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

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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.

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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 (oocytectomy) 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 oocytectomized 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 ($n = 4$/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 ($n = 4$/dose), FGF8 ($n = 5$/dose) or FGF17 ($n = 4$/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 ($n = 4$/dose), and the progression of the oocyte through meiosis ($n = 9$/dose) as well as key genes involved in nuclear maturation ($n = 4$/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, oocytectomized COCs and oocytectomized COCs cultured with denuded oocytes (1 denuded oocyte/µL; $n = 4$/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 µg/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 µg/mL sodium pyruvate, 75 µg/ml amicacin, 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.

Oocytectomy

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
BMP15	F: 5′GTCAGCAGCCAAGAGGTAGTG3′ R: 5′CCCGAGGACATACTCCCTTAC3′	360	59	Caixeta *et al*. (2013*a*)
GDF9	F: 5′TGGTCCTTGCTGAAGCATCTAGA3′ R: 5′ACAGTGTTGTAGAGGTGGCTTCT3′	202	59	Caixeta *et al*. (2013*a*)
KIT	F: 5′ATCATGAAGACCTGCTGGGATGCT3′ R: 5′GGGCTGCAGTTTGCTAAGTTGGAA3′	128	60	AF263827.1
YBX2	F: 5′GTGCTGGCAATCCAAGTCC3′ R: 5′CTTCTCTCCTTCCACGACATC3′	118	60	NM_001098126.2
KL1	F: 5′GTGTGATTTCCTCAACATCAAGTCC3′ R: 5′TGCTACTGCTGTCATTCCTAAGGG3′	111	60	NM_174375.2
KL2	F: 5′AAGGGAAGGCCTCAAATTCCATTGAAGA3′ R: 5′AGCAAACCCGATCACAAGAGA3′	92	60	NM_174375.2
CCNB1	F: 5′GGATACCTATGTGCCCAAGAAG3′ R: 5′CCGATTTCTGGAGGGTACATTT3′	96	60	NM_001045872.1
NPPC	F: 5′TCAGCCTCCTCGCATCT3′ R: 5′ACAGCTGGTGTTGTATCC3′	101	60	NM_174125.2
NPR2	F: 5′ATGACAGCATCAACCTGGACTGGA3′ R: 5′AGCACGAAACGACTATCCACCACA3′	145	60	NM_174126.2
PTGS2	F: 5′AAGCCTAGCACTTTCGGTGGAGAA3′ R: 5′TCCAGAGTGGGAAGAGCTTGCATT3′	168	60	Caixeta *et al*. (2013*b*)
CYC-A	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*. 2013*a*,*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 ΔΔCt 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*. 2013*a*).

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
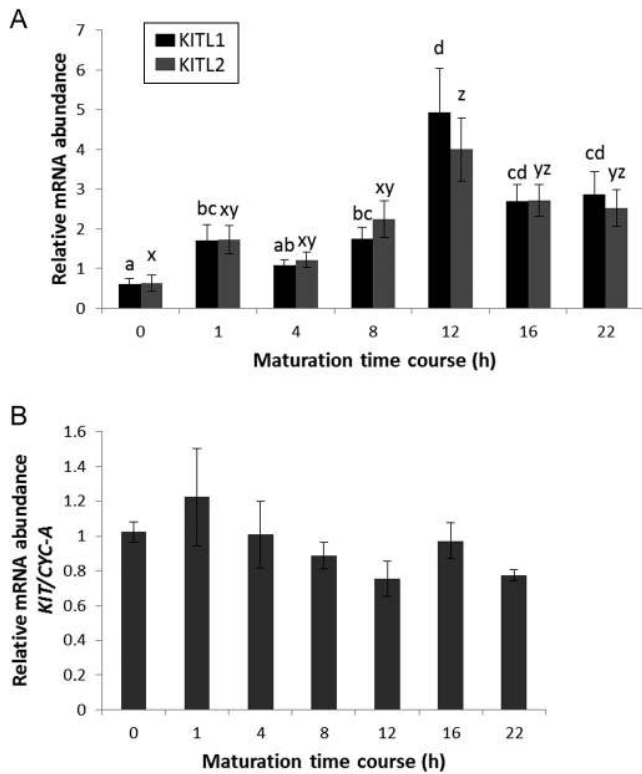
Figure 1 Abundance of *KITL1, KITL2* (A) and *KIT* (B) mRNA in cumulus cells during IVM. Groups of immature COCs represent 0 h. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±s.e.m.) relative to a calibrator sample and were calculated by the ΔΔCt method with 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
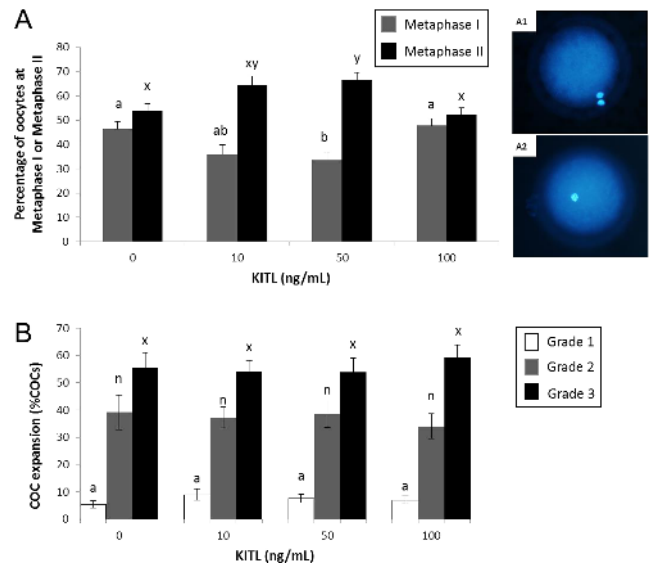

