Effect of kit ligand on natriuretic peptide precursor C and oocyte maturation in cattle

Paula F Lima¹, Cinthia M Ormond¹, Ester S Caixeta¹, Rodrigo G Barros¹, Christopher A Price^{2,*} and José Buratini^{1,*}

¹Departamento de Fisiologia, Instituto de Biociências, Universidade Estadual Paulista, Rubião Junior, Botucatu, São Paulo, Brazil and ²Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Quebec, Canada

Correspondence should be addressed to J Buratini; Email: buratini@ibb.unesp.br

*(C A Price and J Buratini contributed equally to this work)

Abstract

In vitro maturation (IVM) of oocytes in cattle is inefficient, and there is great interest in the development of approaches to improve maturation and fertilization rates. Intraovarian signalling molecules are being explored as potential additives to IVM media. One such factor is kit ligand (KITL), which stimulates the growth of oocytes. We determined if KITL enhances oocyte maturation in cattle. The two main isoforms of *KITL* (*KITL1* and *KITL2*) were expressed in bovine cumulus–oocyte complexes (COC), and levels of mRNA increased during FSH-stimulated IVM. The addition of KITL to the culture medium increased the percentage of oocytes that reached meiosis II but did not affect cumulus expansion after 22 h of IVM. Addition of KITL reduced the levels of mRNA encoding natriuretic peptide precursor C (NPPC), a protein that holds oocytes in meiotic arrest, and increased the levels of mRNA encoding YBX2, an oocyte-specific factor involved in meiosis. Removal of the oocyte from the COC resulted in increased *KITL* mRNA levels and decreased *NPPC* mRNA levels in cumulus cells, and addition of denuded oocytes reversed these effects. Taken together, our results suggest that KITL enhances bovine oocyte nuclear maturation through a mechanism that involves NPPC, and that the oocyte regulates cumulus expression of *KITL* mRNA.

Reproduction (2016) **152** 481–489

Introduction

Ovulation is induced by the luteinizing hormone (LH) surge, which causes the increased expression of epidermal growth factor (EGF)-like proteins in granulosa and cumulus cells, and subsequent induction of prostaglandin-endoperoxide synthase 2 (PTGS2) mRNA/protein and prostaglandin secretion. Prostaglandins induce the expansion of the cumulus complex and proteolytic degradation of the follicle wall, and are involved in oocyte nuclear maturation (Richards 2005, Reizel *et al.* 2010, Prochazka *et al.* 2012, Marei *et al.* 2014).

Before the LH surge, oocytes are held in meiotic arrest by high intraoocyte levels of cAMP that are maintained at least in part by the transfer of cGMP from cumulus cells to the oocyte through gap junctions (Shuhaibar *et al.* 2015). The generation of cGMP is driven by natriuretic peptide precursor C (NPPC), which is secreted from granulosa and cumulus cells and activates natriuretic peptide receptor B (NPR2) on cumulus cells (Zhang *et al.* 2010a, Franciosi *et al.* 2014, De Cesaro *et al.* 2015). Loss of NPPC expression results

in resumption of meiosis (Zhang *et al.* 2010a, Kawamura *et al.* 2011, Robinson *et al.* 2012) and addition of NPPC to cumulus–oocyte complexes (COC) *in vitro* delayed resumption (Zhang *et al.* 2010a, Franciosi *et al.* 2014). During follicle growth, NPPC levels are high, and are decreased by hCG in preovulatory follicles (Liu *et al.* 2014). Thus, growing follicles produce quantities of NPPC sufficient to prevent premature resumption of meiosis, and the preovulatory LH surge relieves this brake on oocyte maturation.

Cytoplasmic maturation must be tightly synchronized with nuclear maturation for the oocyte to achieve developmental competence. Accumulation of mRNA stocks that allow protein production until the embryo becomes transcriptionally competent is a crucial component of cytoplasmic maturation since transcriptional activity progressively decreases as chromatin condensation advances in the germinal vesicle (Hyttel *et al.* 1986, Lodde *et al.* 2007). Y-box binding protein 2 (YBX2) appears to be a key regulator in this context; YBX2 increases mRNA content, mRNA stability and protein synthesis in mouse oocytes

(Yu *et al.* 2004, Medvedev *et al.* 2011). In the bovine oocyte, YBX2 expression decreases as maturation progresses from the germinal vesicle stage to metaphase II (Vigneault *et al.* 2009).

Another factor involved in oocyte maturation is kit ligand (KITL), which is secreted from granulosa cells and acts on receptors (KIT) on the oocyte (Hutt *et al.* 2006, Thomas & Vanderhyden 2006, Celestino *et al.* 2010). Alternative splicing of *KITL* mRNA results in a full-length and a truncated protein, termed KITL1 and KITL2 respectively, and both are biologically active (Brannan *et al.* 1991, Huang *et al.* 1992, Zhou *et al.* 1994, Kawaguchi *et al.* 2007). Although KITL is well known to stimulate growth and survival of oocytes (Packer *et al.* 1994, Ismail *et al.* 1997, Thomas *et al.* 2008), its effects on nuclear maturation are controversial: addition of KITL to denuded rat oocytes reduced the rate of the 1st polar body extrusion after 14 h culture (Ismail *et al.* 1996, 1997), whereas it increased the rate of the 1st polar body extrusion after 22 h in mice (Ye *et al.* 2009).

