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Paradoxical effects of kisspeptin: it enhances oocyte *in vitro* maturation but has an adverse impact on hatched blastocysts during *in vitro* culture

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Abstract. Kisspeptin (Kp) is best known as a multifunctional peptide with roles in reproduction, the cardiovascular system and cancer. In the present study the expression of kisspeptin hierarchy elements (*KISS1*, *GNRH1* and *LHB*) and their receptors (*KISS1R*, *GNRHR* and *LHCGR*, respectively) in porcine ovary and in cumulus–oocyte complexes (COCs) were investigated, as were its effects on the *in vitro* maturation (IVM) of oocytes and their subsequent ability to sustain preimplantation embryo competence after parthenogenetic electrical activation. Kp system elements were expressed and affected IVM of oocytes when maturation medium was supplemented with 10^{-6} M Kp. Oocyte maturation, maternal gene expression (*MOS*, *GDF9* and *BMP15*), blastocyst formation rate, blastocyst hatching and blastocyst total cell count were all significantly increased when oocytes were matured in medium containing Kp compared with the control group (without Kp). A Kp antagonist (p234) at 4×10^{-6} M interfered with this hierarchy but did not influence the threshold effect of gonadotrophins on oocyte maturation. FSH was critical and permissive to Kp action on COCs by increasing the relative expression of *KISS1R*. In contrast, Kp significantly increased apoptosis, the expression of pro-apoptotic gene, *BAK1*, and suppressed trophoblast outgrowths from hatched blastocysts cultured on feeder cells. The present study provides the first functional evidence of the Kp hierarchy in porcine COCs and its role in enhancing oocyte maturation and subsequent developmental competence in an autocrine–paracrine manner. However, Kp supplementation may have a harmful impact on cultured hatched blastocysts reflecting systemic or local regulation during the critical early period of embryonic development.

Additional keywords: embryo development, *KISS1R*, oocyte maturation, p234.

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Introduction

Kisspeptins (Kp) are peptide products of the *KISS1* gene, which was discovered by Lee *et al.* in 1996 as a metastasis-suppressing gene in malignant melanoma cells (Lee *et al.* 1996). Kp is now best known as a multifunctional peptide because of its role in cancer, the cardiovascular system and reproduction (Mead *et al.* 2007). Due to the dynamic and progressive nature of research into this hormone, scientists may propose the term 'kisspeptinology' to reference the science dealing with this fascinating molecule (Dungan *et al.* 2006; Vaudry 2010).

The reproductive dimension of the KISS1/KISS1R system was disclosed in late 2003, when *KISS1R* (previously known as *GPR54* or Kp receptor) gene mutations were first reported in humans and mice suffering hypogonadotrophic hypogonadism (de Roux *et al.* 2003; Seminara *et al.* 2003). From that moment onwards, Kp became known as a master regulator of the reproductive axis in a hierarchical manner (Tena-Sempere 2006*a*) and among the most potent elicitors of GnRH-gonadotrophin secretion in a variety of mammalian species, including humans (Roa *et al.* 2008; Felip *et al.* 2009; Roa *et al.* 2009; Roseweir and Millar 2009; Pineda *et al.* 2010). An ever-growing number of studies worldwide has helped to substantiate the essential functions of Kp in controlling different aspects of reproduction (Roa and Tena-Sempere 2007). It works primarily on the hypothalamus to activate GnRH neurons; in addition, neuroanatomical studies have allowed identification of a discrete population of Kp neurons in different hypothalamic areas (Smith *et al.* 2005*a*, 2005*b*; Kauffman *et al.* 2007).

While the contention that Kp acts primarily at central levels to regulate ovarian function is well defined, the possibility of additional effects at other sites of the hypothalamic–pituitary–ovarian axis cannot be ruled out (Roa and Tena-Sempere 2007). Initial analyses in rodents suggested expression of *KISS1* and *KISS1R* genes in the ovary (Terao *et al.* 2004). In addition, Kp and KISS1R immunoreactivity were recently demonstrated in ovarian tissue sections from cyclic rats, where *KISS1*, but not *KISS1R*, gene expression was shown to fluctuate in a cycle-dependent manner under the regulation of pituitary LH (Castellano *et al.* 2006). Moreover, ovarian expression of *KISS1* and *KISS1R* genes was recently documented in fish (Nocillado *et al.* 2007; Filby *et al.* 2008), hamsters (Shahed and Young 2009), pigs and goats (Inoue *et al.* 2009) and primates (Gaytán *et al.* 2009). Kp stimulates ovulation in rats (Matsui *et al.* 2004), ewes (Caraty *et al.* 2007) and mares (Briant *et al.* 2006). Furthermore, inhibitors of cyclooxygenase-2, known to disturb follicular rupture and ovulation, inhibited *KISS1* gene expression selectively in the rat ovary (Gaytán *et al.* 2009). Together, these reports suggest a local role for Kp in directly controlling the ovulation process, but a clear picture of its local function has not yet fully emerged. In pig, many factors are implicated in the maturation of oocytes *in vivo* or *in vitro*, including intrafollicular or systemic factors, which makes this process physiologically complex (Hunter 2000). This raises the question: is kisspeptin involved in the oocyte maturation process?

Recently, Roseweir *et al.* (2009) discovered a potent Kp antagonist (p234) that revealed the critical roles of Kp in both female puberty and the preovulatory gonadotrophin surge (Pineda *et al.* 2010). No doubt antagonising kisspeptin action will become a powerful investigative tool and set the scene for more extensive physiological and pathophysiological studies as well as therapeutic intervention (Millar *et al.* 2010; Vaudry 2010).

Besides the known anti-metastatic role of Kp, attention has been directed towards Kp system actions on the placenta by Bilban *et al.* (2004), who first identified Kp as an endocrine–paracrine regulator of trophoblast invasion (Terao *et al.* 2004).

The objectives of the present study were to reveal the local existence of a kisspeptin hierarchy in the porcine ovary, examine its effect on *in vitro* maturation of oocytes and on subsequent embryonic development, and also to show the effect of kisspeptin supplementation on blastocyst viability and development after hatching *in vitro*.

Materials and methods

Ovaries and peptides

Ovaries were obtained from sows at a local slaughterhouse and were transported to the laboratory within 1 h of collection in 0.9% NaCl at 25–30°C. Kp (112–121)-NH2 and Kp peptide analogue or Kp antagonist (p234) were obtained from Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA). The peptide sequence of p234 is ac-DANWNGFGDWRF-NH2, as recently described (Roseweir *et al.* 2009; Pineda *et al.* 2010).

Cumulus–oocyte complexes (COCs) and porcine follicular fluid (PFF) recovery

Cumulus–oocyte complexes (COCs) were aspirated from antral follicles (3–6 mm in diameter) with an 18-gauge needle fixed to a 10-mL disposable syringe. The recovered oocytes were washed three times with tissue culture medium (TCM)-199-HEPES (Invitrogen, Carlsbad, CA, USA) and selected for *in vitro* maturation on the basis of visual assessment of morphological features, i.e. a compact multi-layered cumulus mass and a dark, evenly granulated cytoplasm. Porcine follicular fluid (PFF) was aspirated from 6 to 8 mm follicle size, centrifuged at 1600*g* for 1 min at 25°C, the supernatants were collected and filtered sequentially through 1.2 and 0.45 μm pore size syringe filters (Pall Corp., Newquay, UK) and then stored at −20°C until used.