Figure 2 Effect of KITL1 on meiosis progression (A) and cumulus expansion (B) after 22 h of IVM. Oocytes were classified as MI (meiosis I; A1) or TI/MII (oocytes in the transition telophase I/metaphase II and oocytes in metaphase II; A2). The degree of cumulus expansion was classified as grades 1 (poor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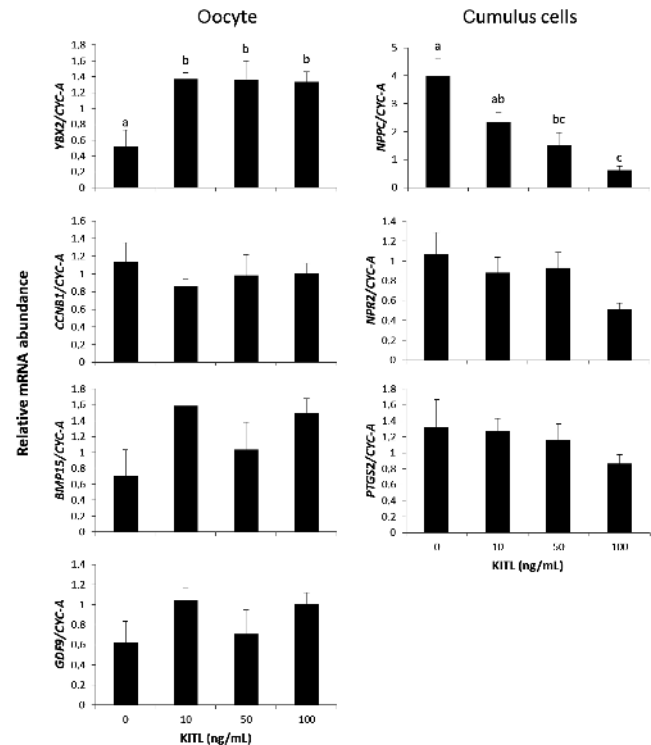

