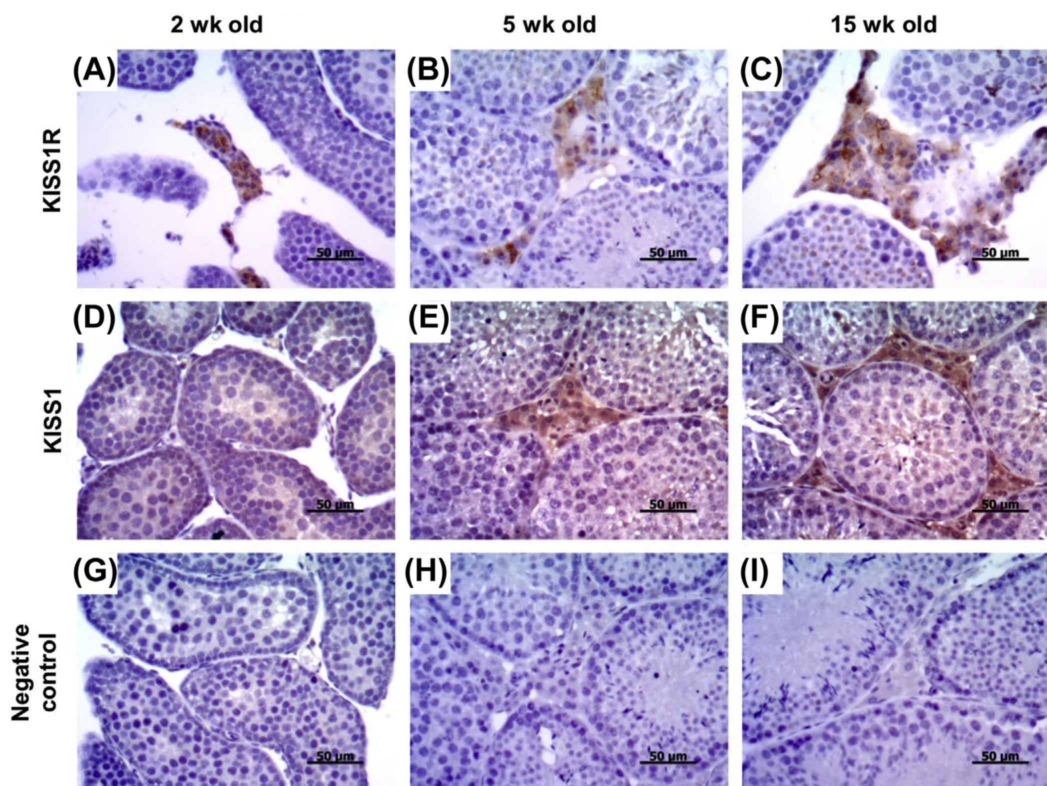

Fig. 6. Immunohistochemical staining of KISS1R and kisspeptin in testicular tissues from ICR mice of different ages. Testicular tissues from (A and D) 2-week-old, (B and E) 5-week-old, and (C and F) 15-week-old ICR mice were stained for KISS1R (upper panel) or KISS1 (middle panel) with specific antibodies. Positive immunostaining appeared brown in color and is indicated with an arrow. (G–I) Sections that were incubated without primary antibody are shown as negative controls. Scale bars = 50 μm. L = Leydig cells; ST = seminiferous tubules.

expression of kisspeptin in the testes. A previous study found the expression of *Kiss1* and *Kiss1r* in Leydig cells.[33] However, kisspeptin10 does not stimulate testosterone production from primary cultured testes tissue.[34] In our study, with primary mouse Leydig cells, we showed that ovine LH significantly stimulated testosterone production after 4 hours' or 24 hours' treatment. However, cotreatment with kisspeptin10 did not affect testosterone production. In adult mouse Leydig cells treated with an antagonist of kisspeptin, peptide234 still did not affect ovine LH-stimulated testosterone production. Moreover, to test if kisspeptin affects activities of steroidogenic enzymes, including CYP11A1 and HSD3B1, primary mouse Leydig cells were treated with kisspeptin in the presence of 22-hydroxycholesterol or pregnenolone. Our results showed that the activity of neither CYP11A1 nor HSD3B1 was altered by kisspeptin. Our results suggest that kisspeptin does not affect steroidogenesis of Leydig cells, which conflicts with those of a previous study, in which kisspeptin stimulated progesterone production in immature rat luteal cells.[35] In that study, luteal cells were collected from juvenile rats, which had been stimulated with PMSG and hCG to promote ovulation. By contrast, with Leydig cells collected from adult male mice without any pretreatment, we showed that kisspeptin10 and its antagonist did not directly affect LH-stimulated testosterone production in adult Leydig cells when the expression of KISS1R was found in these cells. The conflicting results may be due to the ages of the animals, types of cell, or pretreatment of mice with hormones or not prior to acquisition of primary cells, even though luteal cells and Leydig cells share the same steroidogenesis pathway.