Oocyte in vitro *maturation (IVM)*

The COCs were cultured in four-well dishes (50 COCs per well; Falcon, Becton Dickinson Ltd, Plymouth, UK) in basic maturation medium, TCM-199 supplemented with 10 ng mL^{-1} epidermal growth factor (EGF), 0.57 mM cysteine, 0.91 mM sodium pyruvate, 5 μg mL^{-1} insulin and 1% (v/v) Pen-Strep (Invitrogen) at 39°C in a humidified atmosphere of 5% CO_2 for 44 h (2 stages, 22 h each with change of medium for second stage). Different maturation conditions were used: 'undefined conditions' represents the presence of 10% PFF (v/v) in the basic maturation medium while 'defined medium' consisted of basic maturation medium with 1 μg mL^{-1} FSH (Antrin, Teikoku, Japan) or 1 μg mL^{-1} LH (Bioniche, Belleville, ON, Canada) or according to the experimental design in Table 1. After 44 h or at the designated time, oocytes and cumulus cells were separated by pipetting with 0.1% hyaluronidase in Dulbecco's PBS (Invitrogen) supplemented with 0.1% polyvinyl alcohol.

Table 1. Effect of kisspeptin and different treatments in defined and undefined medium on the nuclear maturation of porcine oocytes matured *in vitro*

Values with different superscripts are statistically different at $P \leq 0.05$

	Undefined medium		Defined medium							
	FSH + PFF	FSH + PFF + p234	FSH	FSH + p234	FSH + KP	FSH + KP + p234	KP	FSH + LH	LH	LH + p234
Polar body extrusion % (*n*)	87.56%[a] (176/201)	64.39%[b] (132/205)	53.19%[c] (100/188)	49.74%[c] (97/195)	75.46%[e] (163/216)	50%[c] (96/192)	0%[A] (0/180)	66.31%[b] (126/190)	39.89%[d] (75/188)	38.29%[d] (72/188)

[A]This value is statistically negligible.

Denuded oocytes and separated cumulus cells were subjected to one of the following: (*1*) DNA staining with 25 μg mL^{-1} bisbenzamide (Hoechst 33258) to check first polar body extrusion percentage, (*2*) total RNA extraction for RT-PCR and qPCR, or (*3*) parthenogenetic activation for subsequent culture *in vitro*.

Parthenogenetic activation and in vitro *culture (IVC) of matured oocytes*

Denuded oocytes were kept in a four-well cell culture plate (SPL Lifesciences Co. Ltd, Gyeonggi, Korea) containing mannitol (0.25 M) for 1.5 min to allow oocytes to settle down. The oocytes were then transferred into a special mannitol chamber connected with a BTX Electro Cell Manipulator 2001 (BTX, Inc., San Diego, CA, USA) and activated by a single pulse of 1.5 kV cm^{-1} for 100 μs (Okada *et al.* 2006). Electro-activated oocytes were then washed in TCM and transferred into 500 μL of 4 mM 6-dimethylaminopurine (6-DMAP) and cultured for 4 h to produce diploid zygotes in an atmosphere of 39°C, 5% CO_2, 5% $O_2$and 90% N_2. Presumptive diploid zygotes were washed and transferred into 30-μL microdrops of porcine zygote medium-3 (Yoshioka *et al.* 2002) covered with mineral oil (Sigma-Aldrich Corp., St Louis, MO, USA) and cultured in an atmosphere of 39°C, 5% CO_2, 5% O_2 and 90% N_2. On Day 2, embryos were evaluated for cleavage to the 2-cell stage or beyond. Blastocyst formation and expansion were assessed on Day 6 and hatching up to Day 8.

Total blastocyst cell count

Hatched blastocysts were washed in PBS then nuclei were stained with 25 μg mL^{-1} bisbenzamide for 1 h at 37°C. Stained blastocysts were mounted on a glass slide in a drop of glycerol, gently flattened with a coverglass and visualised for cell counting with a fluorescence microscope using a 346-nm excitation filter. Digital photographs were also taken for counting cell numbers using ImageJ 1.42q software (NIH, Bethesda, MD, USA).

Blastocyst culture

A total of 30 hatched blastocysts were divided into two groups then cultured with or without Kp in a medium consisting of DMEM/F12 supplemented with 10% (v/v) FBS (Invitrogen), 1 mM glutamine (Invitrogen), 25 mM $NaHCO_3$ and 1% (v/v) minimal essential medium (MEM) nonessential amino acid solution (Invitrogen) at 39°C in a humidified atmosphere of 5% CO_2 and 95% air on mouse embryonic fibroblasts treated with mitomycin C (Sigma-Aldrich Corp.) as feeder cells.

Total RNA extraction, semiquantitative and real-time PCR

A total of 60 COCs were used in each batch for total RNA extraction. Three pools of COCs were used for analysis and each pool was repeated three times. Total RNAs were extracted from detached cumulus granulosa cells and from denuded oocytes using the Easy-Spin (DNA-free) Total RNA Extraction Kit (iNtRON Biotechnology Inc., Kyunggi, Korea) according to the manufacturer's instructions. In brief, cumulus granulosa cells were washed twice with PBS (Invitrogen). Denuded oocytes were washed twice with PBS and then treated with diethylpyrocarbonate (DEPC)-treated water and kept at −80°C or used fresh. Total RNAs were then eluted from all samples following the manufacturer's protocol (iNtRON Biotechnology). Reverse transcription was carried out at 50°C for 50 min. Individual RT reactions were performed using random hexamer and superscript III reverse transcriptase (Invitrogen) in a 20-μL reaction volume. An amount of 1–2 μL cDNA was subjected to reverse transcription–polymerase chain reaction (RT-PCR) using Maxime PCR PreMix kit-*i*-starTaq (Intron Biotech., Seoul, Republic of Korea). The PCR amplification was carried out for one cycle of denaturation at 95°C for 5 min and subsequent cycles with denaturation at 95°C, annealing for 30 s, extension at 72°C for 45 s and final extension at 72°C for 5 min. Subsequently 10 μL of PCR product was fractionated on 1% agarose gel (Intron Biotech.) and stained with RedSafe (Intron Biotech.). Based in our optimisation curves testing different numbers of PCR cycles to select exponential amplification conditions, 35 and 30 PCR cycles were chosen for semiquantitative analysis of all candidate genes and *GAPDH*, respectively. Specificity of PCR products was confirmed by direct sequencing (Macrogen, Seoul, Korea). The identity of each product was confirmed by sequence homology analysis using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) GenBank (http://blast.ncbi.nlm.nih.gov/). Quantification of intensity of RT-PCR signals was carried out by densitometric scanning using ImageJ 1.42q software (NIH, USA) and values of the specific targets were normalised to those of the internal control (*GAPDH*) to express arbitrary units of relative expression. In all assays, cDNA template negative and reactions without reverse transcription resulted in negative amplification. Real-Time RT-PCR (RT-qPCR) was done according to the Takara Bio Inc. guidelines. Total 22 μL PCR reaction was made by adding 2 μL cDNA, 1 μL forward primer, 1 μL reverse primer, 8 μL SYBR Premix Ex Taq, 0.4 μL ROX Reference (Takara Bio Inc. Shiga, Japan) and 9.6 μL of nuclease-free water (Ambion Inc., Austin, TX, USA). The reaction was done by using the 7300 Real Time PCR System (Applied Biosystems, Forest City, CA, USA) according to the company's instructions. The thermal profile for real-time RT-PCR was 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 40 s. RT-qPCR was used for comparison of relative gene expression patterns in oocytes (*MOS*, *CCNB1*, *GDF9* and *BMP15*) and blastocysts (*BAK1* and *BCL2L1*) against *GAPDH* expression. Primer sequences, annealing temperatures and approximate sizes of the amplified fragments are listed in Table S1 available as an Accessory publication to this paper.