It is well established that the oocyte regulates cumulus cell function in mice, but the role the oocyte plays in cattle is less clear. The oocyte is required for cumulus glycolysis and expansion in mice (Eppig 2005, Sugiura *et al.* 2005, Vanderhyden 1993) but not in cattle (Ralph *et al.* 1995, Sutton *et al.* 2003). Removal of the oocyte from the COC (oocyectomy) in mice reduced the levels of mRNA encoding certain genes in cumulus cells including *Nppc* (Lee *et al.* 2013), and increased those of other genes including *Kitl* (Joyce *et al.* 1999); a microarray study with bovine oocyectomized cumulus cells did not identify *NPPC* or *KITL* as genes affected by the oocyte (Regassa *et al.* 2011). The inhibitory effect of the murine oocyte on cumulus cell *Kitl* mRNA levels is likely mediated through the secretion of growth/differentiation factor 9 (Gdf9) (Joyce *et al.* 2000), although another oocyte-secreted factor, bone morphogenetic factor (Bmp) 15, has been shown to stimulate cumulus *Kitl* mRNA levels in rodents (Otsuka & Shimasaki 2002, Thomas *et al.* 2005, Miyoshi *et al.* 2012).

It is not known if the bovine oocyte regulates *KITL* or *NPPC* expression in cattle, and although *NPPC* has been shown to delay meiotic resumption in cattle (Franciosi *et al.* 2014), the impact of *KITL* on meiotic resumption in cattle has not been reported. In this study, we tested the hypothesis that *KITL* signalling regulates COC maturation under the influence of the oocyte in the cow. The specific objectives were to determine the regulation of *KITL* expression by the oocyte and putative oocyte-secreted factors, and to measure the effects of *KITL* on cumulus expansion, *NPPC* mRNA levels and oocyte maturation.

Materials and methods

Unless specified, all chemicals and reagents were purchased from Sigma.

Experimental design

The patterns of expression of *KITL1* and *KITL2* mRNA in cumulus cells and of *KIT* mRNA in oocytes were evaluated during IVM at 0, 4, 8, 12, 16 and 22 h ($n=4/\text{time}$). To investigate the effects of oocyte-secreted factors on the abundance of *KITL1* mRNA in cumulus cells, the culture medium was supplemented with graded doses of recombinant BMP15 ($n=4/\text{dose}$), FGF8 ($n=5/\text{dose}$) or FGF17 ($n=4/\text{dose}$; R&D Systems). The effect of *KITL* on COC maturation was determined by culturing COC for 22 h with 0, 10, 50 or 100 ng/mL recombinant *KITL1* (R&D Systems) to evaluate cumulus expansion and abundance of mRNA encoding key proteins involved in expansion ($n=4/\text{dose}$), and the progression of the oocyte through meiosis ($n=9/\text{dose}$) as well as key genes involved in nuclear maturation ($n=4/\text{dose}$). The effect of the oocyte and oocyte-secreted factors on the expression of *KITL* and *NPPC* in cumulus cells was tested by the comparison of mRNA levels from intact COCs, oocyectomized COCs and oocyectomized COCs cultured with denuded oocytes (1 denuded oocyte/ μL ; $n=4/\text{group}$).

In vitro maturation

Ovaries of adult cows (predominantly Nellore, *Bos indicus*) were obtained at an abattoir local to the São Paulo State University campus in Botucatu and transported to the laboratory in saline solution (0.9% NaCl) at 37°C. COCs were aspirated from 3 to 8 mm diameter follicles with an 18 gauge needle and pooled in a 15 mL conical tube. After sedimentation, COCs were recovered and selected using a stereomicroscope. Only COCs with homogeneous cytoplasm and compact multilayer of cumulus cells were used (Grade 1 and 2). COCs were washed and transferred in groups of 20 to a 100 μL drop of maturation medium, TCM199 containing Earle's salts supplemented with 1 $\mu\text{g}/\text{mL}$ porcine FSH (equivalent to 0.002 IU; Folltropin-V Bioniche Animal Health, Belleville ON, Canada), 10 IU/mL LH (Lutropin-V, Bioniche Animal Health), 22 $\mu\text{g}/\text{mL}$ sodium pyruvate, 75 $\mu\text{g}/\text{mL}$ ampicillin, 4 mg/mL BSA and growth factors. Drops were covered with mineral oil and incubated at 38.5°C in 5% CO_2 in humidified air.

Oocyectomy

Oocyte–cumulus complexes were placed in 200 μL drops of TCM199 partially covered with mineral oil, and the cytoplasm of the oocytes was removed with a micromanipulator as described (Buccione *et al.* 1990) with modifications (Paradis *et al.* 2010).

Assessment of cumulus expansion

Cumulus expansion was visually assessed at 22 h of culture according to a subjective scoring system. Grades 1–3 were attributed to increasing degrees of expansion (Grade 1: poor expansion, characterized by few morphological changes compared with before maturation; Grade 2: partial expansion, characterized by fair expansion but notable clusters lacking expansion; Grade 3: complete or nearly complete expansion (Machado *et al.* 2015)).

Table 1 Information of specific primers used for amplification in real-time PCR.

