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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

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Abstract

Oocyte-secreted factors (OSFs) regulate differentiation of cumulus cells and are of pivotal relevance for fertility. Bone morphogenetic protein 15 (BMP15) and fibroblast growth factor 10 (FGF10) are OSFs and enhance oocyte competence by unknown mechanisms. We tested the hypothesis that BMP15 and FGF10, alone or combined in the maturation medium, enhance cumulus expansion and expression of genes in the preovulatory cascade and regulate glucose metabolism favouring hyaluronic acid production in bovine cumulus–oocyte complexes (COCs). BMP15 or FGF10 increased the percentage of fully expanded COCs, but the combination did not further stimulate it. BMP15 increased cumulus cell levels of mRNA encoding a disintegrin and metalloprotease 10 (*ADAM10*), *ADAM17*, amphiregulin (*AREG*), and epiregulin (*EREG*) at 12 h of culture and of prostaglandin (PG)-endoperoxide synthase 2 (*PTGS2*), pentraxin 3 (*PTX3*) and tumor necrosis factor alpha-induced protein 6 (*TNFAIP6* (*TSG6*)) at 22 h of culture. FGF10 did not alter the expression of epidermal growth factor-like factors but enhanced the mRNA expression of *PTGS2* at 4 h, *PTX3* at 12 h, and *TNFAIP6* at 22 h. FGF10 and BMP15 stimulated glucose consumption by cumulus cells but did not affect lactate production or levels of mRNA encoding glycolytic enzymes phosphofructokinase and lactate dehydrogenase A. Each growth factor increased mRNA encoding glucosamine:fructose-6-PO_4 transaminases, key enzymes in the hexosamine pathway leading to hyaluronic acid production, and BMP15 also stimulated hyaluronan synthase 2 (*HAS2*) mRNA expression. This study provides evidence that BMP15 and FGF10 stimulate expansion of *in vitro*-matured bovine COCs by driving glucose metabolism toward hyaluronic acid production and controlling the expression of genes in the ovulatory cascade, the first acting upon *ADAM10*, *ADAM17*, *AREG*, and *EREG* and the second on downstream genes, particularly *PTGS2*.
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Introduction

In vitro maturation (IVM) of cumulus–oocyte complexes (COCs) is a key component of assisted reproduction in cattle, humans, and other species (Gilchrist *et al*. 2008), although its widespread use is hindered because developmental competence of the oocyte is markedly compromised by IVM in cattle, sheep, mice, and humans (Thompson *et al*. 1995, Child *et al*. 2002, Rizos *et al*. 2002, Vanhoutte *et al*. 2009). Clearly, more detailed information is required on the cascade of molecular events that lead to COC maturation for the improvement of IVM protocols. The preovulatory LH surge induces COC expansion *in vivo* (reviewed by Richards *et al*. (2002)), and studies on mice and rats indicate that the effect of LH on the COC is mediated by the epidermal growth factor (EGF)-like family members, amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC), that are secreted by mural granulosa cells and act upon cumulus cells (Park *et al*. 2004, Ashkenazi *et al*. 2005, Conti *et al*. 2006). FSH can also stimulate the expression/synthesis of EGF-like growth factors in murine and bovine cumulus cells (Downs & Chen 2008, Caixeta *et al*. 2012). EGF-like growth factors are synthesized as transmembrane precursors and must undergo proteolytic cleavage ('shedding') by members of the disintegrin and metalloproteinase (ADAM) family (reviewed by Ben-Ami *et al*. (2006)). Once released, the EGF-like factors

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-13-0079
Online version via www.reproduction-online.org

activate the EGF receptor (EGFR) on cumulus cells and stimulate the expression of genes necessary for cumulus expansion, including prostaglandin (PG)-endoperoxide synthase 2 (*PTGS2*), tumor necrosis factor alpha-induced protein 6 (*TNFAIP6* (*TSG6*)), pentraxin 3 (*PTX3*), and hyaluronan synthase 2 (*HAS2*) (Ashkenazi *et al*. 2005, Conti *et al*. 2006, Shimada *et al*. 2006, Su *et al*. 2010).