ELISA

Kp in serum samples from sows (late oestrous stage), follicular fluid and IVM-conditioned medium (44 h from start of IVM) was measured by ELISA Kit specific for porcine kisspeptin (KISS1; Hölzel Diagnostika GmbH, Cologne, Germany) with minimum detectable concentration 0.09 ng mL^{-1}. Kp concentration was measured according to the absorbance or optical density at 450 nm using the standard curve provided in the kit.

Immunofluorescence detection of KISS1R in COCs and early embryos

Oocytes with attached cumulus cells or hatched blastocysts were fixed in 4% paraformaldehyde (w/v) in phosphate buffered saline (PBS), pH 7.4 for 30 min at room temperature. After washing in PBS, oocytes were permeabilised by incubation in PBS containing 0.1% Triton-X100 (v/v) and then were blocked by 1% goat serum (v/v; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature then overnight at 4°C. Primary antibody directed against KISS1R, rabbit polyclonal anti-GPR54 (H-202) (Santa Cruz Biotech. Inc., Seoul, Korea) with dilution 1 : 100, was prepared in 1% goat serum; oocytes were incubated in primary antibody solution for 2 h at room temperature, washed in PBS, then incubated in secondary antibody (Cy3-conjugated goat anti-rabbit IgG; Jackson Immuno Research laboratory Inc., West Grove, PA, USA) diluted 1 : 200 in PBS for 1 h at room temperature before washing in PBS. Blastocyst nuclei were counterstained with Vectashield mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI; Vector Laboratories).

Experimental designs

Experiment 1: expression and production of Kp and its hierarchy elements in pig ovary

As reviewed above, Kp and its receptor KISS1R are found in the porcine ovary so we first checked the expression of Kp hierarchy elements (*KISS1*, *KISS1R*, *GNRH1*, *GNRHR*, *LHB* and *LHCGR*) in whole porcine ovary samples by RT-PCR and measured Kp levels in PFF compared with serum samples.

Experiment 2: expression of Kp hierarchy in COCs during IVM

We checked the expression of this system in cumulus cells and oocytes individually in a time-dependent manner (0, 4, 8, 22, 26, 30 and 44 h) because these hormones might be expressed in a pulsatile manner (Shupnik and Fallest 1994).

Experiment 3: effect of Kp supplementation on IVM of oocytes and their developmental competence

We first calculated the appropriate dosage of Kp supplementation in the presence of minimal FSH level. To supply the least concentration of FSH required for homogeneous cumulus expansion (FSH threshold), we added different amounts of FSH to the basic maturation medium and determined the least concentration capable of supporting homogeneous expansion of cumulus (Fig. S1 available as an Accessory publication to this paper). No significant difference in oocyte nuclear maturation was found among the different concentrations of Kp (0.5, 1, 2 and 4×10^{-6} M) supplemented to the IVM medium containing FSH threshold (0.125 μg mL^{-1}; see Table S2), so we studied the effect of 10^{-6} M Kp supplementation with or without its antagonist, p234 (4×10^{-6} M according to Millar *et al.* 2010 with modification), on (*1*) nuclear maturation (first polar body extrusion), (*2*) temporal gene expression of oocyte maturation markers *MOS*, *CCNB1*, *GDF9* and *BMP15*, (*3*) expression of its subsequent hierarchy (GnRHα and LHβ subunits), and (*4*) the embryos that subsequently developed from these oocytes.

Experiment 4: effect of FSH on KISS1R expression

Given that results from IVM suggested that FSH is critical for Kp action, we studied the effect of FSH on *KISS1R* expression in cumulus cells and oocytes by semi-quantitative RT-PCR.

Experiment 5: effect of Kp supplementation on blastocyst viability and development

We examined the expression of *KISS1R* in parthenogenetically derived pig blastocysts, either expanded or hatched, by RT-PCR and immunofluorescence. The effects of Kp supplementation on blastocyst viability and trophectoderm outgrowths were studied in a model to examine the effect of Kp on early embryonic development.

Statistical analysis

Statistical analysis was done utilising GraphPad Prism 4.02 (Graphpad Software Inc., San Diego, CA, USA). The data were analysed by one-way ANOVA followed by Tukey's test. Significance was determined when the P value was less than 0.05 or 0.01.

Results

Expression of kisspeptin system elements mRNA in porcine ovary and its level in follicular fluid

Our initial screening profile using RT-PCR revealed the expression of all Kp system elements and their receptors (*KISS1*, *KISS1R*, *GNRH1*, *GNRHR*, *LHB* and *LHCGR*) in the porcine ovary (Fig. 1*a*). Measurement of Kp levels by ELISA showed that it was ~10-fold higher in follicular fluid than in serum (335 ± 22.5 pg mL^{-1} vs 25 ± 5.2 pg mL^{-1}, $P \leq 0.05$).

Expression of kisspeptin system elements in porcine COCs during IVM in a time-dependent manner

RT-PCR of COCs during 44 h of IVM showed the expression of Kp system elements with various patterns (Fig. 1*b*, *c*). *KISS1* was only expressed in oocytes after the mid-stage of IVM (22–26 h) and Kp level was estimated by ELISA (111 ± 45.5 pg mL^{-1}) in the IVM-conditioned medium when COCs were cultured in a defined medium without PFF while *KISS1R* was expressed evenly during all of the IVM period. *GNRH1* showed transient expression during the stages of IVM reflecting a pulsatile expression and release while its receptors, GNRHR, tended to be expressed throughout IVM except for the oocyte, which expressed by the 8th hour of the IVM period. *LHB* was expressed in cumulus cells before oocytes (8 h and 22 h, respectively) and its receptor, *LHCGR*, showed even expression in cumulus cells for the entire IVM period, whereas it was expressed later in oocytes. *KISS1R* expression was confirmed by immunofluorescence and it was detected in both cumulus cells and oocytes (Fig. 1*d*, *e*).

