REPRODUCTION
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Kisspeptin modulates fertilization capacity of mouse spermatozoa

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Abstract

Kisspeptin acts as an upstream regulator of the hypothalamus–pituitary–gonad axis, which is one of the main regulatory systems for mammalian reproduction. *Kiss1* and its receptor *Kiss1r* (also known as G protein-coupled receptor 54 (*Gpr54*)) are expressed in various organs, but their functions are not well understood. The purpose of this study was to investigate the expression profiles and functions of kisspeptin and KISS1R in the reproductive tissues of imprinting control region mice. To identify the expression pattern and location of kisspeptin and KISS1R in gonads, testes and ovarian tissues were examined by immunohistochemical or immunofluorescent staining. Kisspeptin and KISS1R were expressed primarily in Leydig cells and seminiferous tubules respectively. KISS1R was specifically localized in the acrosomal region of spermatids and mature spermatozoa. Kisspeptin, but not KISS1R, was expressed in the cumulus–oocyte complex and oviductal epithelium of ovarian and oviductal tissues. The sperm intracellular calcium concentrations significantly increased in response to treatment with kisspeptin 10 in Fluo-4-loaded sperm. The IVF rates decreased after treatment of sperm with the kisspeptin antagonist peptide 234. These results suggest that kisspeptin and KISS1R might be involved in the fertilization process in the female reproductive tract. In summary, this study indicates that kisspeptin and KISS1R are expressed in female and male gametes, respectively, and in mouse reproductive tissues. These data strongly suggest that the kisspeptin system could regulate mammalian fertilization and reproduction.

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Introduction

Mammalian fertilization is a complex process during which a spermatozoon meets and fuses with an oocyte within an oviduct. Mammalian spermatozoa proceed through multiple modifications and reactions to successfully fertilize oocytes, including maturation in the epididymis, capacitation in the female reproductive ducts, hyperactivation of motility, and an acrosome reaction (Naz & Rajesh 2004, Yoshida *et al*. 2008). Only capacitated spermatozoa recognize and bind to the oocyte zona pellucida and complete the acrosome reaction (Fraser 2010). Oviducts present a complex environment involving specific signals from oviductal and follicular fluids that promote sperm capacitation (Lee *et al*. 1976, Hunter 2005). These signals activate multiple events, including increasing intracellular calcium concentration ($[Ca^{2+}]_i$), pH, and cAMP levels, and inducing phosphorylation of specific tyrosine residues (Visconti *et al*. 1995, 1999, Fraser 2010). However, the activation mechanisms of receptors and channels on the sperm plasma membrane are poorly understood.

Kisspeptins belong to the arginine–phenylalanine amide peptide family and were originally identified as products of the metastasis suppressor gene *Kiss1* (Kotani *et al*. 2001, Muir *et al*. 2001, Ohtaki *et al*. 2001). The kisspeptin precursor contains 145 amino acids (kisspeptin 145), which is cleaved into peptides containing 54 amino acids (kisspeptin 54), 14 amino acids (kisspeptin 14), 13 amino acids (kisspeptin 13), or 10 amino acids (kisspeptin 10). Kisspeptin peptides share a common C-terminal decapeptide Arg–Phe–NH_2 motif and have similar biological activities (Colledge 2008). Kisspeptins and the kisspeptin receptor KISS1R (also known as the G protein-coupled receptor 54) play key roles in mammalian reproduction by regulating gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus (de Roux *et al*. 2003, Seminara *et al*. 2003). Mutations in *Kiss1* or *Kiss1r* in mice cause hypogonadotropic hypogonadism, a syndrome

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characterized by deficient production of gonadotropins and sex steroids, which prevents complete sexual maturation.

Kiss1 and/or *Kiss1r* transcripts or proteins have been detected in the hypothalamus and the peripheral reproductive tissues, suggesting additional regulatory roles for kisspeptin in reproduction. In this context, regional- and cycle-specific patterns of kisspeptin expression have been described in the rat oviduct. Kisspeptin has been proposed to be a physiological regulator that prevents ectopic implantation (Gaytan *et al*. 2007). However, the distribution of KISS1R in rodent oviducts remains unclear. Both kisspeptin and its receptor are expressed in human and rat ovaries. The *Kiss1* gene is upregulated by a preovulatory luteinizing hormone (LH) surge in rats, suggesting a potential role in ovulation (Castellano *et al*. 2006, Gaytan *et al*. 2009). *Kiss1* and *Kiss1r* gene expression has been reported in human and rodent testes (Ohtaki *et al*. 2001, Terao *et al*. 2004, Zhao *et al*. 2010); however, protein expression and function were incompletely understood. Recently, kisspeptin and KISS1R were detected in human spermatozoa and found to affect motility (Pinto *et al*. 2012). A previous study showed that kisspeptin was present in the oviduct, suggesting that kisspeptin may participate in capacitation, acrosome reaction, and/or fertilization. To test this hypothesis, we examined the mRNA and/or protein expression patterns for the kisspeptin/KISS1R system in mouse gonads. This work provides evidence that kisspeptin regulates fertilization by mouse spermatozoa.

Materials and methods

Animals and materials

Adult male (10–16 weeks old) and female (8–12 weeks old) imprinting control region (ICR) mice were purchased from the National Taiwan University, maintained in a 12 h light (0800–2000 h):12 h darkness (2000–0800 h) cycle at 22 ± 1 °C, and provided a chow diet and water *ad libitum* for the duration of the study. Initial RT-PCR, histology, and kisspeptin and KISS1R expression patterns were examined in 12 mice. The intracellular concentration of free calcium ($[Ca^{2+}]_i$) in spermatozoa was examined in nine male mice. Ten mice of each gender were used in the IVF experiment. All experimental protocols were approved by the Institutional Animal Care and Use Committee, College of Medicine, National Taiwan University. All procedures conformed to the National Institutes of Health Guide for the care and use of laboratory animals.

All nucleotide primers were obtained from Bio Basic (Markham, ON, Canada). The oligopeptide kisspeptin 10 and its antagonist, peptide 234 (Roseweir *et al*. 2009), were purchased from Kelowna International Scientific (Taipei, Taiwan). All other reagents were purchased from Sigma–Aldrich or Invitrogen unless otherwise specified.

Isolation of seminiferous tubules and interstitial cells

The method for isolation of seminiferous tubules and interstitial cells was described previously (Tsui *et al*. 2012). Briefly, mouse testis tunica albuginea was removed and the testicular contents were washed once with isolation buffer 1×Hanks' balanced salt solution (Invitrogen), containing 0.1% (w/v) BSA and 200 U/ml collagenase type 1 (Worthington, Lakewood, CA, USA). Then, the testicular contents were transferred to fresh isolation buffer and incubated at 37 °C with platform shaking at 50 r.p.m. for 5 min. The testicular contents were passed through a 250-mesh nylon filter and separated into seminiferous tubules and interstitium suspended in filtrate. Interstitial cells were collected by centrifugation at 300 ***g*** for 5 min, and upper media were removed. Both seminiferous tubules and interstitial cells were frozen in liquid nitrogen and stored at −80 °C until use.