HAS2 is necessary for the synthesis of hyaluronic acid, the major component of cumulus extracellular matrix, and reduction of HAS2 by siRNA in the mouse COC inhibited expansion (Sugiura *et al*. 2009). Hyaluronic acid is produced by glucose metabolism via the hexosamine biosynthetic pathway in cumulus cells, for which the rate-limiting step is the conversion of fructose-6-phosphate to glucosamine-6-phosphate by the enzymes glucosamine:fructose-6-PO_4 transaminases (GFPTs). The final step in this pathway leads to hyaluronic acid production, catalyzed by HAS2. Although the hexosamine biosynthetic pathway is critical for expansion, most of the glucose consumed by cumulus cells is metabolized via the glycolytic pathway by the rate-limiting enzyme phosphofructokinase (PFK) for the production of energy (reviewed by Sutton-McDowall *et al*. (2010)).

Cumulus expansion and glucose metabolism are modulated in part by oocyte-secreted factors (OSFs) including bone morphogenetic protein 15 (BMP15) and fibroblast growth factor 8 (FGF8) (Valve *et al*. 1997, Juengel *et al*. 2004, Gilchrist *et al*. 2008). BMP15 treatment during IVM stimulated cumulus expansion in mice (Yoshino *et al*. 2006) and blastocyst production in cattle (Hussein *et al*. 2006, 2011). Exogenous BMP15 in IVM may improve oocyte developmental competence by enhancing oocyte oxidative phosphorylation, mediated via the cumulus cells (Sutton-McDowall *et al*. 2012). In mice, BMP15 and FGF8 together, but not separately, increased abundance of mRNA encoding glycolytic enzymes PFKP and lactate dehydrogenase A (LDHA), as well as glycolytic activity in cumulus cells (Sugiura *et al*. 2007). However, FGF8, either alone or in combination with BMP15 or growth differentiation factor 9, did not alter competence of mouse cumulus cells to undergo expansion (Sugiura *et al*. 2010). Another FGF of interest is FGF10, which was localized to the bovine oocyte, and its receptors (FGFR1B and FGFR2B) to cumulus cells (Buratini *et al*. 2007, Cho *et al*. 2008). Interestingly, supplementation of IVM medium with FGF10 enhanced cumulus expansion and embryo development in cattle (Zhang *et al*. 2010), although the mechanism of action is mostly unknown.

The objective of this study was to examine the roles of FGF10 and BMP15 in the preovulatory/expansion cascade in cattle. We first tested the hypothesis that BMP15, alone or in combination with FGF10, enhances cumulus expansion in cattle. Then, to gain insight into the mechanisms of action of BMP15 and FGF10 in cumulus expansion, we tested the hypothesis that these growth factors regulate glucose metabolism and the expression of genes in the ovulatory cascade necessary for expansion (*ADAM10*, *ADAM17*, *AREG*, *EREG*, *BTC*, *EGFR*, *PTGS2*, *PTX3*, *TNFAIP6*, and *HAS2*).

Materials and methods

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

In vitro *maturation*

Ovaries of adult cows (predominantly Nellore, *Bos indicus*; around 90 ovaries per collection) were obtained from an abattoir local to the Sao Paulo State University campus in Botucatu and transported to the laboratory in saline solution (0.9% NaCl) containing antibiotics (penicillin G, 100 IU/ml, and streptomycin, 100 μg/ml) at 35–37 °C. COCs were aspirated from 3–8 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 homogenous cytoplasm and at least five compact layers of cumulus cells were used. 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 (see below). Drops were covered with mineral oil and incubated at 38.5 °C in 5% CO_2 in humidified air.