Effect of kisspeptin supplementation of IVM medium on oocyte nuclear maturation, gene expression and developmental competence

Using different formulations of IVM medium produced variable effects on nuclear maturation of oocytes. The presence of

follicular fluid (undefined conditions) containing Kp as shown previously (Table 1), supported higher nuclear maturation rates (87.56%) than other formulations (Table 1). FSH alone supported 53.19% of oocytes to mature while maturation rates with addition of LH or Kp were statistically different from FSH alone (66.31% and 75.46%, respectively). LH alone could support 39.89% of oocytes to mature while Kp alone was ineffective. Furthermore, Kp together with FSH at 0.125 μg mL^{-1} increased oocyte maturation over baseline (0%) but there was no significant increase when the Kp concentration was increased (Table S2). However, results with the addition of Kp antagonist (p234) to undefined maturation conditions (PFF-containing medium) were statistically differed from culturing without it (Table 1). IVM of oocytes in a defined medium with Kp affected the oocyte quality and increased the rate of developmental competence of such oocytes after parthenogenetic electrical activation and IVC (Table 2); there was a statistical difference between the two groups that were cultured with or without Kp in cleavage rate, blastocyst formation, blastocyst hatching and in blastocyst total cell count. To elucidate the molecular events that

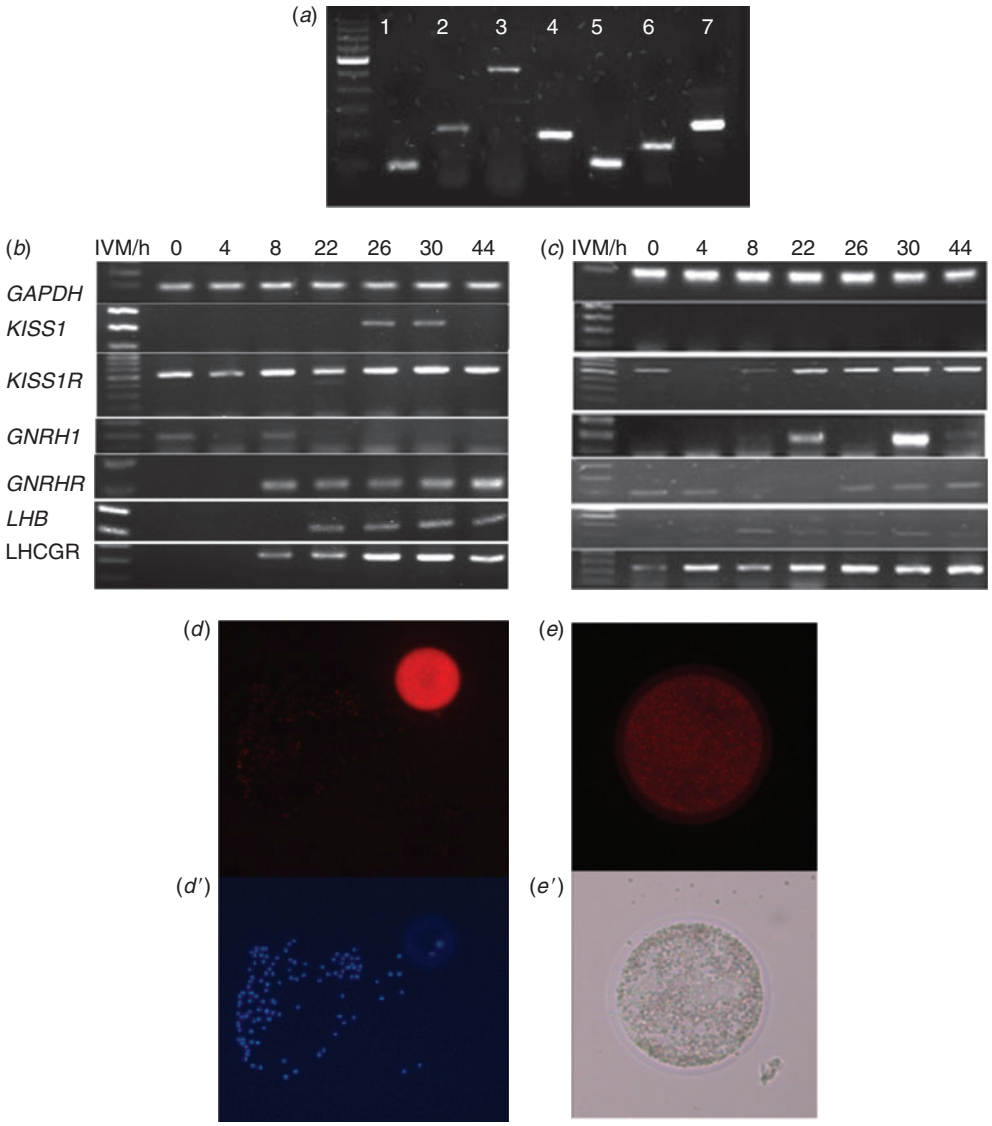

Fig. 1. Expression of kisspeptin hierarchy elements in porcine ovary and COCs. Photomicrograph of gel electrophoresis of RT-PCR products from porcine (*a*) ovary, (*b*) oocytes and (*c*) cumulus cells during IVM in a time-dependent manner. Lane 1, *GAPDH*; Lane 2, *KISS1*; Lane 3, *KISS1R*; Lane 4, *GNRH1*; Lane 5, *GNRHR*; Lane 6, *LHB* and Lane 7, *LHCGR*. (See the product size in Table S1 available as an Accessory publication to this paper). (*d*–*e*′) Localisation of KISS1R in *in vitro*-matured oocytes by indirect immunofluorescence. (*d*,*d*′) Intact COC showing fluorescent signals on both cumulus cells and oocytes against nuclear staining with DAPI. (*e*,*e*′) High resolution of oocytes showing fluorescent spots on the ooplasm membrane. Images were taken under (*e*) UV microscope and (*e*′) visible light.

might be associated with oocyte maturation in the presence or absence of Kp we used real-time qPCR to compare the temporal expression of genes *MOS*,*CCNB1*, *GDF9* and *BMP15* as markers of oocyte maturation and developmental competence (Fig. 2). Gene expression behaviour of both groups was similar along the IVM timeline (i.e. *CCNB1* and *GDF9* progressively increased whereas *MOS* and *BMP15* decreased at the end of IVM time) except for *MOS*, which showed a significant increase at the end of the IVM period in the Kp-treated group. However, a significant increase in the relative temporal expression of *MOS*, *GDF9* and *BMP15* genes was found in Kp-treated IVM compared with control but no difference in the relative temporal expression of *CCNB1* was detected between the two groups.

Table 2. Developmental competence of electrically activated oocytes after IVM in defined medium with or without Kp and following embryo culture

Values with different superscripts are statistically different at $P \leq 0.05$

Parameter	IVM treatment first stage/second stage	
	Kp-negative	Kp-positive
Oocyte number	117	121
Cleavage rate (Day 2)	85 (72.64%)[a]	104 (85.95%)[b]
Blastocysts, % of cleaved embryos (Day 6)	20 (23.52%)[a]	41 (39.42%)[b]
Blastocyst hatching, % of blastocysts (Day 8)	4 (20%)[a]	14 (34.14%)[b]
Hatched blastocyst total cell count ($n = 4$) (mean ± s.e.m.)	50.88 ± 2.5[a]	61.62 ± 2.3[b]

Effect of Kp on GNRH1 and LHB expression

The relative expression of *GNRH1* and *LHB* was studied after IVM of COCs in medium with or without Kp at the end of the first stage of IVM. Expression of oocyte *LHB* was increased over the control group while its expression in cumulus cells showed no difference between the groups. *GNRH1* was only detected in oocytes cultured in Kp-containing medium but not in cumulus cells in both groups (Fig. 3 with diagram).