Semiquantitative RT-PCR analysis of mRNA transcripts

Total RNA was extracted from seminiferous tubules and interstitial cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To synthesize cDNA, total RNA (1 μg) was mixed with 2.5 μM oligo(dT) and 500 μM deoxynucleotide triphosphate, and denatured at 65 °C for 5 min. The sample was cooled on ice, then combined with 40 IU RNaseOUT RNase inhibitor (Invitrogen), 5 mM dithiothreitol, and 200 IU SuperScript III reverse transcriptase (Invitrogen), and incubated at 50 °C for 60 min. The reaction was inactivated by heating to 70 °C for 15 min, and cDNA products were stored at 4 °C. PCR consisted of a denaturing cycle at 95 °C for 2 min, followed by a variable number of amplification cycles defined by denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 40 s. A final extension cycle of 72 °C for 2 min was included. The primer sequences were as follows: *Kiss1* (forward, 5′-TCGTTAATGCCTGGGAAAAG-3′ and reverse, 5′-TTCCAGTTGTAGGTGGACAGG-3′; accession number, NM_178260.3); *Kiss1r* (forward, 5′-GTCGGAAACTCATTGGTCATCTAC-3′ and reverse, 5′-AGCGGGAACACAGTCACATAC-3′; accession number, NM_053244.5); and *Gapdh* (forward, 5′-GCCAAAAGGGTCATCATCTC-3′ and reverse, 5′-CTTACTCCTTGGAGGCCATGT-3′; accession number, NM_008084.2). Primer pairs were so designed that each primer was separated by at least one intron. Based on optimal amplification conditions for semiquantitative analysis of the specific targets, 40 PCR cycles were performed for *Kiss1* and *Kiss1r*, and 25 PCR cycles were performed for *Gapdh*. RT-PCR signal intensities were quantified using VisionWorksLS Image Analysis Software (UVP, Upland, CA, USA). Relative gene expression values were normalized with respect to those of the internal control (*Gapdh*) and presented as the fold change compared with that of control seminiferous tubules.

Immunoblot analysis

Tissues were ground with a mechanical homogenizer in cold lysis buffer (2% SDS, 50 mM Tris, pH 6.8, 5 mM EDTA, 1% 2-mercaptoethanol, and 5% glycerol), and whole-tissue

extracts were prepared. Samples containing 30–60 μg protein were separated by 15% SDS–PAGE. The separated proteins were transferred onto a PVDF membrane. The membrane was blocked by incubating in PBS containing 0.01% Tween 20 (PBST) and 2.5% BSA for 8 h at room temperature, followed by incubation with rabbit polyclonal antibodies specific for kisspeptin 145 (1:100 dilution; product no. ab19028, Abcam, Cambridge, UK) and KISS1R (1:500 dilution; product no. sc-134499; Santa Cruz Biotechnology) in PBST for 18 h at room temperature. Then, the membrane was washed four times with PBST and incubated for 2 h with peroxidase-conjugated goat anti-rabbit IgG (1:25 000 dilution; Jackson ImmunoResearch Laboratory, West Grove, PA, USA). The membrane was washed with PBST and bound antibodies were visualized by the ECL system (Merck Millipore) using Kodak X-OMAT film (Eastman Kodak Co.). The same extracts were separated with 15% SDS–PAGE and stained with Coomassie blue as a loading control.

Immunohistochemistry and immunofluorescence analysis

Formalin-fixed mouse tissues were embedded in paraffin, sectioned into 5-μm thick sections, and mounted on poly-L-lysine-coated slides. Following deparaffinization in xylene, tissues were rehydrated by passing through descending concentrations of ethanol and washing with PBS at room temperature. Retrieval of antigen-binding sites was achieved by incubating twice for 10 min in 10 mM sodium citrate buffer, pH 6.0, containing 0.05% (v/v) Tween 20, that was heated in a microwave oven. After quenching endogenous peroxidase activity with 1% (v/v) H_2O_2 in methanol for 30 min, the sections were rinsed three times for 5 min each with PBS. Slides were incubated in a blocking buffer (PBS containing 3% (v/v) normal goat serum and 0.2% (v/v) Triton X-100) for 3 h. Then, the slides were incubated with the following primary antibodies diluted in blocking buffer for 24 h at 4 °C in a moist chamber: rabbit anti-kisspeptin 10 (1:800 dilution; product no. T-4771, Bachem, Torrance, CA, USA); rabbit anti-kisspeptin 145 (1:100 dilution; Abcam); and rabbit anti-KISS1R (1:100 dilution; product no. ab12698, Abcam). Negative controls for antibodies were established using blocking buffer alone. Slides were washed five times for 5 min each with PBS at room temperature and incubated with biotinylated goat anti-rabbit secondary antibodies for 60 min in a moist chamber. The sections were rinsed again before incubating with an avidin–biotin–HRP complex in the Vectastain Universal ELITE ABC kit (Vector Laboratories, Burlington, ON, Canada) for 30 min according to the manufacturer's instructions. After additional rinsing, slides were incubated for 10–20 min at room temperature with diaminobenzidine to visualize immunostaining. Finally, slides were rinsed with distilled water twice for 10 min each, counterstained with hematoxylin for 30 s, and hydrated with ethanol and xylene before 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).

To evaluate the location of KISS1R, testicular sections and sperm smear slides were examined using triple-labeled immunofluorescent staining with acrosomal marker zona pellucida 3 receptor (ZP3R) and Hoechst 33342 (Sigma–Aldrich). Tissue slides were prepared as described above, with an additional treatment using 25 μg/ml trypsin at 37 °C for 10 min to retrieve the sp56 antigen. Slides were incubated with the diluted rabbit anti-KISS1R antibody and mouse anti-sp56 antibody (1:100 dilution; product no. 55101, QED Bioscience, San Diego, CA, USA) for 24 h. Slides were washed five times for 5 min each with PBS at room temperature, followed by a 1-h incubation with DyLight 488-conjugated goat anti-rabbit and DyLight 594-conjugated goat anti-mouse secondary antibodies (1:400 dilution; Jackson ImmunoResearch Laboratory), and 2 μg/ml Hoechst 33342 for nuclear staining, in blocking buffer at room temperature. For sperm smear samples, slides were rinsed with methanol for 5 min and processed by blocking and incubating with antibodies as described above. After washing three times for 5 min each with PBS, slides were mounted with Prolong Gold antifade reagent (Invitrogen) and observed with a confocal microscope TCS SP5 II (Leica, Wetzlar, Germany).