Gene	Primer sequence	Fragment size (bp)	Annealing temperature (°C)	Reference
<i>BMP15</i>	F: 5'GTCAGCAGCCAAGAGGTAGTG3' R: 5'CCCGAGGACATACTCCCTTAC3'	360	59	Caixeta et al. (2013a)
<i>GDF9</i>	F: 5'TGGTCCTTGCTGAAGCATCTAGA3' R: 5'ACAGTGTGTAGAGGTGGCTTCT3'	202	59	Caixeta et al. (2013a)
<i>KIT</i>	F: 5'ATCATGAAGACCTGCTGGGATGCT3' R: 5'GGGCTGCAGTTTGCTAAGTTGGAA3'	128	60	AF263827.1
<i>YBX2</i>	F: 5'GTGCTGGCAATCCAAGTCC3' R: 5'CTTCTCTCCTCCACGACATC3'	118	60	NM_001098126.2
<i>KL1</i>	F: 5'GTGATGATTTCCTCAACATCAAGTCC3' R: 5'TGCTACTGCTGTCTTCTAAGGG3'	111	60	NM_174375.2
<i>KL2</i>	F: 5'AAGGGAAGGCCTCAAATTCATTGAAGA3' R: 5'AGCAAACCCGATCACAAGAGA3'	92	60	NM_174375.2
<i>CCNB1</i>	F: 5'GGATACCTATGTGCCAAGAAG3' R: 5'CCGATTCTGGAGGGTACATT3'	96	60	NM_001045872.1
<i>NPPC</i>	F: 5'TCAGCCTCCTCGCATCT3' R: 5'ACAGCTGGTGTGTATCC3'	101	60	NM_174125.2
<i>NPR2</i>	F: 5'ATGACAGCATCAACCTGGACTGGA3' R: 5'AGCACGAAACGACTATCCACCACA3'	145	60	NM_174126.2
<i>PTGS2</i>	F: 5'AAGCCTAGCACTTCGGTGGAGAA3' R: 5'TCCAGAGTGGGAAGAGCTTGCAAT3'	168	60	Caixeta et al. (2013b)
<i>CYC-A</i>	F: 5'GCCATGGAGCGCTTTGG3' R: 5'CCACAGTCAGCAATGGTGATCT3'	65	60	Machado et al. (2009)

F, forward primer; R, reverse primer.

Meiosis progression: oocyte nuclear maturation

Meiosis progression was assessed after 22 h of maturation. Oocytes were denuded by pipetting in PBS and stained for 20 min with Hoechst 33342 (1 mg/mL Invitrogen H-1399) under a UV excitation using epifluorescence microscopy, and stage of meiosis were determined and classified as metaphase I (MI) or telophase I/metaphase II (TI/MII).

Gene expression analysis

After culture, cumulus cells and oocytes from 20 COCs were mechanically separated by repeated pipetting in PBS without calcium and magnesium. Cumulus cells were transferred to 1.5 mL tubes, collected by centrifugation for 5 min at 700 g and frozen at -80°C in 350 μL of RNA extraction lysis buffer from the RNeasy kit (Qiagen). Total RNA was extracted using the RNeasy kit as recommended by the manufacturer. After purification, RNA samples were eluted in 30 μL of RNase-free water. Total RNA concentrations were measured by spectrophotometry using a NanoDrop ND 1000 (Thermo Scientific). Total RNA (100 ng/reaction for cumulus cells and 9 μL of the RNA sample for oocytes) was incubated with DNase I (1 U/ μg ; Invitrogen) and then reverse transcribed using oligo-dT primers and Omniscript (for cumulus cells) or Sensiscript (for oocytes) reverse transcriptases (Qiagen). The reagents were incubated at 37°C for 60 min and then at 93°C for 3 min for enzyme inactivation. Relative real-time RT-PCR analysis was performed with an ABI 7500 thermocycler using Power SYBR Green PCR Master Mix (Applied Biosystems). The final volume of the PCR mix was 25 μL , and thermocycling conditions were: 95°C for 10 min (1 cycle), denaturing at 95°C for 10 s followed by annealing for 1 min (40 cycles). Primers for the housekeeping gene *CYCA* and for target genes *PTGS2*, *BMP15* and *GDF9* were as previously used and validated (Caixeta et al. 2013a,b). Primers to amplify bovine *YBX2*, *CCNB1*, *KIT*, *KITL1*, *KITL2*, *NPPC* and *NPR2* were designed

with PrimerQuest Tool and are shown in Table 1. The relative expression values for each gene were calculated using the $\Delta\Delta\text{Ct}$ method with efficiency correction and using one control sample as calibrator (Pfaffl 2001). Cyclophilin-A was used as the housekeeping gene as previously validated in our laboratory (Caixeta et al. 2013a).

Statistical analysis

Maturation and cumulus expansion data were arcsine transformed before analysis, and gene expression data were transformed to logarithms when not normally distributed. The effects of treatments with growth factors on cumulus cell expansion, maturation and gene expression were tested by analysis of variance (ANOVA), and means were compared with the Tukey–Kramer HSD test using JMP software (SAS Institute, Cary, NC, USA). Differences were considered significant when $P < 0.05$.

Results

KITL1, *KITL2* and *KIT* mRNA levels in COCs during IVM

To determine whether *KITL* undergoes alternative splicing in cattle, primers were designed to span the potential splice junction, and PCR performed on cumulus cell RNA. Agarose gel electrophoresis revealed two bands, and sequencing identified one product lacking 84 nucleotides of exon 3 that corresponds to *KITL2*, and another product including exon 3 corresponding to *KITL1*. Subsequent real-time PCR was performed with primers specific to each splice variant. Average Ct values for *KITL1* and *KITL2* mRNA in cumulus cells were 28.8 and 29.3 cycles respectively. Relative abundance of *KITL1* and *KITL2* mRNA increased during IVM with

Figure 1 Abundance of *KITL1*, *KITL2* (A) and *KIT* (B) mRNA in cumulus cells during IVM. Groups of immature COCs represent 0 h. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values ($\pm$ S.E.M.) relative to a calibrator sample and were calculated by the $\Delta\Delta C_t$ method with efficiency correction. Bars with different letters are significantly different (a, b, c for *KITL1*/x, y, z for *KITL2*; $P < 0.05$). Data were derived from four independent replicates.

significantly greater levels at 12 h compared with 0–8 h (Fig. 1). The abundance of *KIT* mRNA in the oocyte did not vary during IVM.