The effects of graded doses of recombinant human BMP15 (R&D Systems, Minneapolis, MN, USA; 0, 10, 50, and 100 ng/ml; four replicates/dose) or recombinant human FGF10 (R&D Systems; 0, 0.5, 10, and 50 ng/ml; four replicates/dose) on cumulus expansion were tested after 22 h of culture. To test potential synergism between BMP15 and FGF10 in the regulation of cumulus expansion, an additional experiment was performed with minimally effective doses of BMP15 alone (100 ng/ml), FGF10 alone (10 ng/ml), and BMP15 (100 ng/ml) plus FGF10 (10 ng/ml; all treatments were done in four replicates). Cumulus expansion was visually assessed according to a subjective scoring system. Grades 1–3 were attributed to increasing degrees of expansion (1 – poor expansion, characterized by a few morphological changes compared with before maturation; 2 – partial expansion, characterized by fair expansion but notable clusters lacking expansion; 3 – complete or nearly complete expansion; Zhang *et al*. (2010)).

To test the effects of BMP15 and FGF10 and of their combination on gene expression, maturation medium was supplemented with growth factors as described above (four replicates). All treatments were tested after 4,

12, and 22 h of culture; these time points were chosen based on a previous time-course study (Caixeta *et al.* 2012). Different times of culture were assessed in different experiments and thus treatments were compared within each time point but not between time points.

Gene expression analysis

After culture, cumulus cells and oocytes were mechanically separated from all 20 COCs cultured per group by repeated pipetting in PBS. Cumulus cells were transferred to 1.5 ml tubes, washed twice by centrifugation for 5 min at 700 ***g***, and 350 µl of the RNA extraction lysis buffer was added to the cell pellets. Samples were stored at −80 °C until RNA extraction.

Total RNA was extracted from cumulus cells using the RNeasy kit (Qiagen) as recommended by the manufacturer. After purification, RNA samples were eluted in 30 µl of RNAse-free water. Total RNA concentrations were measured by spectrophotometer using a NanoDrop ND 1000 (Thermo Scientific, Wilmington, DE, USA). Total RNA (100 ng/reaction) was incubated with DNAse I to prevent interference of DNA contamination with the PCR analysis (1 U/µg; Invitrogen) and then reverse transcribed using Oligo-dT primers and Omniscript reverse transcriptase (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). The primer sequences, amplicon sizes, and annealing temperatures for each target gene are given in Table 1. Reactions were optimized to provide maximum amplification efficiency for each gene. The specificity of the PCR products was assessed by melting curve analyses and amplicon size was determined by electrophoresis in 2% agarose gels.