Sperm isolation

The method of sperm preparation for intracellular Ca^{2+} measurement was described previously (Lee *et al.* 2005). Preparation of sperm for smear slides and $[Ca^{2+}]_i$ measurements was performed in modified HEPES medium (HM) (120 mM NaCl, 2 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 0.36 mM NaH_2PO_4, 15 mM $NaHCO_3$, 10 mM HEPES, 5.6 mM glucose, 1.1 mM sodium pyruvate, 18.5 mM sucrose, 100 IU/ml penicillin, and 100 μg/ml streptomycin). The media pH values were adjusted to 7.3–7.4 by aerating in 5% CO_2 in an incubator at 37 °C for 24 h before use. The cauda epididymis was cut into pieces, and motile sperm were allowed to swim in the media at 37 °C for 20 min. Sperm were separated on a 1-ml 75% (v/v) Percoll gradient, centrifuged at 300 ***g*** for 30 min, and collected to measure $[Ca^{2+}]_i$. Cells were washed twice with HM by centrifugation at 100 ***g*** for 5 min and resuspended in HM. Sperm smear slides were prepared by smearing sperm suspensions onto poly-L-lysine-coated slides followed by air-drying. Slides were fixed in cold methanol for 2 min, air-dried again, and stored at −20 °C. For IVF experiments, spermatozoa isolated from cauda epididymis were allowed to swim in commercial human tubal fluid (HTF; Merck Millipore) at 37 °C for 20 min, and diluted to a final concentration of 1×10^6 sperm/ml for further procedures.

Measurement of intracellular free Ca^{2+} in spermatozoa

Changes in intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured in noncapacitated sperm loaded with Fluo-4 (Invitrogen) in 96-well black plates using a Synergy H1 Hybrid Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA) at 37°C. Sperm (1×10^8 cells/ml) were loaded with 15 μM Fluo-4 AM, which is a cell-permeable fluorescent Ca^{2+} indicator, and incubated in the dark at 37 °C for 45 min. Excess dye was removed by three centrifugation steps at 100 ***g*** for 5 min at room temperature. Pellets were resuspended in HM, with or without 1.8 mM $CaCl_2$ for normal conditions or low Ca^{2+} conditions, and adjusted to 5×10^6 cells/ml. Each well was filled with 100 μl

sperm suspension; fluorescence was excited at 485 nm and emission was recorded at 520 nm. Fluorescence was recorded before and after addition of 100 µl (1:1 dilution) solutions containing 25–200 µM kisspeptin 10. The change in $[Ca^{2+}]_i$ was measured as $F'–F_0/F_0$ (%), where F' is the fluorescence intensity at the experimental time point and F_0 is the average fluorescence intensity before treatment (−5 to 0 min). The statistical result was calculated as the average change in $[Ca^{2+}]_i$ during the sustained plateau fluorescence phase (27–30 min).

IVF

Noncapacitated or capacitated sperm were incubated in HTF and treated with peptide 234 during capacitation or acrosome reaction to test its effect on fertilization. In the control, 0.9% saline was substituted for the peptide 234 solution. Detailed procedures are described below. For the capacitation step, motile sperm (1×10^6 sperm/ml) were placed in capacitated conditional medium (HTF containing 0.3% fatty acid free BSA) and supplemented with 50 µM peptide 234 or with saline for 2 h at 37 °C in culture chambers. Then, the fertilization step was performed by adding cumulus–oocyte complexes (COCs; see next paragraph for preparation details) to the wells and incubating with or without 50 µM peptide 234 for 6 h at 27 °C. Then, eggs were washed three times with potassium simplex optimized medium plus amino acids (KSOM+AAs; Merck Millipore) and cultured in fresh KSOM+AAs. Morphological assessment of cleavage was performed to verify successful fertilization, which was defined as cleavage into the two-cell embryo stage. The fertilization rate was determined by counting the number of two-cell embryos at 24 h.

Preparation of COCs was as follows: female mice were treated with 10 IU pregnant mares' serum gonadotropin (PMSG; Sigma–Aldrich) and 10 IU human chorionic gonadotropin (hCG; China Chemical & Pharmaceutical, Taipei, Taiwan.) by i.p. injection 48 h apart to induce superovulation. Bilateral oviducts were excised 12–14 h after hCG injection, and COCs were released from the ampulla into HTF that contained sperm with mineral oil.

Statistical analysis

Each experiment was replicated at least three times. Data were expressed as means+S.E.M. Data were analyzed by Student's *t*-test or one-way ANOVA followed by Duncan's method with Sigma Plot Software (Systat Software, San Jose, CA, USA); $P<0.05$ indicated significance.

Results

Expression of Kiss1 *and* Kiss1r *genes in seminiferous tubules and interstitial cells*

Previous studies reported that *Kiss1* and *Kiss1r* are expressed in the testes (Ohtaki *et al.* 2001, Funes *et al.* 2003). To explore the expression patterns of these genes, testicular tissues from adult mice were separated into seminiferous tubules and interstitial cells, and analyzed by semiquantitative RT-PCR and immunoblot assay (Fig. 1A and D). *Kiss1* transcript levels were 3.3-fold higher in interstitial cells than in seminiferous tubules ($n=3$, $P<0.05$; Fig. 1B). By contrast, *Kiss1r* transcript levels were 14.3-fold lower in interstitial cells than in seminiferous tubules ($n=3$, $P<0.001$; Fig. 1C). These results suggest that kisspeptin (*Kiss1*) and KISS1R (*Kiss1r*) were expressed predominantly in interstitial cells and seminiferous tubules, respectively, in mouse testes.