Effect of FSH on KISS1R *expression*

As our results showed the essential role of FSH, to study the effect of FSH on *KISS1R* expression, COCs were cultured in medium with or without FSH for the first quarter of IVM (after 11 h). The magnitude of oocyte *KISS1R* relative expression was increased 4-fold in FSH-containing medium compared with the negative control group that indicated a permissive effect of FSH to Kp action (Fig. 4 with diagram).

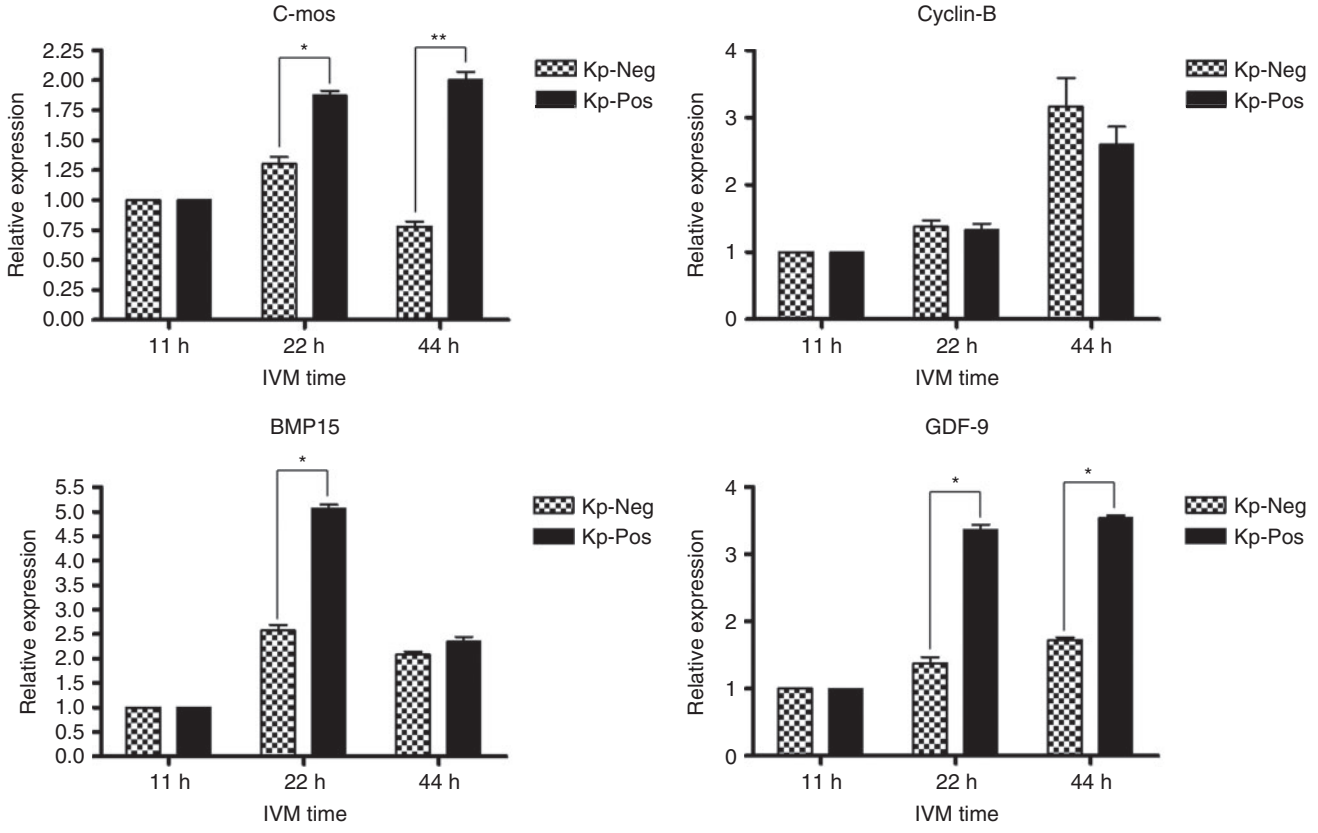

Fig. 2. Effect of kisspeptin supplementation on temporal expression of C-mos, cyclin-B1, BMP15 and GDF9 mRNAs during pig oocyte *in vitro* maturation (IVM) by real-time PCR. The relative gene abundance was normalised to *GAPDH* levels. The mRNA expression in denuded oocytes at 11 h was arbitrarily set as one-fold. Significant difference is indicated as *, $P < 0.05$ and **, $P < 0.001$.

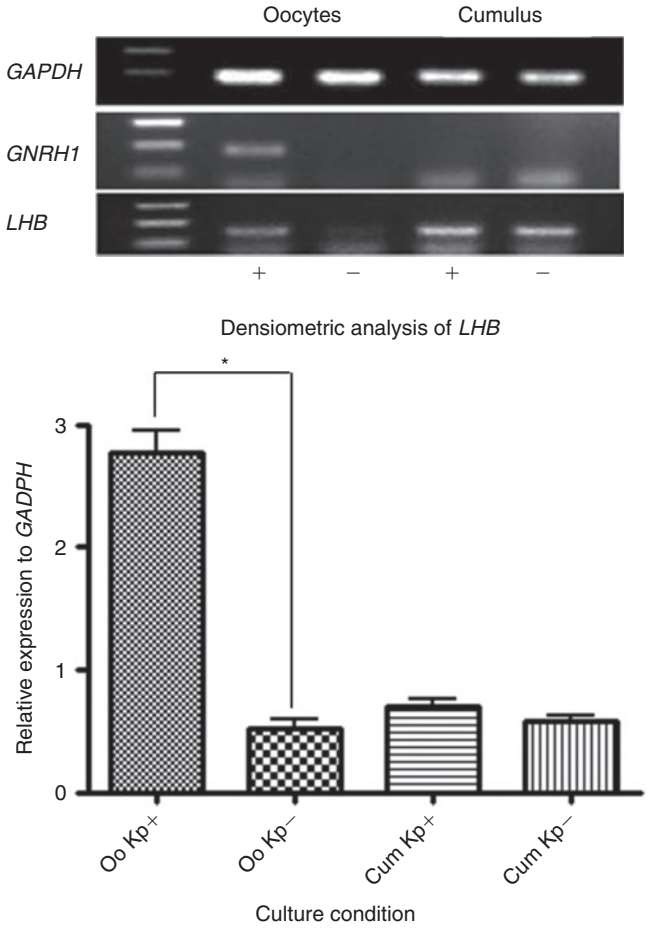

Fig. 3. Effect of kisspeptin on *LHB* and *GNRH1* expression in oocytes and cumulus cells after IVM of COCs for 22 h. The diagram shows the densiometric relative expression of *KISS1R* in oocytes and cumulus cells. (+) Kp-positive and (–) Kp-negative IVM medium. Significant difference is indicated as *, $P < 0.05$.