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

Genes	Primer sequence	Fragment size (bp)	Annealing temperature (°C)	Reference
CYC-A	F: 5′-GCCATGGAGCGCTTTGG-3′ R: 5′-CCACAGTCAGCAATGGTGATCT-3′	65	60	Machado *et al.* (2009)
GAPDH	F: 5′-GGCGTGAACCACGAGAAGTATAA-3′ R: 5′-CCCTCCACGATGCCAAAGT-3′	119	62	Machado *et al.* (2009)
H2AFZ	F: 5′-GAGGAGCTGAACAAGCTGTTG-3′ R: 5′-TTGTGGTGGCTCTCAGTCTTC-3′	74	60	Machado *et al.* (2009)
AREG	F: 5′-CTTTCGTCTCTGCCATGACCTT-3′ R: 5′-CGTTCTTCAGCGACACCTTCA-3′	100	60	Portela *et al.* (2011)
EREG	F: 5′-ACTGCACAGCATTAGTTCAAACTGA-3′ R: 5′-TGTCCATGCAAACAGTAGCCATT-3′	100	60	Portela *et al.* (2011)
BTC	F: 5′-GCCCCAAGCAGTACAAGCAT-3′ R: 5′-GCCCCAGCATAGCCTTCATC-3′	100	59	AF140597
EGFR	F 5′-AAAGTTTGCCAAGGGACAAG-3′ R: 5′-AAAGCACATTTCCTCGGATG-3′	253	53	Caixeta *et al.* (2009)
PTGS2	F: 5′-AAGCCTAGCACTTTCGGTGGAGAA-3′ R: 5′-TCCAGAGTGGGAAGAGCTTGCATT-3′	168	60	NM_174445.2
HAS2	F: 5′-ACACAGACAGGCTGAGGACAACTT-3′ R: 5′-AAGCAGCTGTGATTCCAAGGAGGA-3′	133	60	NM_174079.2
PTX3	F: 5′-CCTCAGCTATCGGTCCATAA-3′ R: 5′-ATTGAAGCCTGTGAGGTCTGC-3′	294	54	Caixeta *et al.* (2009)
TNFAIP6	F: 5′-GCAAAGGAGTGTGGTGGTGTGTTT-3′ R: 5′-ACTGAGGTGAATGCGCTGACCATA-3′	135	60	BC151789.1
ADAM10	F: 5′-ACCCCCCAAAGTCTCTCACA-3′ R: 5′-AATCATGCGGAGATCCAAAGTT-3′	210	60	Li *et al.* (2009*b*)
ADAM17	F: 5′-TGGGATGTGAAGATGTTGCTAGA-3′ R: 5′-ATCCAAGTGTTCCCATATCAAAATC-3′	105	60	Portela *et al.* (2011)
GFPT1	F: 5′-GTTGAAACATGGCCCTCTGGCTTT-3′ R: 5′-TGCCTAGCAACCACTTGCTGTAGA-3′	117	60	NM_001109961.1
GFPT2	F: 5′-GCCTTGTACCCAAGTGCTTTGCTT-3′ R: 5′-TGCACGGTATTGGAAGAGTCTGCT-3′	123	60	NM_001076883.1
GLUT1	F: 5′-CAGGAGATGAAGGAGGAGAGC-3′ R: 5′-CACAAATAGCGACACGACAGT-3′	258	59	BC119940.1
GLUT4	F: 5′-ATTGTGGCCATCTTTGGCTTCGTG-3′ R: 5′-AACCCATGCCGATGATGAAGTTGC-3′	160	60	AY458600.1
PFKP	F: 5′-TCAGAGAACCGTGCCTGGAAGAAA-3′ R: 5′-TGACCACAAGCTCCTTGATCTGCT-3′	112	59	NM_001193220.1
LDHA	F: 5′-TCTGGATTCAGCTCGCTTCCGTTA-3′ R: 5′-TTCTTCAGGGAGACACCAGCAACA-3′	147	60	BC146210.1

F, forward primer; R, reverse primer.

To select the most stable housekeeping gene, cyclophilin-A (*CYCA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and histone H2AFZ (*H2AFZ*) amplification profiles were compared using the geNorm applet for Microsoft Excel (medgen.ugent.be/genorm; Vandesompele *et al*. (2002)); the most stable housekeeping gene was *CYCA*.

The relative expression values for each gene were calculated using the $\Delta\Delta Ct$ method with efficiency correction and using one control sample as calibrator (Pfaffl 2001). Mean efficiency values for each gene were calculated from the amplification profile of individual samples with LinRegPCR software (Ramakers *et al*. 2003). Each sample was run in duplicate.

Glucose metabolism

Glucose and lactate concentrations were determined in spent media (including media that were not used for IVM), following 22-h culture with and without BMP15 (100 ng/ml) and FGF10 (10 ng/ml). At the completion of culture, spent media were collected, snap frozen with liquid nitrogen, and stored at −80 °C. Glucose and lactate levels were measured using a Hitachi 912 chemical analyzer (F. Hoffmann-La Roche Ltd.) from three experimental replicates. To determine glucose uptake, the measured glucose concentration was subtracted from the concentration of glucose in media blanks (drops of media, cultured without cells). The base medium (TCM199) did not contain lactate. Glucose uptake and lactate production were expressed as pmol/COC per h (Sutton-McDowall *et al*. 2012).

Statistical analysis

Cumulus expansion data were transformed to radians using arcsine transformation, and gene expression data were transformed to base 10 logarithms when not normally distributed. The effects of treatments with BMP15 and/or FGF10 on cumulus cell expansion, gene expression, and glucose and lactate levels were tested by ANOVA, and means were compared with the Tukey–Kramer HSD test. The analyses were performed with JMP software (SAS Institute, Cary, NC, USA) and the results are presented as means ± S.E.M. Differences were considered significant when $P<0.05$.