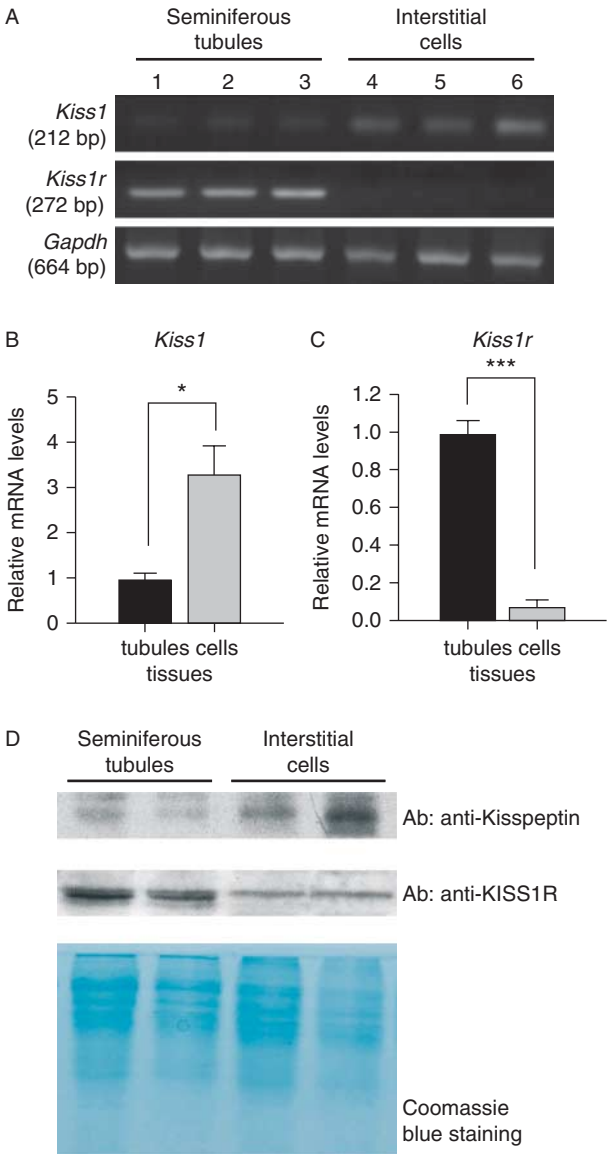

Figure 1 Expression profiles of kisspeptin (*Kiss1*) and KISS1R (*Kiss1r*) in mouse testicular tissues. (A) Seminiferous tubules (lanes 1–3) and interstitial cells (lanes 4–6) were isolated from the testes of three adult ICR mice and analyzed by RT-PCR for *Kiss1* and *Kiss1r* expression. (B and C) *Kiss1* and *Kiss1r* mRNA expression levels in seminiferous tubules (tubules) and interstitial cells (cells) were quantified using imaging analysis software. (D) Seminiferous tubules and interstitial cells from two mice were analyzed for kisspeptin and KISS1R protein expression by immunoblot assay. Loading controls are shown as Coomassie blue staining. *$P<0.05$ and ***$P<0.001$. Error bars are means+S.D.

Immunodetection of KISS1R and kisspeptin in seminiferous tubules and interstitial cells

Gene expression analyses were complemented by immunohistochemical assays to determine the expression and distribution patterns for KISS1R and kisspeptin proteins in testicular tissues from adult mice. Strong KISS1R immunoreactivity was detected in spermatids (Fig. 2A), but not in spermatogonia, spermatocytes, or Sertoli cells in seminiferous tubules (Fig. 3A and B). KISS1R was rarely detected in Leydig cells of the interstitial testicular compartment (Fig. 2D), but ~12.4±4.9% of total Leydig cells (mean±S.D.; $n=7$; at least 300 cells counted in each mouse testis) expressed KISS1R. By contrast, intense kisspeptin immunoreactivity was detected in Leydig cells of the interstitial testicular compartment (Fig. 2E). Kisspeptin immunostaining was undetectable in the majority of cells in the seminiferous tubules, but it was detected in the residual bodies of elongated spermatids, which are the transitional cells from which spermatozoa develop during spermiogenesis (Fig. 2B). Strong immunostaining for KISS1R and kisspeptin was present in the trophoblast giant cells and syncytiotrophoblast, respectively, which served as positive controls (Fig. 2G and H). As negative controls for the immunohistochemical procedure, adjacent sections were incubated without primary antibodies (Fig. 2C, F and I).

KISS1R was specifically expressed in spermatids (Fig. 2A) and not in spermatogonia or spermatocytes (Fig. 3A and B). To verify this observation, KISS1R immunostaining was performed on spermatids at different maturation stages, including round and elongated spermatids (Fig. 3). We performed triple-fluorescence analysis on testicular sections and sperm smear slides, which were collected from cauda epididymis of adult ICR mice (Fig. 4). Samples were stained for KISS1R (green), the acrosomal marker zona pellucida 3 receptor ZP3R (red), and nucleus (blue) and visualized by confocal scanning laser microscopy. KISS1R colocalized with the acrosome but not with the cellular membrane in spermatids of different developmental stages. By contrast, triple-fluorescence analysis for kisspeptin (green), ZP3R (red), and nucleus (blue) using sperm smear slides showed undetectable kisspeptin expression in mouse spermatozoa (Supplementary Fig. 1, see section on supplementary data given at the end of this article). These results strongly suggest that KISS1R is expressed in mouse spermatids and mature spermatozoa.

Immunodetection of kisspeptin in epididymis

These results indicate that kisspeptin may have a role in spermatozoa. Epididymides provide an optimum environment for spermatozoal maturation and storage. Epididymal fluid accompanies ejaculated spermatozoa to seminal plasma and may protect spermatozoa (Reyes-Moreno *et al.* 2002). Strong kisspeptin immunoreactivity was detected in epididymal epithelium,

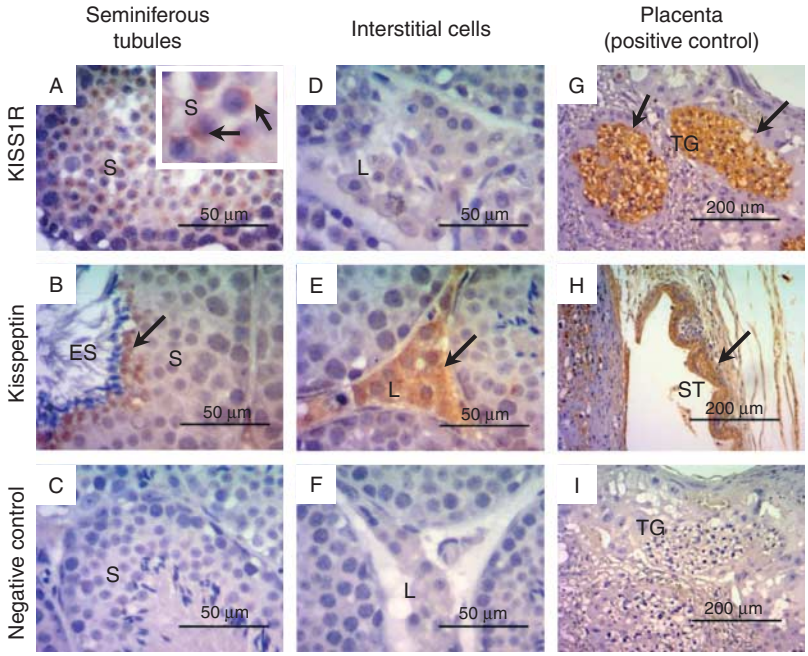

Figure 2 Immunohistochemical staining of KISS1R and kisspeptin in mouse testicular tissues. (A and B) Seminiferous tubules, (D and E) interstitial cells, and (G and H) placenta (immunostaining positive control) of adult ICR mice were stained for KISS1R (upper panel) or kisspeptin (middle panel) with specific antibodies. Positive immunostaining appeared brown in color and is indicated with an arrow. The insert panel shows the original image at 2.5× magnification. (C, F and I) Sections that were incubated without primary antibody are shown as negative controls. ES, elongated spermatid; S, spermatid; L, Leydig cell; TG, trophoblast giant cell; and ST, syncytiotrophoblast. Scale bars=50 µm (A, B, C, D, E and F) or 200 µm (G, H and I).