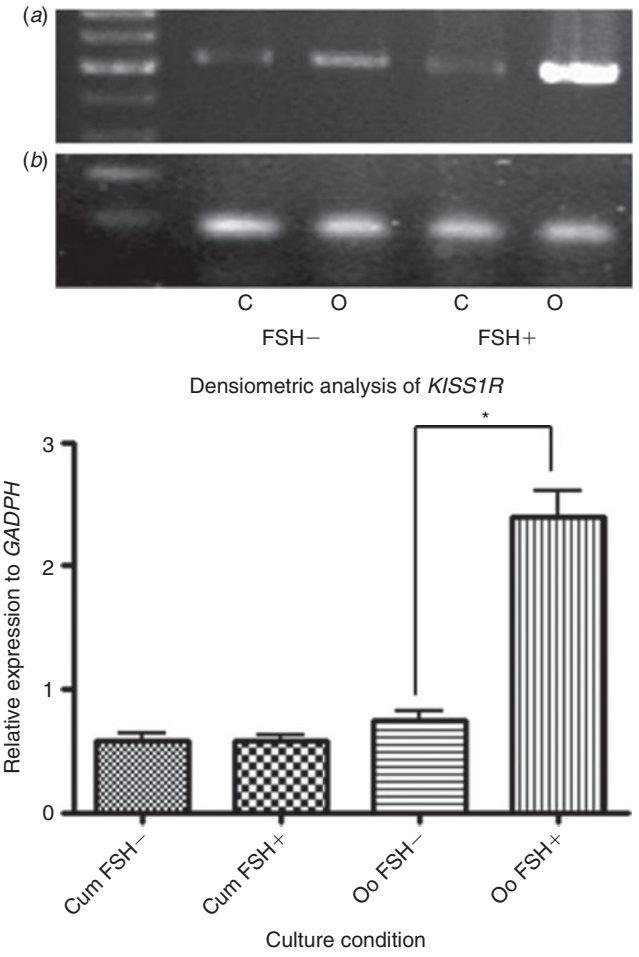

Fig. 4. Effect of FSH treatment on *KISS1R* expression in cumulus cells (C) and oocytes (O) cultured for 11 h in IVM medium. (*a*) *KISS1R*, (*b*) *GAPDH*. The diagram shows the densiometric relative expression of *KISS1R* in oocytes and cumulus cells. Significant difference is indicated as *, $P < 0.05$.

Expression of KISS1R in porcine parthenogenetic blastocysts and effect of Kp supplementation on their viability and trophectoderm outgrowths

Screening of *KISS1R* expression by RT-PCR showed its expression in blastocysts after hatching but not in expanded blastocysts (Fig. 5*a*). KISS1R expression was confirmed by immunofluorescence, where it was detected in the hatched blastocysts (Fig. 5*b*). Surprisingly, culturing hatched blastocysts on feeder cells in medium containing Kp hindered trophoblastic outgrowths after their attachment to the feeder cell layer and caused degeneration of trophoblasts (Fig. 5*d*). In addition, Kp caused an increase in cystic embryoid body formation in blastocysts cultured on medium supplemented with Kp compared with that of controls (Fig. 6*b*, *c*). We analysed the expression of *BAK1* (proapoptotic) and *BCL2L1* (antiapoptotic, previously *Bcl-xL2*) genes to explore the possible mechanism of hatched blastocyst degeneration caused by Kp. Expression of *BAK1* was found to be significantly increased (3.5-fold) in Kp-treated blastocysts compared with controls, with no difference in *BCL2 L1* expression (Fig. 6*d*).

Discussion

During the past decade Kp and KISS1R have received much attention in reproductive biology because compelling experimental evidence has strongly suggested their involvement in the control of the development and function of the hypothalamo–hypophyseal–gonadal (HPG) axis. From genetic, molecular and pharmacological approaches, this essential function is assumed to be primarily conducted at central hypothalamic levels, in which neuroanatomical and physiological studies have proposed Kp neurons as gatekeepers of the reproductive axis in a hierarchical manner (Dungan *et al.* 2006; Tena-Sempere 2006*a*, 2006*b*; Roa *et al.* 2008; Pineda *et al.* 2010; Smith *et al.* 2011). However, it is conspicuous that expression of the *KISS1*/*KISS1R* system has been reported in different reproductive organs, including gonads, in diverse species, including mammals and fishes (Ohtaki *et al.* 2001; Terao *et al.* 2004; Castellano *et al.* 2006; Nocillado *et al.* 2007; Filby *et al.* 2008; Gaytán *et al.* 2009; Inoue *et al.* 2009; Shahed and Young 2009; Kirby *et al.* 2010).