Although kisspeptin showed no effect on primary Leydig cell steroidogenesis, we found that testosterone levels increased from the age of 2 weeks (prepuberty) to that of 15 weeks (adult), with a maximum at 4 weeks (puberty). One study showed that chronic administration of kisspeptin10 by implantation into the yellowtail kingfish promoted the expression of *fshb* and *lhb* mRNA in the pituitary and induced development of spermatogonia and secondary spermatocytes.[36] Similar to the levels of testosterone, the expression of *Lhcgr*, *Star*, and *Cyp11a1* in the present study increased and reached a plateau around the age of 4 weeks, explaining the increasing testosterone level during puberty.

Expression of *Kiss1* and *Esr1* also increased from 2 weeks to 4 weeks. Chianese et al[37] reported that estradiol treatment induced *Kiss1r* expression in frog testes, while treatment with kisspeptin increased the expression of ERa. Our results are consistent with their observations, as *Kiss1* and *Esr1* expression increased coinstantaneously.

As mentioned previously, the expression of *Kiss1r* is low in intertitial cells and that of *Kiss1* is higher.[38] In our study, with immnuohistochemistry staining, we found the expression of

KISS1R in the testes of 2-, 5-, and 15-week-old mice. This result was confirmed with our real-time PCR data, which showed that the expression of *Kiss1r* did not alter in 2–15-week-old mice. Expression of *Kiss1* was found to increase during 2–4 weeks to attain its highest level and maintained that expression level until the age of 15 weeks. The expression pattern was also shown in immunohistochemistry staining; the signal of KISS1 was not found in the testes of 2-week-old mice but was found in the testes of 5- and 15-week-old mice.

In general, our results indicated that the gonadosomatic index reached the highest level in 4-week-old mice, whereas testosterone rapidly increased from the age of 3 weeks or 3 weeks. We also found that *Kiss1* and *Kiss1r* were specifically expressed in mouse Leydig cells, and expression levels of *Kiss1* was increased after puberty. However, increased expression of KISS1 did not appear to be involved in the regulation of steroidogenesis. In male chub mackerel (*Scomber japonicus*), administration of Kiss1 1-15 increased the gonadosomatic index, as well as the number of spermatozoa, spermatocytes, and spermatids.[39] Based on these reports and our immunohistochemistry staining showing strong expression of kisspeptin in 5- and 15-week-old mice but weak expression in the testes of 2-week-old mice, we propose that kisspeptin expression in Leydig cell may be correlated with the maturation of Leydig cells or the development of the testes during puberty. Further investigations are required to determine if KISS1 is directly involved in testicular development.

Acknowledgments

This work was supported by grants from National Science Council, Taiwan, Republic of China (NSC101-2313-B-002-016 and NSC101-2313-B-002-028).

References

1. Gottsch ML, Clifton DK, Steiner RA. From KISS1 to kisspeptins: an historical perspective and suggested nomenclature. *Peptides* 2009;**30**:4–9.
2. Colledge WH. GPR54 and kisspeptins. *Results Probl Cell Differ* 2008;**46**:117–43.
3. Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, et al. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology* 2004;**145**:4073–7.
4. Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. Regulation of *Kiss1* gene expression in the brain of the female mouse. *Endocrinology* 2005;**146**:3686–92.
5. Smith JT, Dungan HM, Stoll EA, Gottsch ML, Braun RE, Eacker SM, et al. Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology* 2005;**146**:2976–84.
6. Horikoshi Y, Matsumoto H, Takatsu Y, Ohtaki T, Kitada C, Usuki S, et al. Dramatic elevation of plasma metastin concentrations in human pregnancy: metastin as a novel placenta-derived hormone in humans. *J Clin Endocrinol Metab* 2003;**88**:914–9.
7. Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, et al. Metastasis suppressor gene *KiSS-1* encodes peptide ligand of a G-protein-coupled receptor. *Nature* 2001;**411**:613–7.
8. Matsui H, Takatsu Y, Kumano S, Matsumoto H, Ohtaki T. Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. *Biochem Biophys Res Commun* 2004;**320**:383–8.
9. Navarro VM, Castellano JM, Fernandez-Fernandez R, Barreiro ML, Roa J, Sanchez-Criado JE, et al. Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. *Endocrinology* 2004;**145**:4565–74.
10. Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, et al. Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. *Endocrinology* 2005;**146**:1689–97.
11. Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, et al. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci U S A* 2005;**102**:1761–6.
12. Plant TM, Marshall GR. The functional significance of FSH in spermatogenesis and the control of its secretion in male primates. *Endocr Rev* 2001;**22**:764–86.
13. Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM. Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. *Proc Natl Acad Sci U S A* 2005;**102**:2129–34.
14. Dhillo WS. Kisspeptin: a novel regulator of reproductive function. *J Neuroendocrinol* 2008;**20**:963–70.
15. Richards JS. New signaling pathways for hormones and cyclic adenosine 3′,5′-monophosphate action in endocrine cells. *Mol Endocrinol* 2001;**15**:209–18.
16. Stocco DM. StAR protein and the regulation of steroid hormone biosynthesis. *Annu Rev Physiol* 2001;**63**:193–213.
17. Lavoie HA, King SR. Transcriptional regulation of steroidogenic genes: STARD1, CYP11A1 and *HSD3B*. *Exp Biol Med (Maywood)* 2009;**234**:880–907.
18. Stocco DM, Clark BJ. Regulation of the acute production of steroids in steroidogenic cells. *Endocr Rev* 1996;**17**:221–44.
19. Tsui KH, Wang JY, Wu LS, Chiu CH. Molecular mechanism of isocupressic acid suppresses MA-10 cell steroidogenesis. *Evid based Complement Altern Med* 2012;**2012**:190107. http://dx.doi.org/10.1155/2012/190107.
20. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, et al. The metastasis suppressor gene *KiSS-1* encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem* 2001;**276**:34631–6.
21. Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ, Calamari A, et al. AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. *J Biol Chem* 2001;**276**:28969–75.
22. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A* 2003;**100**:10972–6.
23. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno Jr JS, Shagoury JK, et al. The *GPR54* gene as a regulator of puberty. *N Engl J Med* 2003;**349**:1614–27.
24. Li Q, Roa A, Clarke IJ, Smith JT. Seasonal variation in the gonadotropin-releasing hormone response to kisspeptin in sheep: possible kisspeptin regulation of the kisspeptin receptor. *Neuroendocrinology* 2012;**96**:212–21.
25. Migaud H, Ismail R, Cowan M, Davie A. Kisspeptin and seasonal control of reproduction in male European sea bass (*Dicentrarchus labrax*). *Gen Comp Endocrinol* 2012;**179**:384–99.
26. Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, et al. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. *Biochem Biophys Res Commun* 2003;**312**:1357–63.
27. Thompson EL, Murphy KG, Patterson M, Bewick GA, Stamp GW, Curtis AE, et al. Chronic subcutaneous administration of kisspeptin-54 causes testicular degeneration in adult male rats. *Am J Physiol Endocrinol Metab* 2006;**291**:E1074–82.
28. Castellano JM, Navarro VM, Fernandez-Fernandez R, Roa J, Vigo E, Pineda R, et al. Expression of hypothalamic KiSS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats. *Diabetes* 2006;**55**:2602–10.