Figure 3 Effect of KITL1 on mRNA levels of genes regulating COC maturation in cumulus cells (*NPPC*, *NPR2* and *PTGS2*) and in the oocyte (*CCNB1*, *YBX2*, *BMP15* and *GDF9*). Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±s.e.m.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different ($P < 0.05$). Data were derived from four independent replicates.
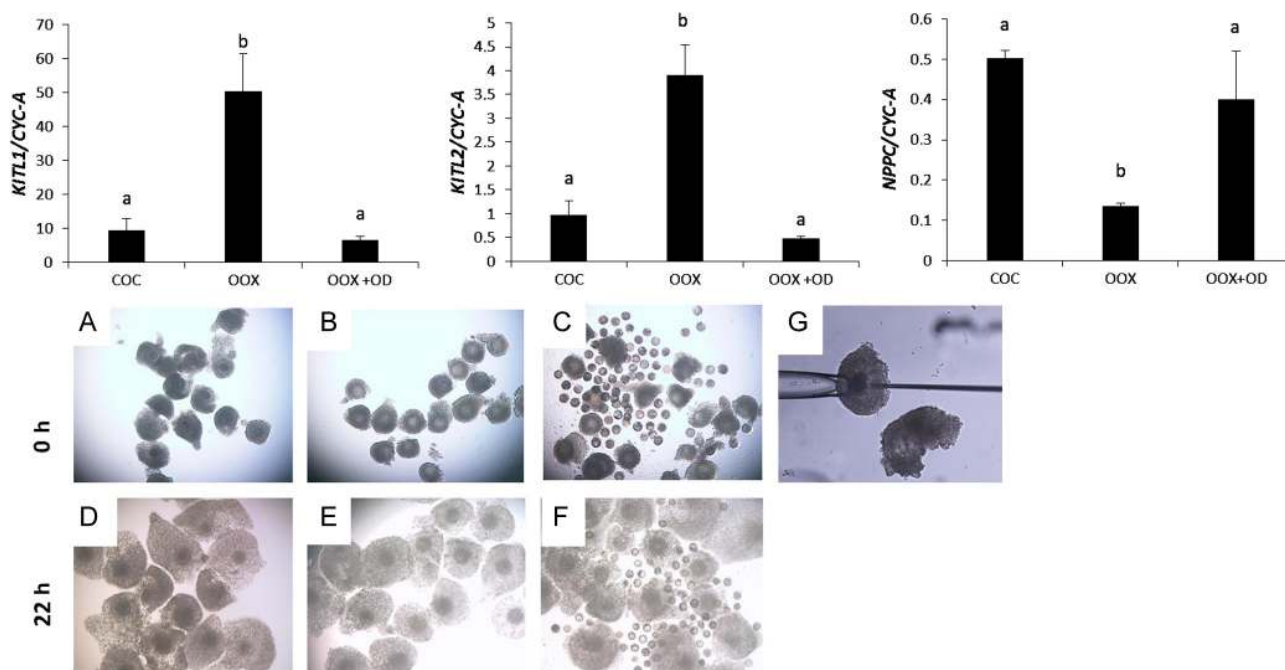
Figure 4 Effects of oocyte removal and oocyte replacement on KITL1, KITL2 and NPPC mRNA levels in cumulus cells. COCs were cultured intact (COC), oocytectomized (OOX) or oocytectomized with denuded oocytes (OOX+DO) for 22 h. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (*P* < 0.05). Data were derived from four independent replicates. Ilustrative 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
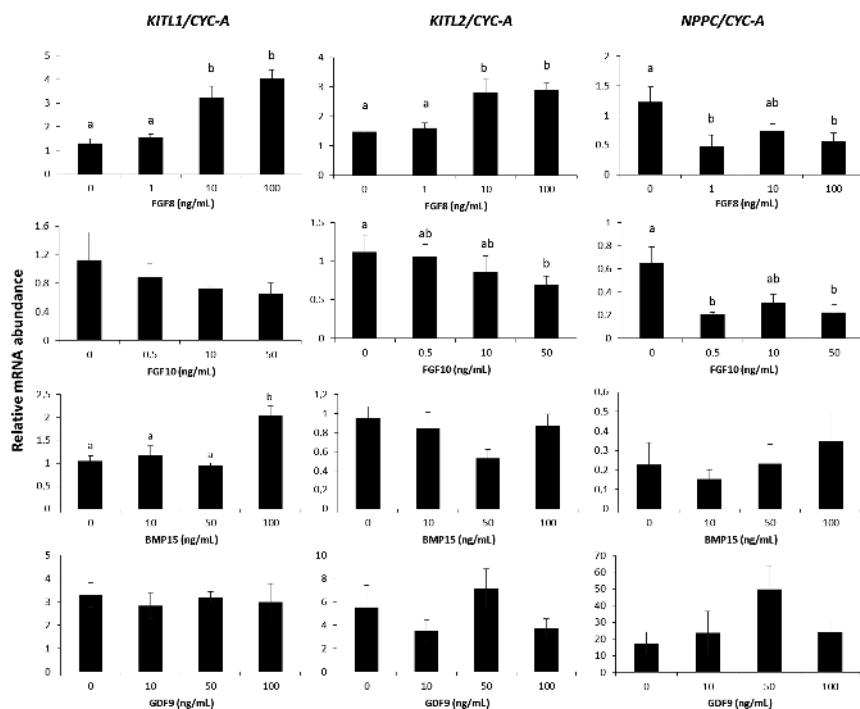

Figure 5 Effects of putative oocyte-secreted factors on *KITL1, KITL2* and *NPPC* mRNA levels in cumulus cells. COCs were cultured with graded doses of FGF8, FGF10, BMP15 and GDF9 for 22 h of IVM. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (*P* < 0.05). Data were derived from four independent replicates.
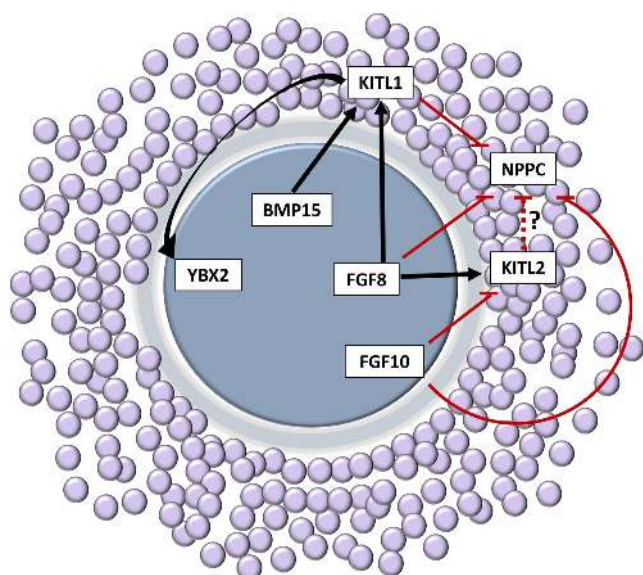
Figure 6 Illustrative model of the paracrine interaction of KITL, NPPC, YBX2 and oocyte-derived factors (BMP15, FGF8 and FGF10) in the bovine COC.

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 oocytectomy 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 GFD9 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 oocytectomy 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*. 2010*b*).

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*. 2010*a*, 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.

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