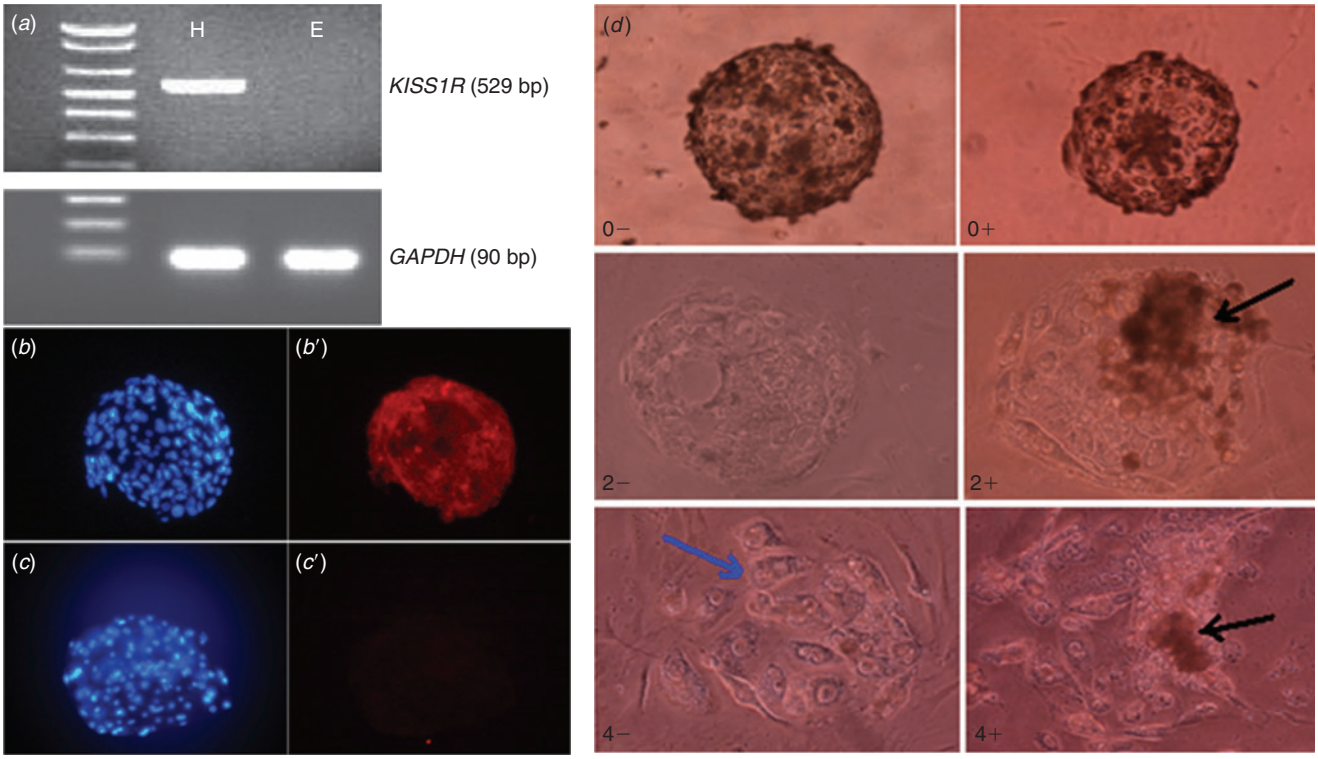

Fig. 5. Expression of *KISS1R* and effect of Kp supplementation on parthenogenetically derived blastocysts. (*a*) mRNA expression of *KISS1R* in parthenogenetically derived blastocysts (H, hatched and E, expanded). (*b*–*c*′) Localisation of *KISS1R* in hatched blastocysts. (*b*,*b*′) Procedures with primary and secondary antibodies (*c*,*c*′) Procedures with secondary antibody only as a negative control. Images show nuclear staining with DAPI and fluorescent signals of *KISS1R*. (*d*) Effect of kisspeptin supplementation on trophoblast outgrowths from parthenogenesis-derived blastocysts cultured on mouse embryonic feeder cells for 0, 2 and 4 days with (+) or without (–) Kp. Normal appearance of cuboidal-shaped trophoblast indicated by blue arrows. Black arrows indicate degeneration of trophoblasts.

Based on preliminary evidence showing prominent expression of *KISS1* and *KISS1R* genes in the pig ovary (Inoue *et al.* 2009) and the cloning of these genes encoding both Kp and its receptors in the pig (Li *et al.* 2008; Tomikawa *et al.* 2010), in the present study we aimed to define the profile of ovarian *KISS1* gene expression and its hierarchy (*KISS1R*, *GNRH1*, *GNRHR*, *LHB* and *LHCGR*), particularly in cumulus cells and oocytes, and its role during maturation of oocytes *in vitro*. We then followed the early expression of *KISS1R* in early embryos and studied the effect of Kp on their development.

Our present results conclusively demonstrate, for the first time, that the genes encoding Kp and its hierarchy elements are temporally expressed in pig cumulus–oocyte complexes throughout IVM. Kp supplementation enhanced oocyte maturation but interestingly, we showed that Kp could have an adverse impact on early embryo development if it is supplemented after blastocyst hatching.

First, we screened the expression of Kp hierarchy elements genes in the whole porcine ovary by RT-PCR and used this as a positive control for the next steps. The Kp system was expressed in the pig ovary (Fig. 1*a*). Therefore, COCs were isolated from immature follicles and matured *in vitro* for 44 h; cumulus cells and oocytes were then separated and used for RT-PCR to detect Kp hierarchy elements in a time-dependent sampling (0, 4, 8, 22, 26, 30 and 44 h). Interestingly, all the system elements were expressed in COC with variable patterns during the IVM process. *KISS1* was only detected in oocytes but *KISS1Rs* were expressed throughout the IVM period in both oocytes and cumulus cells, which may suggest a continuous and direct action of Kp on oocytes and cumulus cells in an autocrine–paracrine fashion. Analysis of PFF revealed the presence of Kp at high concentrations compared with serum levels (335 ± 22.5 pg mL^{-1} vs 25 ± 5.2 pg mL^{-1}, $P \leq 0.05$) so it may be involved *in vivo* during follicular development and suggests an intrafollicular or systemic origin of action. Also, we cannot exclude an action of Kp to stimulate GnRH and LH biosynthesis by both cumulus cells and oocytes because they are considered to be subordinates in this hierarchy. The GnRHα subunit was expressed in both cumulus cells and oocytes intermittently throughout IVM, suggesting a pulsatile release of this hormone (Gharib *et al.* 1990; Shupnik and Fallest 1994; Haisenleder *et al.* 2001; El Majdoubi *et al.* 2003; Krsmanovic *et al.* 2009), whereas its receptors, *GNRHR*, were expressed evenly throughout IVM, suggesting a direct action of GnRH on cumulus cells or oocytes (Hillensjö and LeMaire 1980). Perhaps this expression profile reflects an action of Kp by releasing GnRHα from cumulus cells or oocytes but with an intrinsic pulsatile nature. Moreover, *LHB* was expressed in oocytes in a pulsatile fashion starting after 8 h

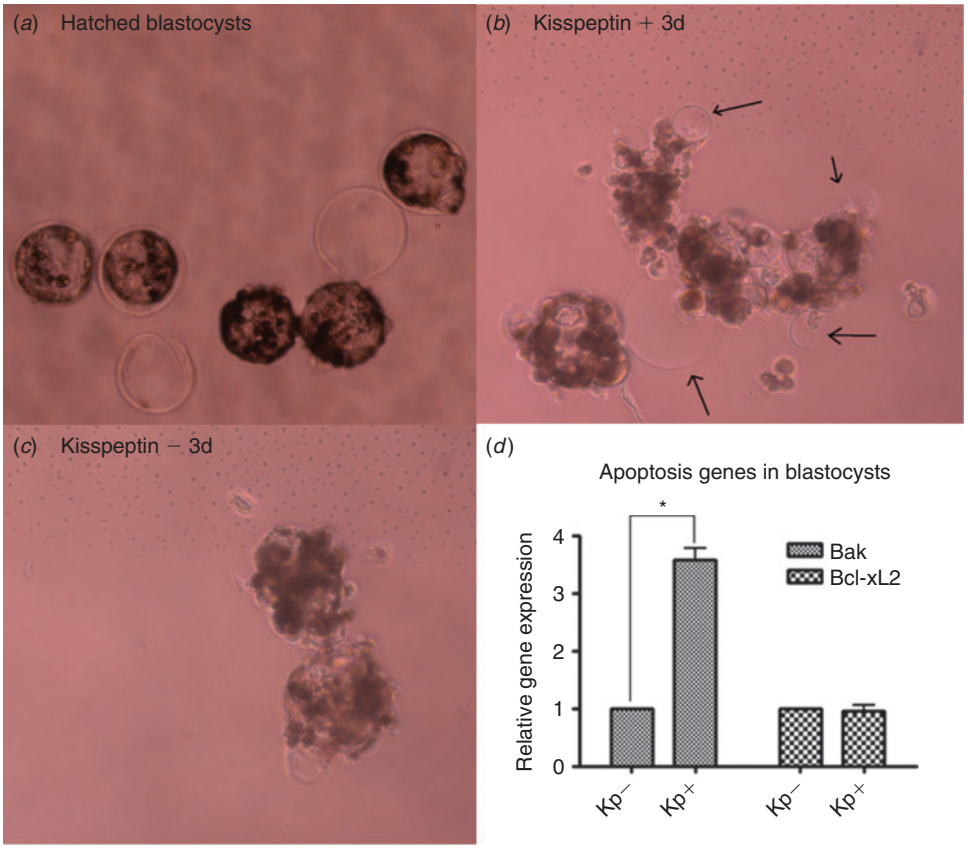

Fig. 6. Apoptotic effect of Kp supplementation on porcine hatched parthenogenetically derived blastocysts. (*a*) Normal hatched blastocysts cultured without feeder cells (Day 0). (*b*) Blastocysts cultured in medium supplemented with Kp for 3 days; black arrows indicate cystic embryoid bodies. (*c*) Blastocysts cultured in medium without Kp for 3 days. (*d*) Relative expression of apoptosis-related genes (Bak) and (Bcl-xL2) in hatched blastocysts cultured for 24 h in medium with (Kp+) or without (Kp–) kisspeptin. Significant difference is indicated as *, $P < 0.05$.