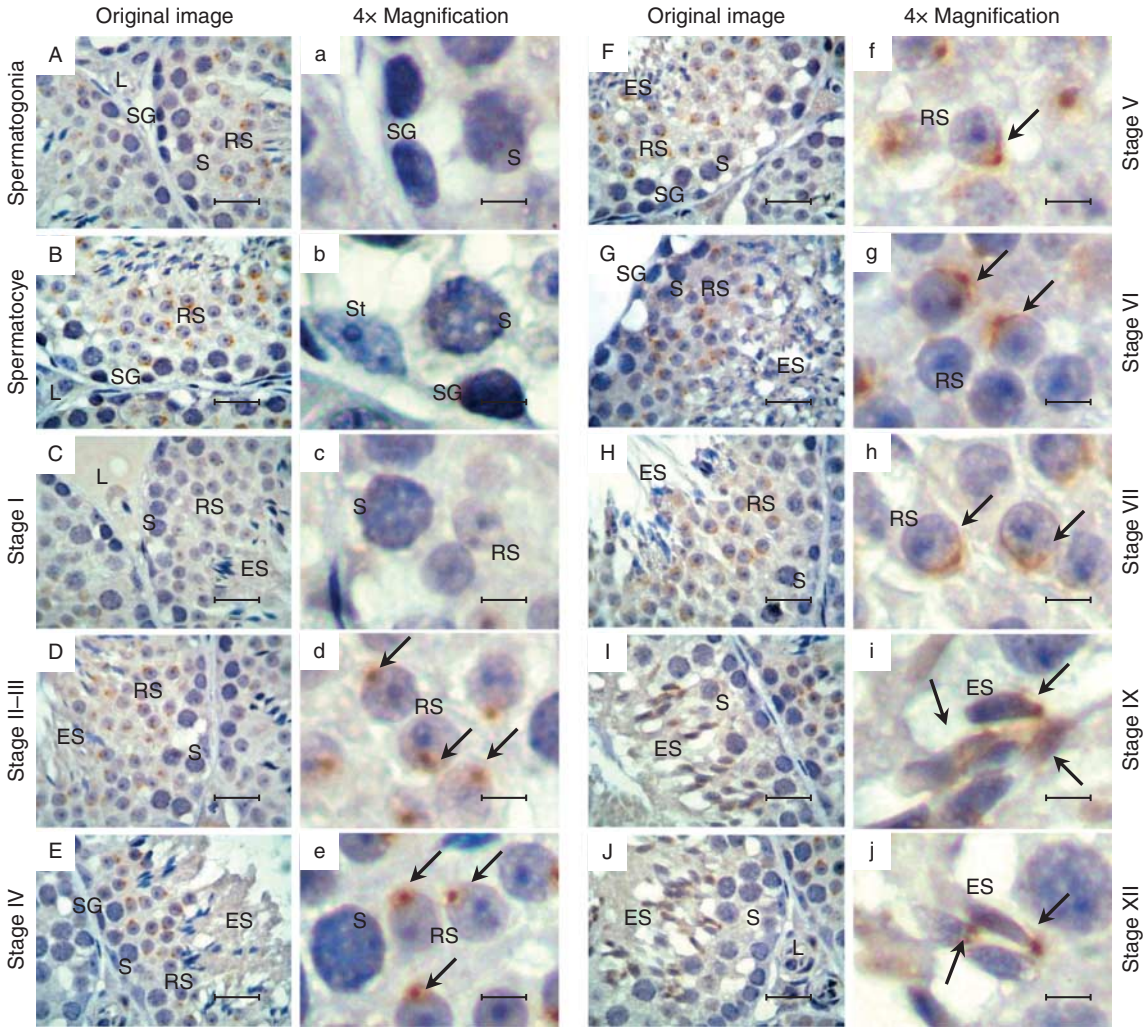

Figure 3 Immunohistochemical staining of KISS1R in spermatids at different maturation stages in mouse testicular tissue. Testicular tissue sections from adult ICR mice were stained for KISS1R with specific antibodies. KISS1R (brown) was specifically expressed during different stages of spermatid maturation. (A, B, C, D, E, F, G, H, I and J) Images were captured at 1000× magnification by light microscopy and (a, b, c, d, e, f, g, h, i and j) central view of the original image at 4× magnification. Immunoreactivity appeared brown in color and is indicated with an arrow. ES, elongated spermatid; RS, round spermatid; S, spermatocyte; SG, spermatogonia; St, Sertoli cell; and L, Leydig cell. Scale bars=20 μm (A, B, C, D, E, F, G and H) or 5 μm (a, b, c, d, e, f, g and h).

including caput, corpus, and cauda epididymes, but was not detected in the surrounding smooth muscle (Fig. 5). This is the first report of kisspeptins in epididymis.

Immunodetection of kisspeptin in ovaries and oviducts

To collect ovarian follicles and oviducts during a preovulatory stage, mice were killed following pretreatment with PMSG for 48 h. Kisspeptin immunoreactivity was detected in mouse ovarian tissues, specifically in follicles, corpora lutea, and interstitial glands. Kisspeptin was detected in the granulosa layer of preovulatory follicles, cumulus cells around oocytes, oocyte cytoplasm (Fig. 6A and B), and theca cells (data not shown). After ovulation, kisspeptin immunoreactivity was clearly detected in COCs (Supplementary Fig. 2, see section on supplementary data given at the end of this article). In addition, strong kisspeptin immunolabeling indicated the presence of kisspeptin in the ciliated oviduct epithelium (Fig. 6E and F). By contrast, KISS1R was undetected in granulosa cells and oocytes of preovulatory follicles and in the ciliated oviduct epithelium (Fig. 6C and G), but it was detected in the interstitial gland (data not shown).