Effect of KITL on cumulus expansion and oocyte maturation

Addition of recombinant KITL1 to IVM medium did not affect cumulus expansion, but significantly stimulated the progression of meiosis as indicated by the increased proportion of oocytes reaching meiosis II (Fig. 2). Furthermore, KITL1 increased the levels of mRNA encoding the germ cell-specific marker *YBX2*, but did not alter those encoding *CCNB1*, *BMP15* and *GDF9* in the oocyte, or *NPR2* in cumulus cells (Fig. 3).

Addition of KITL1 decreased the abundance of *NPPC* mRNA in cumulus cell and had no effect on cumulus cell *NPR2* or *PTGS2* mRNA levels.

Effects of the oocyte or oocyte-secreted factors on KITL mRNA levels

Removal of the oocyte significantly increased the abundance of mRNA encoding *KITL1* and *KITL2* in cumulus

Figure 2 Effect of KITL1 on meiosis progression (A) and cumulus expansion (B) after 22 h of IVM. Oocytes were classified as MI (meiosis I; A1) or MII (oocytes in the transition telophase I/metaphase II and oocytes in metaphase II; A2). The degree of cumulus expansion was classified as grades 1 (poor expansion), 2 (partial expansion) and 3 (full expansion). Different letters within meiosis stages indicate significant differences ($P < 0.05$).

Figure 3 Effect of KITL1 on mRNA levels of genes regulating COC maturation in cumulus cells (*NPPC*, *NPR2* and *PTGS2*) and in the oocyte (*CCNB1*, *YBX2*, *BMP15* and *GDF9*). Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values ($\pm$ S.E.M.) relative to a calibrator sample by the $\Delta\Delta C_t$ method with efficiency correction. Bars with different letters are significantly different ($P < 0.05$). Data were derived from four independent replicates.

Figure 4 Effects of oocyte removal and oocyte replacement on KITL1, KITL2 and NPPC mRNA levels in cumulus cells. COCs were cultured intact (COC), oocyctomized (OOX) or oocyctomized with denuded oocytes (OOX+DO) for 22 h. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values ($\pm$ S.E.M.) relative to a calibrator sample by the $\Delta\Delta$ Ct method with efficiency correction. Bars with different letters are significantly different ($P < 0.05$). Data were derived from four independent replicates. Illustrative photomicrographs of culture groups before and after IVM (A and D: intact COCs; B and E: OOX; C and F: OOX plus denuded oocytes), and of the oocytectomy procedure (G).

cells, and addition of denuded oocytes reversed this effect (Fig. 4). In the same samples, oocytectomy significantly decreased NPPC mRNA levels, which again was restored to control levels by coculture with denuded oocytes (Fig. 4).

The addition of FGF8 to intact COC in IVM medium for 22 h increased cumulus cell KITL1 and KITL2 but decreased NPPC mRNA levels, addition of BMP15 increased only KITL1 mRNA levels, addition of FGF10

Figure 5 Effects of putative oocyte-secreted factors on KITL1, KITL2 and NPPC mRNA levels in cumulus cells. COCs were cultured with graded doses of FGF8, FGF10, BMP15 and GDF9 for 22 h of IVM. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values ($\pm$ S.E.M.) relative to a calibrator sample by the $\Delta\Delta$ Ct method with efficiency correction. Bars with different letters are significantly different ($P < 0.05$). Data were derived from four independent replicates.

Figure 6 Illustrative model of the paracrine interaction of KITL, NPPC, YBX2 and oocyte-derived factors (BMP15, FGF8 and FGF10) in the bovine COC.

decreased *KITL2* and *NPPC* mRNA abundance and addition of GDF9 had no effect on *KITL1*, *KITL2* or *NPPC* mRNA expression (Fig. 5).

Discussion

In mice, the oocyte is critical for several cumulus cell functions including expansion, and regulates the levels of mRNA encoding *Nppc* and *Kitl*, two proteins involved in resumption of meiosis. In cattle, the oocyte is not necessary for expansion, and effects of the oocyte on cumulus cell *NPPC* and *KITL* mRNA levels have not previously been reported. In this study, we demonstrate that, surprisingly, the bovine oocyte regulates these two genes in a manner very similar to that previously observed in the mouse, and we show that KITL enhances nuclear maturation in the cow. We also link KITL and NPPC for the first time, demonstrating that KITL downregulates *NPPC* mRNA levels in cumulus cells, and suggesting that this is a mechanism by which KITL increases the maturation of the oocyte. A model depicting the paracrine interaction involving KITL, NPPC, YBX2 and oocyte-derived factors in the bovine COC is proposed (Fig. 6).

One of the more interesting results of this study is the similarity in the response of cumulus cell *KITL* and *NPPC* mRNA levels to oocyte removal observed here in cattle with that previously observed in mice (Joyce *et al.* 1999, Lee *et al.* 2013). This appears to be unusual in that cumulus function in cattle, such as metabolism and expansion (Ralph *et al.* 1995, Sutton *et al.* 2003) and appears not to be impacted by the removal of the oocyte. Equally novel is the ability of denuded bovine oocytes to completely restore *KITL* and *NPPC* mRNA levels to those observed

in intact COCs, suggesting the role of an oocyte-secreted factor (OSF). A previous study demonstrated the role of the bovine oocyte in maintaining cumulus cell health (Hussein *et al.* 2005); collectively, these data point to a role for the oocyte in cumulus function in cattle, but one that differs from that in the mouse.