29. Gaytan F, Gaytan M, Castellano JM, Romero M, Roa J, Aparicio B, et al. KiSS-1 in the mammalian ovary: distribution of kisspeptin in human and marmoset and alterations in KiSS-1 mRNA levels in a rat model of ovulatory dysfunction. *Am J Physiol Endocrinol Metab* 2009;**296**:E520−31.
30. Thompson EL, Amber V, Stamp GW, Patterson M, Curtis AE, Cooke JH, et al. Kisspeptin-54 at high doses acutely induces testicular degeneration in adult male rats via central mechanisms. *Br J Pharmacol* 2009;**156**:609−25.
31. Ramzan F, Qureshi IZ. Intraperitoneal kisspeptin-10 administration induces dose-dependent degenerative changes in maturing rat testes. *Life Sci* 2011;**88**:246−56.
32. Irfan S, Ehmcke J, Wahab F, Shahab M, Schlatt S. Intratesticular action of kisspeptin in rhesus monkey (*Macaca mulatta*). *Andrologia* 2014;**46**:610−7.
33. Anjum S, Krishna A, Sridaran R, Tsutsui K. Localization of gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH), kisspeptin and GnRH receptor and their possible roles in testicular activities from birth to senescence in mice. *J Exp Zool A Ecol Genet Physiol* 2012;**317**:630−44.
34. Mei H, Doran J, Kyle V, Yeo SH, Colledge WH. Does kisspeptin signaling have a role in the testes? *Front Endocrinol (Lausanne)* 2013;**4**:198.
35. Peng J, Tang M, Zhang BP, Zhang P, Zhong T, Zong T, et al. Kisspeptin stimulates progesterone secretion via the Erk1/2 mitogen-activated protein kinase signaling pathway in rat luteal cells. *Fertil Steril* 2013;**99**. 1436−43, e1.
36. Nocillado JN, Zohar Y, Biran J, Levavi-Sivan B, Elizur A. Chronic kisspeptin administration stimulated gonadal development in pre-pubertal male yellowtail kingfish (*Seriola lalandi*; Perciformes) during the breeding and non-breeding season. *Gen Comp Endocrinol* 2013;**191**:168−76.
37. Chianese R, Ciaramella V, Fasano S, Pierantoni R, Meccariello R. Kisspeptin receptor, GPR54, as a candidate for the regulation of testicular activity in the frog *Rana esculenta*. *Biol Reprod* 2013;**88**:73.
38. Hsu MC, Wang JY, Lee YJ, Jong DS, Tsui KH, Chiu CH. Kisspeptin modulates fertilization capability of mouse spermatozoa. *Reproduction* 2014;**147**:835−45.
39. Selvaraj S, Ohga H, Nyuji M, Kitano H, Nagano N, Yamaguchi A, et al. Subcutaneous administration of Kiss1 pentadecapeptide accelerates spermatogenesis in prepubertal male chub mackerel (*Scomber japonicus*). *Comp Biochem Physiol A Mol Integr Physiol* 2013;**166**:228−36.