Effect of kisspeptin on intracellular Ca^{2+} concentration in spermatozoa

The presence of KISS1R in mouse spermatozoa strongly suggests that the kisspeptin system plays a role in modulating sperm function, which is often associated with changes in $[Ca^{2+}]_i$ levels. Therefore, we analyzed

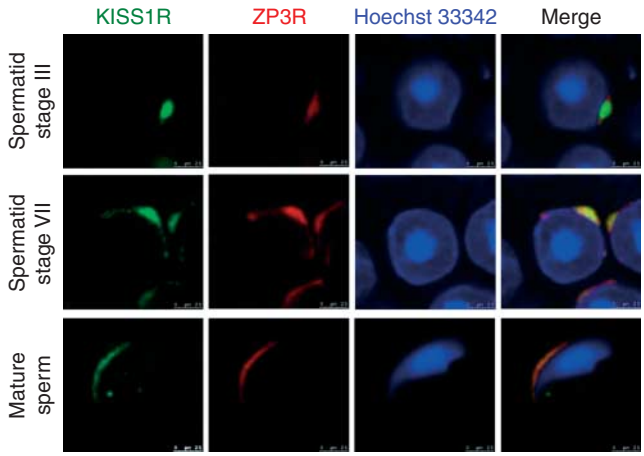

Figure 4 Immunofluorescent staining of KISS1R and ZP3R in early- and middle-stage spermatids and mature sperm. Triple-fluorescence analysis was performed on testicular sections and sperm collected from cauda epididymis of adult ICR mice by staining for KISS1R (green), ZP3R (red), and nucleus (blue). Images were viewed by confocal scanning laser microscopy. Scale bars = 2.5 μm.

the effects of kisspeptin on $[Ca^{2+}]_i$ in Fluo-4-loaded mouse spermatozoa. Treatment with 25, 50, and 100 μM kisspeptin 10 caused concentration-dependent $[Ca^{2+}]_i$ increases, with the fluorescence intensity increasing by 22.2±4.5, 85.5±14.4, and 129.9±24.9%, respectively, in mouse spermatozoa (Fig. 7A and B). Increases in the fluorescence intensity were rapidly induced and reached a plateau within 5–10 min after kisspeptin exposure. Previous studies suggested that activated KISS1R evoked intracellular calcium release via the G protein q/11 ($G_{q/11}$)–phospholipase C (PLC)–inositol 1,4,5-trisphosphate receptor ($InsP_3R$) signaling pathway in the transfected Chinese hamster ovary K1 cell line and in GnRH neurons (Kotani *et al.* 2001, Liu *et al.* 2008). We examined the nature of kisspeptin 10-induced calcium increase in mouse spermatozoa. In low-Ca^{2+} medium, the $[Ca^{2+}]_i$ displayed concentration-dependent increases of 71.1±18.3 and 206.6±14.1% in response to 50 and 100 μM kisspeptin 10 respectively (Fig. 7C and D). These results suggest that kisspeptin induces $[Ca^{2+}]_i$ mobilization via release of intracellular calcium.

Effect of kisspeptin antagonist on the fertilization capacity of spermatozoa

Taken together, these results suggest that fertilization may involve systemic modulation of kisspeptin, with KISS1R expressed on spermatozoa, and kisspeptin secreted by epididymal epithelium, oviductal epithelium, oocytes, and cumulus cells. We examined the fertilization capacity of spermatozoa in the absence of a kisspeptin signal during capacitation or acrosome reaction. The fertilization capacity of spermatozoa was estimated by performing IVF. Spermatozoa from cauda epididymis were incubated in capacitated conditional medium without (control group, addition of saline only) or with 50 μM peptide 234 (P234 group), a kisspeptin antagonist, for 2 h during the capacitation step, and then these spermatozoa were used for IVF assays. The fertilization rates of sperm treated with peptide 234 were significantly reduced (Fig. 8B; $n=7$; $P<0.05$) compared with those of controls. However, the fertilization rates of capacitated sperm treated with peptide 234 during the fertilization step were not significantly different from those of the control group (Fig. 8C; $n=3$; $P=0.21$). These results indicate that the fertilization capacities of mouse spermatozoa are reduced by a kisspeptin antagonist during capacitation.

Discussion

Previous studies reported that kisspeptin and its receptor, KISS1R, play important roles in regulating the hypothalamus–pituitary–gonad (HPG) axis, which controls reproductive functions such as puberty onset and seasonal breeding. However, *Kiss1* and *Kiss1r* are also expressed in other tissues in addition to the hypothalamus (Ohtaki *et al.* 2001, Funes *et al.* 2003). The function of kisspeptin in these tissues was unclear. In this study, we provide evidence that KISSR is expressed in mouse spermatozoa and kisspeptin signals may regulate the fertilization capacity of spermatozoa.

To the best of our knowledge, this is the first report of kisspeptin and KISS1R expression in mouse seminiferous

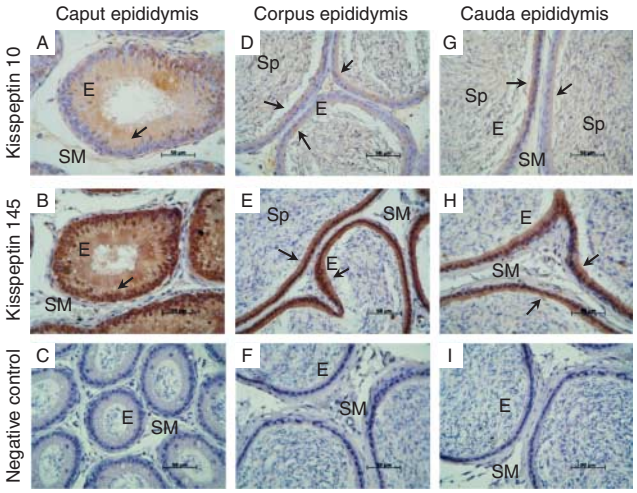

Figure 5 Immunohistochemical staining of kisspeptin 10 and kisspeptin 145 in mouse epididymis. Adult mouse caput (A and B), corpus (D and E), and cauda (G and H) epididymis sections were stained with an antibody against kisspeptin 10, which reacted with all active forms of kisspeptin, and an antibody against kisspeptin 145, which reacted with the kisspeptin precursor. Immunoreactivity appeared brown in color and is indicated with an arrow. (C, F and I) Sections incubated without primary antibody are shown as negative controls. E, epithelium; SM, smooth muscle; and Sp, sperm mass. Scale bar = 50 μm.