In rodents, Gdf9 is an abundant OSF (Crawford & McNatty 2012) and inhibits *Kitl* mRNA levels in cumulus cells (Joyce *et al.* 2000). The murine oocyte may therefore maintain low *Kitl* expression levels through the secretion of Gdf9 (Joyce *et al.* 2000, Otsuka and Shimasaki 2002, Thomas *et al.* 2005, Miyoshi *et al.* 2012), and thus removal of the oocyte increased *Kitl* mRNA levels (Joyce *et al.* 1999). In the cow, however, it is believed that BMP15 is the more abundant OSF compared with GDF9 (Crawford & McNatty 2012) and so the oocyte may not control cumulus *KITL* mRNA in the same manner. Moreover, a recent study points to a physiological and more effective action for the promature form of the heterodimer BMP15-GDF9 named cumulin in relation to BMP15 and GDF9 homodimers in monovular species (Mottershead *et al.* 2015). In this study, FGF8 and BMP15 stimulated *KITL* mRNA levels, as in the mouse, and FGF10 inhibited *KITL* mRNA abundance. Therefore, the increase in *KITL* mRNA levels following oocyte removal in cattle may be at least in part attributable to the loss of FGF10, and not to the influence of oocyte-derived FGF8 and BMP15 homodimer. The reduction in *NPPC* mRNA levels induced by FGF8 is likely mediated by KITL signalling given the stimulatory effect of FGF8 on *KITL* expression and the inhibitory effect of KITL on *NPPC* expression. In contrast, FGF10 appears to inhibit *NPPC* expression by a different mechanism since it decreased *KITL2* mRNA levels without affecting those of *KITL1*. The decrease in *NPPC* expression induced by FGF10 is consistent with its previously reported stimulatory effect on oocyte nuclear maturation (Zhang *et al.* 2010b).

In this study, both *KITL1* and *KITL2* mRNA levels increased with time during cumulus expansion. This is consistent with the increase in *Kitl* mRNA levels observed in granulosa and cumulus cells in mice in response to eCG (Ismail *et al.* 1996, Ye *et al.* 2009). The effects of KITL on oocyte maturation are not yet clear. Addition of KITL to denuded rat oocytes reduced the rate of the 1st polar body extrusion after 14 h culture (Ismail *et al.* 1996), whereas it increased the rate of the 1st polar body extrusion after 22 h in mice (Ye *et al.* 2009) and the rate of oocytes reaching MII in cattle (present study). The importance of this effect is not clear, as in mice, treatment with KITL did not enhance the rate of blastocyst development (Ye *et al.* 2009). The KITL-induced increase in rates of maturation in the mouse was associated with increased levels of Ccnb1 protein in MI oocytes (Ye *et al.* 2009), whereas in this study, KITL did not alter *CCNB1* mRNA levels in MII oocytes. As *CCNB1* is involved in meiosis 1, it is likely that the time point chosen in this study was too late to detect an effect of KITL.

Addition of KITL1 to COC decreased the abundance of mRNA encoding NPPC, and this may offer an explanation for the mechanism of action of KITL. Resumption of meiosis is triggered by a decrease in cAMP levels in the oocyte, which is the result of decreased transfer of cAMP and cGMP from cumulus cells (Norris *et al.* 2009). There is no evidence that KITL alters cumulus cGMP levels, and KITL did not alter FSH-stimulated cAMP levels in rat granulosa–oocyte cocultures (Miyoshi *et al.* 2012). However, NPPC has been demonstrated to increase cumulus cell cGMP levels and maintain meiotic arrest in rodents and cattle (Zhang *et al.* 2010a, Franciosi *et al.* 2014); therefore, the decrease in NPPC mRNA caused by KITL observed here might be expected to lead to a decrease in NPPC signalling and of cGMP levels, and thus the resumption of meiosis. We are not aware of any other reports demonstrating the regulation of NPPC levels by KITL. In the present dataset, although the highest dose of KITL promoted the maximal decrease in NPPC mRNA abundance, it did not alter oocyte nuclear maturation. A possible explanation for this intriguing observation is that high doses of KITL may precociously and intensively suppress NPPC/NPR2 signalling leading to abrupt closure of gap junctions, which could be detrimental to meiosis resumption.

A further novel finding of this study is the stimulatory effect of KITL on oocyte YBX2 mRNA levels. In this study, we used oocytes from follicles 3–8 mm in diameter, which are predominantly at advanced stages of the germinal vesicle and with low transcriptional activity (Lodde *et al.* 2007, 2008), and oligo-dT primers in the RT-PCR strategy. Therefore, this result more likely reflects an increase in adenylated YBX2 mRNA. In female mice, *Ybx2* is expressed exclusively in the oocyte and encodes a very abundant protein that stabilizes maternal mRNA, sustains protein synthesis and is required for normal spindle formation (Yu *et al.* 2001, 2002, 2004, Medvedev *et al.* 2011). Mice null for *Ybx2* are infertile (Yang *et al.* 2005) and adult mice are characterized by degeneration and loss of oocytes. Interestingly, in juvenile *Ybx2*^{-/-} mice, with a less pronounced loss of oocytes, follicles were unable to ovulate in response to exogenous gonadotrophin (Yang *et al.* 2005), suggesting that *Ybx2* may play some role in oocyte signalling to the cumulus cells and the mechanism of ovulation. In cattle, YBX2 protein levels decrease from the germinal vesicle stage to meiosis II (Vigneault *et al.* 2009). Although speculative, the present data suggest that KITL may enhance YBX2 action in the bovine oocyte, contributing for RNA stability and protein production during the maternal-embryo transition and for normal spindle formation/meiosis completion.