of IVM culture in cumulus cells, suggesting its involvement in later stages of oocyte maturation and cumulus expansion. *LHRs* were expressed in oocytes early in IVM, which may indicate an action of either systemic or ovarian LH *in vivo* while cumulus LHRs were expressed evenly during IVM, suggesting the necessity for LH in cumulus cell expansion. Many studies have shown the expression of *GNRH1*, *GNRHR* and *LHB* in the ovary of pigs and other species (Pati and Habibi 2002; Okazaki *et al.* 2003; Takekida *et al.* 2003; Schirman-Hildesheim *et al.* 2005; Cárdenas *et al.* 2008; Litichever *et al.* 2009; Procházka *et al.* 2009) and are consistent with our present results to support the concept of a local-acting network or hierarchy.

We studied the effect of various factors on IVM of oocytes as indicated by the extrusion of the first polar body. According to our hypothesis, Kp activates its subordinate GnRHα, thereby increasing LH synthesis, which, in turn, will affect oocyte maturation. In Table 1, addition of Kp to the maturation medium with FSH increased oocyte maturation more than addition of LH (75.46% vs 66.31%), which may reflect a direct action of Kp on oocytes; also, we cannot exclude its possible role in activating hierarchy subordinates. Kp increased oocyte maturation even without cumulus cells, which may indicate a direct effect of Kp on oocytes (see Table S3). The presence of p234, a Kp antagonist, can interfere with Kp action but cannot affect the threshold effect of both LH and FSH. PFF yielded more oocyte maturation than other additives (87.56%), while p234 reduced this action (64.39%) by interfering with Kp that already exists in PFF and may be secreted either from oocytes or the ovary. One may propose the involvement of Kp in the intrafollicular complex of oocyte maturation *in vitro* or *in vivo*.

To further explore the underlying molecular mechanisms in oocyte maturation that might caused by Kp, expression patterns of transcripts C-mos, cyclin-B1, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) were obtained, as they are considered to be markers of female germ cells (Sagata *et al.* 1988; Kubelka *et al.* 1995; Pennetier *et al.* 2004). C-mos is a proto-oncogene identified as a regulator of oocyte maturation in human (Hashiba *et al.* 2001), mouse (O'Keefe *et al.* 1989) and pig (Newman and Dai 1996; Ohashi *et al.* 2003). As a mitogen-activated protein kinase (MAPK), Mos activates extracellular signal-regulated protein kinase (ERK) 1 and 2. A functional study suggested that Mos also mediates the activity of maturation/M phase promoting factor (MPF), another essential regulator of meiosis resumption

formed by cyclin B1 and Cdc2 kinase, through the MAPK pathway and stabilisation of cyclin B1 (Newman and Dai 1996). GDF9 and BMP15 belong to the transforming growth factor-b (TGF-b) superfamily, which contains many members with important roles in regulating fertility (reviewed in Juengel *et al.* 2004). GDF9 and BMP15 are currently identified as oocyte-secreted factors involved in folliculogenesis and oocyte maturation, as well as in cooperative regulation of granulosa cells (McNatty *et al.* 2005). The relative elevation in the temporal expression of *MOS*, *GDF9* and *BMP15* (Fig. 2) might explain the increase in oocyte maturation and blastocyst yield found in Kp-treated COCs.

To confirm the involvement of Kp in stimulating its hierarchical subordinates, Kp increased the magnitude of *LHB* relative expression by 2.5-fold and increased the pulsatile frequency of *GNRH1* expression in oocytes (Fig. 3). From these results, we can hypothesise the local-acting Kp hierarchy as follows; Kp, either of oocytic or intrafollicular origin, works on its receptor KISS1R to increase *GNRH1* pulsatile expression, which, in turn, increases LHβ biosynthesis.

Furthermore, Kp affected the developmental competence of embryos resulting from oocytes matured *in vitro* and electrically activated as indicated by cleavage rate, blastocyst yield, hatching and total cell count (Table 2). Also, one might propose an effect of Kp on cytoplasmic maturation of oocytes because Kp was found to increase Ca^{2+} oscillations in *KISS1R*-expressing cells (Liu *et al.* 2008; Castaño *et al.* 2009; Constantin *et al.* 2009; Kroll *et al.* 2011) thereby implicating it in cytoplasmic and nuclear maturation of oocytes (Sun *et al.* 2009; Zhang *et al.* 2010). Further investigations are required in this regard.

The absence of FSH from the IVM medium resulted in failure of oocyte maturation even with addition of Kp, confirming the critical role of gonadotrophins for *in vitro* oocyte maturation in pigs. FSH or LH is necessary for driving completion of the first meiosis and for cumulus cell viability and expansion (Hsueh *et al.* 1984, 1994; Chun *et al.* 1996). To confirm the necessity of FSH for Kp action (Fig. 4), addition of FSH to the maturation medium led to a prominent increase in *KISS1R* expression, reflecting the permissive effect of FSH for Kp action.

We followed up expression of the Kp system in subsequent embryo development. *KISS1R* was expressed in blastocysts only after hatching (Fig. 5*a*, *b*). The action of Kp on the placenta was studied early on and it is known as an inhibitor of placental invasion (Bilban *et al.* 2004; Hiden *et al.* 2007). Studies showed that alterations in serum levels of Kp are associated with placental disorders (Dhillo *et al.* 2006; Torricelli *et al.* 2008; Armstrong *et al.* 2009). Interestingly, in our experiments, Kp supplementation affected both trophoblastic viability and outgrowths; as shown in Fig. 5*d*, Kp suppressed trophoblastic outgrowths derived from blastocyst culture on feeder cells and caused degeneration of the trophectoderm starting from the second day of culture. Moreover, blastocysts cultured on medium supplemented with Kp caused an increase in cystic embryoid body formation that was mainly caused by high apoptotic rates within the embryonic cells (Boyd *et al.* 1984; Brown *et al.* 2006). To further explore the underlying molecular mechanisms in blastocyst degeneration caused by Kp, expression of *BAK1* as a pro-apoptotic gene was found to be significantly increased (3.5-fold) in 24 h Kp-treated blastocysts than control ones, with no difference in *BCL2L1* expression as an anti-apoptotic gene (Fig. 6*d*). These results together confirm the apoptotic action of Kp as recently revealed (Navenot *et al.* 2009*a*, 2009*b*; Martinez-Fuentes *et al.* 2011).

In conclusion, this is the first report showing the existence and interaction of the Kp hierarchy in porcine COCs and its enhancing role in oocyte maturation and subsequent embryo developmental competence in an autocrine–paracrine manner. However, Kp supplementation may have a paradoxical impact on hatched blastocyst development reflecting systemic or local regulation during this early critical period.

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