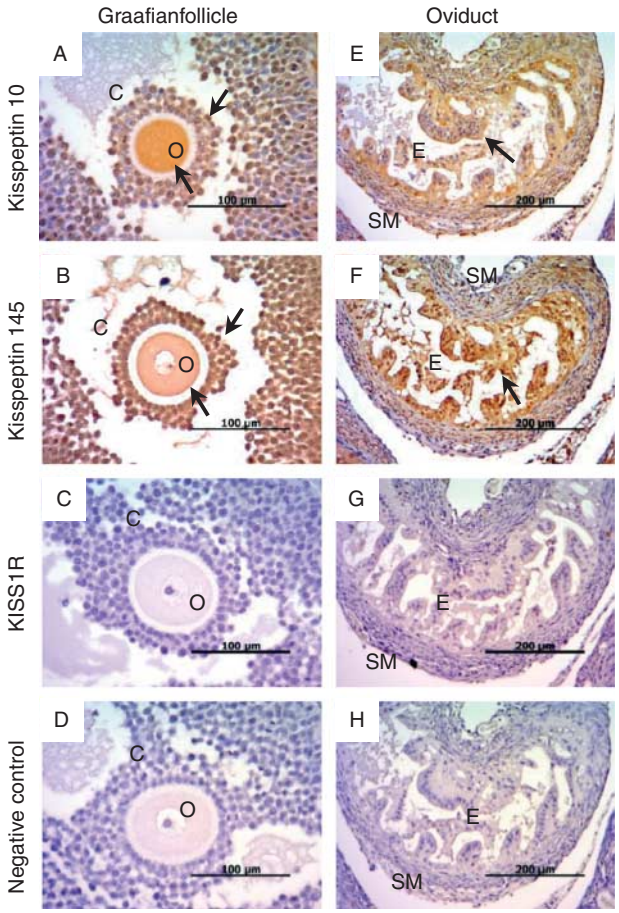

Figure 6 Immunohistochemical staining of kisspeptin 10, kisspeptin 145, and KISS1R in reproductive tissues from female mice. Ovarian (A, B and C) and oviductal (E, F and G) tissues were stained with anti-kisspeptin 10 (which reacted with all active forms of kisspeptin), anti-kisspeptin 145 (which reacted with kisspeptin precursor), and anti-KISS1R (which reacted with the kisspeptin receptor) antibodies. Immunoreactivity appeared brown in color and is indicated with an arrow. (D and H) Sections incubated without primary antibodies are shown as negative controls. O, oocyte; C, cumulus cell; E, epithelium; and SM, smooth muscle. Scale bars = 100 μm (A, B, C and D) or 200 μm (E, F, G and H).

tubules and interstitial cells. Our results show that Leydig cells primarily express kisspeptin, with lower levels of KISS1R expression. Primary mouse Leydig cells treated with kisspeptin 10 or kisspeptin antagonist with or without ovine LH did not show altered testosterone secretion (Supplementary Fig. 3, see section on supplementary data given at the end of this article), suggesting that kisspeptin may not affect steroidogenesis in Leydig cells. However, continuous s.c. administration or single high dose of kisspeptin 54 causes testicular degeneration in rats (Thompson *et al*. 2006, 2009, Ramzan & Qureshi 2011). Pretreatment with the GnRH receptor antagonist cetrorelix blocks kisspeptin-induced testicular degeneration, suggesting a GnRH-mediated process through acute hyperstimulation of the HPG axis and not a direct effect of kisspeptin on testes (Thompson *et al*. 2009). This study suggests that the kisspeptin/KISS1R system may regulate important testicular functions in an autocrine or paracrine way, but is not involved in steroidogenesis in Leydig cells.

The results of this study indicate that kisspeptin is robustly produced by Leydig cells in the interstitium and KISS1R is expressed by germ cells such as spermatid and mature sperm. Interactions between interstitial cells and germ cells mediated by the kisspeptin system may exist. However, KISS1R immunostaining was observed only in the acrosomal region and not at the plasma membrane of developing spermatids, suggesting that kisspeptin in Leydig cells may not affect spermiogenesis. The blood–testis barrier must be considered to assess when kisspeptin may gain access to these germ cells to exert its effects. Further experiments are needed to clarify whether kisspeptin acts on germ cells in testis tissue. Mature mouse sperm from cauda epididymis retain KISS1R at the acrosomal region, consistent with previous results in human sperm (Pinto *et al*. 2012). The current results reveal that kisspeptin 10 treatment induces concentration-dependent $[Ca^{2+}]_i$ increases. Therefore, it is highly likely that KISS1R may translocate from the acrosomal membrane to the plasma membrane during late spermiogenesis or maturation in the epididymis. This requires confirmation using high-magnification immunoelectron microscopy.

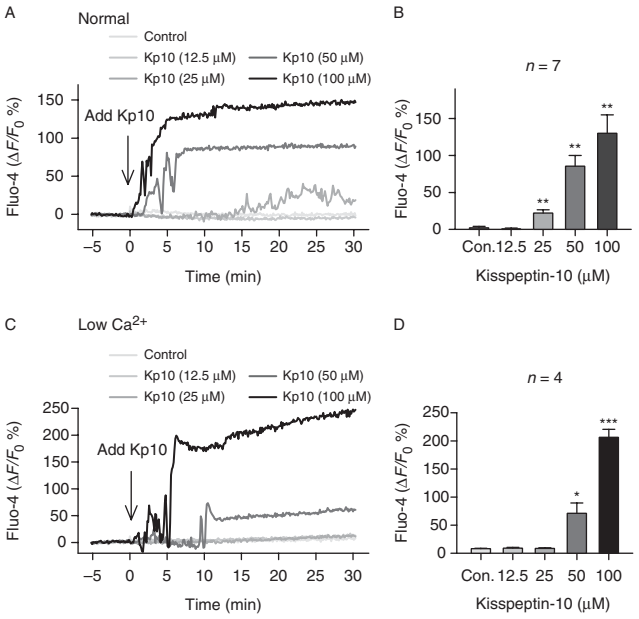

Figure 7 Effect of kisspeptin 10 on intracellular Ca^{2+} levels in mouse spermatozoa. (A and C) Changes in $[Ca^{2+}]_i$ levels in spermatozoa treated with different doses of kisspeptin 10 (Kp10) in (A) normal or (C) low-Ca^{2+} media. (B and D) Statistical analysis of curves during the sustained plateau phase in four or seven independent experiments. Mouse spermatozoa from cauda epididymis were loaded with Fluo-4 AM, and the changes of Fluo-4 fluorescence intensity were recorded using a 96-well multiplate reader. $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ vs control group. Error bars are means + S.E.M.