In summary, the present data show that *KITL* mRNA levels increase during IVM in cattle, and that addition of KITL1 to COC enhances resumption of meiosis. Two potential mechanisms by which KITL may increase oocyte maturation are by inhibiting the expression

of NPPC, a factor that maintains meiotic arrest, and by increasing expression of YBX2, an oocyte-specific protein essential for ovulation and oocyte health. In addition, we provide evidence that the oocyte regulates the expression of *KITL* and *NPPC* in cumulus cells, suggesting a novel mechanism by which the oocyte may influence its own fate.

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.

Funding

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil).

References

- Brannan CI, Lyman SD, Williams DE, Eisenman J, Anderson DM, Cosman D, Bedell MA, Jenkins NA & Copeland NG 1991 Steel-Dickie mutation encodes a c-kit ligand lacking transmembrane and cytoplasmic domains. *PNAS* **88** 4671–4674. (doi:10.1073/pnas.88.11.4671)
- Buccione R, Vanderhyden BC, Caron PJ & Eppig JJ 1990 FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Developmental Biology* **138** 16–25. (doi:10.1016/0012-1606(90)90172-F)
- Caixeta ES, Machado MF, Ripamonte P, Price C & Buratini J 2013a Effects of FSH on the expression of receptors for oocyte-secreted factors and members of the EGF-like family during in vitro maturation in cattle. *Reproduction, Fertility, and Development* **25** 890–899. (doi:10.1071/RD12125)
- Caixeta ES, Sutton-McDowall ML, Gilchrist RB, Thompson JG, Price CA, Machado MF, Lima PF & Buratini J 2013b Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during in vitro maturation of bovine cumulus-oocyte complexes. *Reproduction* **146** 27–35. (doi:10.1530/REP-13-0079)
- Celestino JJ, Bruno JB, Lima-Verde IB, Matos MH, Saraiva MV, Chaves RN, Martins FS, Almeida AP, Cunha RM, Lima LF *et al.* 2010 Steady-state level of kit ligand mRNA in goat ovaries and the role of kit ligand in preantral follicle survival and growth in vitro. *Molecular Reproduction and Development* **77** 231–240.
- Crawford JL & McNatty KP 2012 The ratio of growth differentiation factor 9: bone morphogenetic protein 15 mRNA expression is tightly co-regulated and differs between species over a wide range of ovulation rates. *Molecular and Cellular Endocrinology* **348** 339–343. (doi:10.1016/j.mce.2011.09.033)
- De Cesaro MP, Macedo MP, Santos JT, Rosa PR, Ludke CA, Rissi VB, Gasperin BG & Gonçalves PB 2015 Natriuretic peptides stimulate oocyte meiotic resumption in bovine. *Animal Reproduction Science* **159** 52–59. (doi:10.1016/j.anireprosci.2015.05.012)
- Eppig J 2005 Mouse oocytes control metabolic co-operativity between oocytes and cumulus cells. *Reproduction, Fertility, and Development* **17** 1–2. (doi:10.1071/RDv17n2paperabs)
- Franciosi F, Cotichio G, Lodde V, Tessaro I, Modina SC, Fadini R, Dal Canto M, Renzini MM, Albertini DF & Luciano AM 2014 Natriuretic peptide precursor C delays meiotic resumption and sustains gap junction-mediated communication in bovine cumulus-enclosed oocytes. *Biology of Reproduction* **91** 61. (doi:10.1095/biolreprod.114.118869)
- Huang EJ, Nocka KH, Buck J & Besmer P 1992 Differential expression and processing of two cell associated forms of the kit-ligand: KL-1 and KL-2. *Molecular Biology of the Cell* **3** 349–362. (doi:10.1091/mbc.3.3.349)

- Hussein TS, Froiland DA, Amato F, Thompson JG & Gilchrist RB 2005 Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. *Journal of Cell Science* **118** 5257–5268. (doi:10.1242/jcs.02644)
- Hutt KJ, McLaughlin EA & Holland MK 2006 Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis. *Molecular Human Reproduction* **12** 61–69. (doi:10.1093/molehr/gal010)
- Hyttel P, Xu KP, Smith S & Greve T 1986 Ultrastructure of in-vitro oocyte maturation in cattle. *Journal of Reproduction and Fertility* **78** 615–625. (doi:10.1530/jrf.0.0780615)
- Ismail RS, Okawara Y, Fryer JN & Vanderhyden BC 1996 Hormonal regulation of the ligand for c-kit in the rat ovary and its effects on spontaneous oocyte meiotic maturation. *Molecular Reproduction and Development* **43** 458–469. (doi:10.1002/(SICI)1098-2795(199604)43:4<458::AID-MRD8>3.0.CO;2-O)
- Ismail RS, Dubé M & Vanderhyden BC 1997 Hormonally regulated expression and alternative splicing of kit ligand may regulate kit-induced inhibition of meiosis in rat oocytes. *Developmental Biology* **184** 333–342. (doi:10.1006/dbio.1997.8531)
- Joyce IM, Pendola FL, Wigglesworth K & Eppig JJ 1999 Oocyte regulation of kit ligand expression in mouse ovarian follicles. *Developmental Biology* **214** 342–353. (doi:10.1006/dbio.1999.9437)
- Joyce IM, Clark AT, Pendola FL & Eppig JJ 2000 Comparison of recombinant growth differentiation factor-9 and oocyte regulation of KIT ligand messenger ribonucleic acid expression in mouse ovarian follicles. *Biology of Reproduction* **63** 1669–1675. (doi:10.1095/biolreprod63.6.1669)
- Kawaguchi N, Horiuchi K, Becherer JD, Toyama Y, Besmer P & Blobel CP 2007 Different ADAMs have distinct influences on Kit ligand processing: phorbol-ester-stimulated ectodomain shedding of Kitl1 by ADAM17 is reduced by ADAM19. *Journal of Cell Science* **120** 943–952. (doi:10.1242/jcs.03403)
- Kawamura K, Cheng Y, Kawamura N, Takae S, Okada A, Kawagoe Y, Mulders S, Terada Y & Hsueh AJ 2011 Pre-ovulatory LH/hCG surge decreases C-type natriuretic peptide secretion by ovarian granulosa cells to promote meiotic resumption of pre-ovulatory oocytes. *Human Reproduction* **26** 3094–3101. (doi:10.1093/humrep/der282)
- Lee KB, Zhang M, Sugiura K, Wigglesworth K, Uliasz T, Jaffe LA & Eppig JJ 2013 Hormonal coordination of natriuretic peptide type C and natriuretic peptide receptor 3 expression in mouse granulosa cells. *Biology of Reproduction* **88** 42. (doi:10.1095/biolreprod.112.104810)
- Liu X, Xie F, Zamah AM, Cao B & Conti M 2014 Multiple pathways mediate luteinizing hormone regulation of cGMP signalling in the mouse ovarian follicle. *Biology of Reproduction* **91** 9. (doi:10.1095/biolreprod.113.116814)
- Lodde V, Modina S, Galbusera C, Franciosi F & Luciano AM 2007 Large-scale chromatin remodeling in germinal vesicle bovine oocytes: interplay with gap junction functionality and developmental competence. *Molecular Reproduction and Development* **74** 740–749. (doi:10.1002/mrd.20639)
- Lodde V, Modina S, Maddox-Hyttel P, Franciosi F, Lauria A & Luciano AM 2008 Oocyte morphology and transcriptional silencing in relation to chromatin remodeling during the final phases of bovine oocyte growth. *Molecular Reproduction and Development* **75** 915–924. (doi:10.1002/mrd.20824)
- Machado MF, Portela VM, Price CA, Costa IB, Ripamonte P, Amorim RL & Buratini J 2009 Regulation and action of fibroblast growth factor 17 in bovine follicles. *Journal of Endocrinology* **202** 347–353. (doi:10.1677/JOE-09-0145)
- Machado MF, Caixeta ES, Sudiman J, Gilchrist RB, Thompson JG, Lima PF, Price CA & Buratini J 2015 Fibroblast growth factor 17 and bone morphogenetic protein 15 enhance cumulus expansion and improve quality of in vitro-produced embryos in cattle. *Theriogenology* **84** 390–398. (doi:10.1016/j.theriogenology.2015.03.031)
- Marei WF, Abayasekara DR, Wathes DC & Fouladi-Nashta AA 2014 Role of PTGS2-generated PGE2 during gonadotrophin-induced bovine oocyte maturation and cumulus cell expansion. *Reproductive Biomedicine Online* **28** 388–400. (doi:10.1016/j.rbmo.2013.11.005)
- Medvedev S, Pan H & Schultz RM 2011 Absence of MSY2 in mouse oocytes perturbs oocyte growth and maturation, RNA stability, and the transcriptome. *Biology of Reproduction* **85** 575–583. (doi:10.1095/biolreprod.111.091710)
- Miyoshi T, Otsuka F, Nakamura E, Inagaki K, Ogura-Ochi K, Tsukamoto N, Takeda M & Makino H 2012 Regulatory role of kit ligand-c-kit interaction and oocyte factors in steroidogenesis by rat granulosa cells. *Molecular and Cellular Endocrinology* **358** 18–26. (doi:10.1016/j.mce.2012.02.011)
- Mottershead DG, Sugimura S, Al-Musawi SL, Li JJ, Richani D, White MA, Martin GA, Trotta AP, Ritter LJ, Shi J *et al.* 2015 Cumulin, an Oocyte-secreted heterodimer of the transforming growth factor- β family, is a potent activator of granulosa cells and improves oocyte quality. *Journal of Biological Chemistry* **290** 24007–24020. (doi:10.1074/jbc.M115.671487)
- Norris RP, Ratzan WJ, Freudzon M, Mehlmann LM, Krall J, Movsesian MA, Wang H, Ke H, Nikolaev VO & Jaffe LA 2009 Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development* **136** 1869–1878. (doi:10.1242/dev.035238)
- Otsuka F & Shimasaki S 2002 A negative feedback system between oocyte bone morphogenetic protein 15 and granulosa cell kit ligand: its role in regulating granulosa cell mitosis. *PNAS* **99** 8060–8065. (doi:10.1073/pnas.122066899)
- Packer AI, Hsu YC, Besmer P & Bachvarova RF 1994 The ligand of the c-kit receptor promotes oocyte growth. *Developmental Biology* **161** 194–205. (doi:10.1006/dbio.1994.1020)
- Paradis F, Moore HS, Pasternak JA, Novak S, Dyck MK, Dixon WT & Foxcroft GR 2010 Pig preovulatory oocytes modulate cumulus cell protein and gene expression in vitro. *Molecular and Cellular Endocrinology* **320** 87–96. (doi:10.1016/j.mce.2010.01.034)
- Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29** e45. (doi:10.1093/nar/29.9.e45)
- Prochazka R, Blaha M & Nemcova L 2012 Signaling pathways regulating FSH- and amphiregulin-induced meiotic resumption and cumulus cell expansion in the pig. *Reproduction* **144** 535–546. (doi:10.1530/REP-12-0191)
- Ralph JH, Telfer EE & Wilmot I 1995 Bovine cumulus cell expansion does not depend on the presence of an oocyte secreted factor. *Molecular Reproduction and Development* **42** 248–253. (doi:10.1002/mrd.1080420214)
- Regassa A, Rings F, Hoelker M, Cinar U, Tholen E, Looft C, Schellander K & Tesfaye D 2011 Transcriptome dynamics and molecular cross-talk between bovine oocyte and its companion cumulus cells. *BMC Genomics* **12** 57. (doi:10.1186/1471-2164-12-57)
- Reizel Y, Elbaz J & Dekel N 2010 Sustained activity of the EGF receptor is an absolute requisite for LH-induced oocyte maturation and cumulus expansion. *Molecular Endocrinology* **24** 402–411. (doi:10.1210/me.2009-0267)
- Richards JS 2005 Ovulation: new factors that prepare the oocyte for fertilization. *Molecular and Cellular Endocrinology* **234** 75–79. (doi:10.1016/j.mce.2005.01.004)
- Robinson JW, Zhang M, Shuhaibar LC, Norris RP, Geerts A, Wunder F, Eppig JJ, Potter LR & Jaffe LA 2012 Luteinizing hormone reduces the activity of the NPR2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes. *Developmental Biology* **366** 308–316. (doi:10.1016/j.ydbio.2012.04.019)
- Shuhaibar LC, Egbert JR, Norris RP, Lampe PD, Nikolaev VO, Thunemann M, Wen L, Feil R & Jaffe LA 2015 Intercellular signaling via cyclic GMP diffusion through gap junctions restarts meiosis in mouse ovarian follicles. *PNAS* **112** 5527–5532. (doi:10.1073/pnas.1423598112)
- Sugiura K, Pendola FL & Eppig JJ 2005 Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Developmental Biology* **279** 20–30. (doi:10.1016/j.ydbio.2004.11.027)
- Sutton ML, Cetica PD, Beconi MT, Kind KL, Gilchrist RB & Thompson JG 2003 Influence of oocyte-secreted factors and culture duration on the metabolic activity of bovine cumulus cell complexes. *Reproduction* **126** 27–34. (doi:10.1530/rep.0.1260027)
- Thomas FH & Vanderhyden BC 2006 Oocyte-granulosa cell interactions during mouse follicular development: regulation of kit ligand expression and its role in oocyte growth. *Reproductive Biology and Endocrinology* **4** 19. (doi:10.1186/1477-7827-4-19)
- Thomas FH, Ethier JF, Shimasaki S & Vanderhyden BC 2005 Follicle-stimulating hormone regulates oocyte growth by modulation of expression of oocyte and granulosa cell factors. *Endocrinology* **146** 941–949. (doi:10.1210/en.2004-0826)