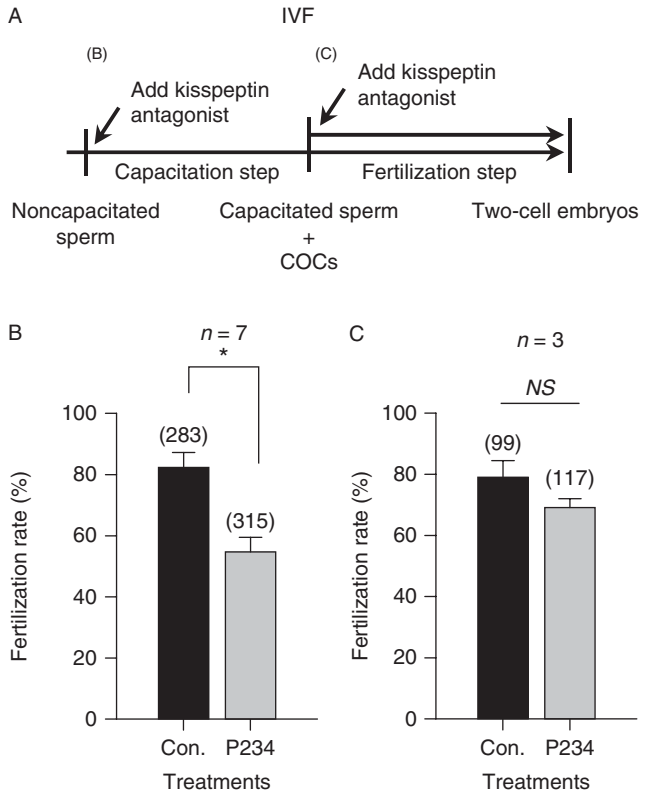

Figure 8 Effect of kisspeptin antagonist peptide 234 (P234) on the fertilization capacities of mouse spermatozoa. (A) Flowchart shows the experimental design to examine the fertilization capacities of spermatozoa in the absence of kisspeptin signal during capacitation or fertilization. (B) Noncapacitated spermatozoa were preincubated with 50 μM P234 in capacitated medium and added with cumulus–oocyte complexes (COCs) for fertilization. (C) Capacitated spermatozoa were incubated in human tubal fluid (HTF) with P234 and added with COCs for fertilization. The data reflect the average of three or seven independent experiments for each treatment, with each treatment including at least 30 eggs. The numbers in parentheses show the total number of eggs. *$P<0.05$ vs control treatment; NS, nonsignificant difference. Error bars are means+S.E.M.

Intracellular calcium in spermatozoa is important for regulating motility, capacitation, the acrosome reaction, and other processes (Costello *et al.* 2009). Increased $[Ca^{2+}]_i$ after kisspeptin treatment in KISS1R-expressing CHO cells and GnRH neurons has been observed previously (Kotani *et al.* 2001, Liu *et al.* 2008). The relationship between kisspeptin and changes in $[Ca^{2+}]_i$ in mouse spermatozoa was confirmed by results showing that kisspeptin 10 (25–100 μM) induced concentration-dependent increases in $[Ca^{2+}]_i$ in mouse spermatozoa. The effective concentrations of kisspeptin may depend on cell type, because effective doses range from 10 nM in mouse GnRH neurons, to 1 μM in bovine anterior pituitary cells, to 25 μM in mouse sperm (Fig. 7; Han *et al.* 2005, Kadokawa *et al.* 2008). The K_d of rat KISS1R is quite low (nM level; Kotani *et al.* 2001); it is possible that higher concentrations of kisspeptin 10 might act on other membrane receptors. However, the current data for effect of kisspeptin 10 on mouse spermatozoa are similar to those using human spermatozoa (10 μM; Pinto *et al.* 2012). The effective progesterone concentration to elevate $[Ca^{2+}]_i$ is species dependent; micromolar concentrations of progesterone are required to stimulate an increase in $[Ca^{2+}]_i$ in mouse sperm, whereas nanomolar concentrations are sufficient in human sperm (Fukami *et al.* 2003, Lishko *et al.* 2011). In the low-Ca^{2+} media, kisspeptin 10 still induced higher levels of intracellular calcium (Fig. 7C and D). Therefore, it might mobilize intracellular calcium pools such as those in the acrosome and mitochondria. Further work is needed to elucidate whether the $G_{q/11}$–PLC–$InsP_3R$ pathway plays an important role in kisspeptin-induced intracellular calcium mobilization.

Expression of kisspeptins and the kisspeptin 145 precursor has been observed in the ciliated epithelium of oviducts, oocytes, and cumulus cells of reproductive tissues of female rats, marmosets, and humans (Castellano *et al.* 2006, Gaytan *et al.* 2007, 2009). *Kiss1* gene expression increases in ovaries during preovulation, suggesting that kisspeptin regulates folliculogenesis, ovulation, and/or luteinization (Castellano *et al.* 2006, Gaytan *et al.* 2009). Kisspeptin may help to prevent ectopic pregnancy, because increased kisspeptin expression is also found during preovulation (Gaytan *et al.* 2007). However, the present results show that KISS1R is not expressed in cumulus cells, oocytes, or oviductal epithelium in mouse ovaries and oviducts, but is detected at the acrosomal region in spermatozoa. Therefore, these data suggest that the kisspeptin/KISS1R system might be involved in regulating the fertilization process in gametes.

The source of kisspeptins, required to regulate sperm function, may be epididymal epithelium (Fig. 5) in addition to Leydig cells but not sperm (Supplementary Fig. 1). After several washing steps, sperm showed no change in $[Ca^{2+}]_i$ during prolonged incubation in control groups, but displayed increased $[Ca^{2+}]_i$ in response to treatment with 25 μM kisspeptin 10. Therefore, we inferred that epididymal epithelium secreted kisspeptins to modulate sperm function. The seminal plasma is composed of multiple ions, energy substrates, and organic compounds such as citric acid, lipids, amino acids, peptides, and low- and high-molecular weight proteins. These components support the survival of spermatozoa in the female reproductive tract and facilitate successful fertilization (Juyena & Stelletta 2012). We propose that seminal plasma contains kisspeptins, which are secreted by the epididymal epithelium (Fig. 5), seminal vesicle, and/or prostate (Ohtaki *et al.* 2001, Curtis *et al.* 2010). The precise source of seminal kisspeptins needs to be elucidated through future experiments.

This is the first report to describe the effects of kisspeptin on the fertilization capacity of sperm. The present data show that treatment with a kisspeptin

antagonist during capacitation, but not during fertilization, reduces the fertilization rates of spermatozoa. This suggests that kisspeptin does not regulate the acrosome reaction, which is consistent with that described previously in human spermatozoa (Pinto *et al*. 2012). This study supports the idea that kisspeptin regulates the fertilization capacities of spermatozoa during capacitation and may protect spermatozoal function in reproductive tracts such as epididymis and oviduct.

In summary, we report that the kisspeptin/KISS1R system may regulate the fertilization process of spermatozoa in mice. KISS1R was expressed at the acrosomal region in spermatozoa, and kisspeptin was expressed on cumulus cells, oocytes, and oviductal epithelium. Kisspeptin induced a concentration-dependent $[Ca^{2+}]_i$ increase, whereas a kisspeptin antagonist reduced fertilization by spermatozoa. Future studies will focus on the specific functions and signaling pathways activated by kisspeptin in spermatozoa.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0368.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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