- Thomas FH, Ismail RS, Jiang JY & Vanderhyden BC 2008 Kit ligand 2 promotes murine oocyte growth in vitro. *Biology of Reproduction* **78** 167–175. (doi:10.1095/biolreprod.106.058529)
- Vanderhyden BC 1993 Species differences in the regulation of cumulus expansion by an oocyte-secreted factor(s). *Journal of Reproduction and Fertility* **98** 219–227. (doi:10.1530/jrf.0.0980219)
- Vigneault C, McGraw S & Sirard MA 2009 Spatiotemporal expression of transcriptional regulators in concert with the maternal-to-embryonic transition during bovine in vitro embryogenesis. *Reproduction* **137** 13–21. (doi:10.1530/REP-08-0077)
- Yang J, Medvedev S, Yu J, Tang LC, Agno JE, Matzuk MM, Schultz RM & Hecht NB 2005 Absence of the DNA-/RNA-binding protein MSY2 results in male and female infertility. *PNAS* **102** 5755–5760. (doi:10.1073/pnas.0408718102)
- Ye Y, Kawamura K, Sasaki M, Kawamura N, Groenen P, Gelpke MD, Rauch R, Hsueh AJ & Tanaka T 2009 Kit ligand promotes first polar body extrusion of mouse preovulatory oocytes. *Reproductive Biology and Endocrinology* **7** 26. (doi:10.1186/1477-7827-7-26)
- Yu J, Hecht NB & Schultz RM 2001 Expression of MSY2 in mouse oocytes and preimplantation embryos. *Biology of Reproduction* **65** 1260–1270. (doi:10.1095/biolreprod65.4.1260)
- Yu J, Hecht NB & Schultz RM 2002 RNA-binding properties and translation repression in vitro by germ cell-specific MSY2 protein. *Biology of Reproduction* **67** 1093–1098. (doi:10.1095/biolreprod67.4.1093)
- Yu J, Deng M, Medvedev S, Yang J, Hecht NB & Schultz RM 2004 Transgenic RNAi-mediated reduction of MSY2 in mouse oocytes results in reduced fertility. *Developmental Biology* **268** 195–206. (doi:10.1016/j.ydbio.2003.12.020)
- Zhang M, Su YQ, Sugiura K, Xia G & Eppig JJ 2010a Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science* **330** 366–369. (doi:10.1126/science.1193573)
- Zhang K, Hansen PJ & Ealy AD 2010b Fibroblast growth factor 10 enhances bovine oocyte maturation and developmental competence in vitro. *Reproduction* **140** 815–26. (doi:10.1530/REP-10-0190)
- Zhou JH, Hikono H, Ohtaki M, Kubota T & Sakurai M 1994 Cloning and characterization of cDNAs encoding two normal isoforms of bovine stem cell factor. *Biochimica et Biophysica Acta* **1223** 148–150. (doi:10.1016/0167-4889(94)90084-1)

Received 23 March 2016

First decision 23 May 2016

Revised manuscript received 11 July 2016

Accepted 4 August 2016