Results

Effects of BMP15 and FGF10 on cumulus expansion

COCs were exposed to graded doses of BMP15 and FGF10 and cumulus expansion was recorded. The percentage of grade 3 COCs (complete or nearly complete cumulus expansion) was increased by BMP15 at 100 ng/ml (Fig. 1A) and by FGF10 at 10 ng/ml (Fig. 1B). A combination of BMP15 with FGF10 did not further enhance cumulus expansion compared with either BMP15 or FGF10 alone (Fig. 1C).

Effects of BMP15 and FGF10 on cumulus expansion-related gene expression

To gain insight into the mechanisms by which BMP15 and FGF10 enhance cumulus expansion, we examined

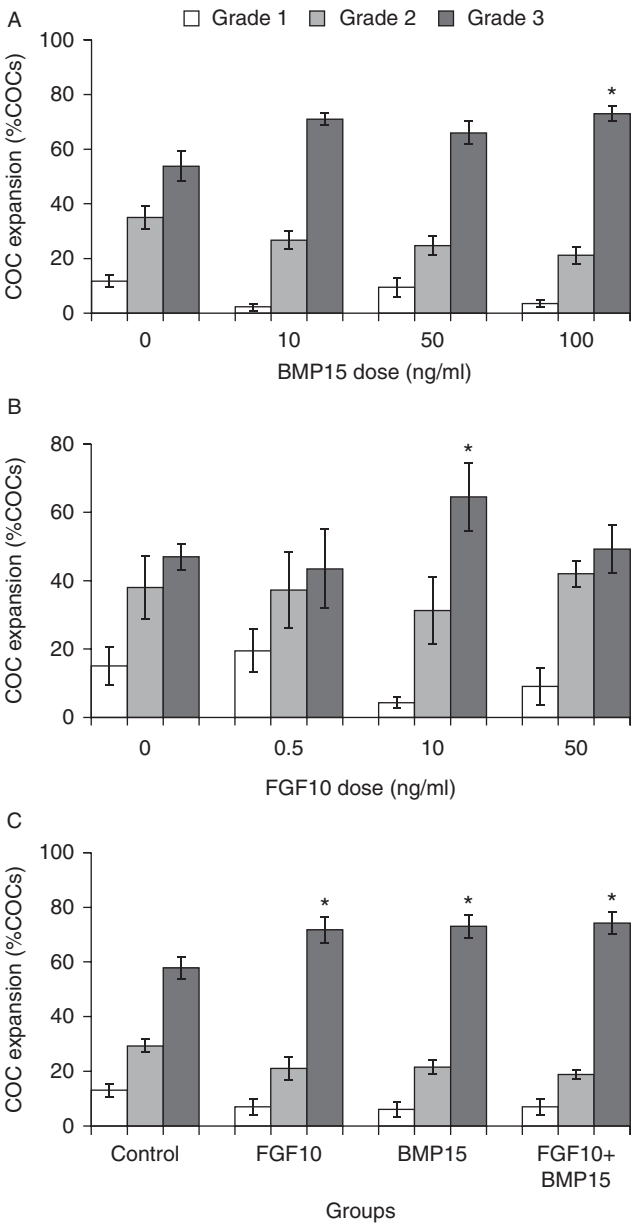

Figure 1 Effects of BMP15 (A), FGF10 (B), and the combination of both (C) on cumulus expansion. COCs were cultured with increasing doses of BMP15 and FGF10, and with a combination of BMP15 (100 ng/ml) and FGF10 (10 ng/ml). After culture for 22 h, the degree of expansion was classified as grade 1 (poor expansion, few morphological changes), 2 (partial expansion), or 3 (complete expansion). Asterisks denote means that were significantly different from control values ($P<0.05$). Data were derived from four independent replicates for each treatment.

whether these factors regulate expression of key genes involved in the ovulatory cascade. BMP15 caused a dose-dependent increase in *ADAM10*, *ADAM17*, *AREG*, and *EREG* mRNA levels at 12 h of culture and of *ADAM17*, *AREG*, *EREG*, *PTGS2*, *PTX3*, and *TNFAIP6* mRNA levels at 22 h of culture (Fig. 2). BMP15 had no effect on *BTC* and *EGFR* mRNA abundance (data not shown).

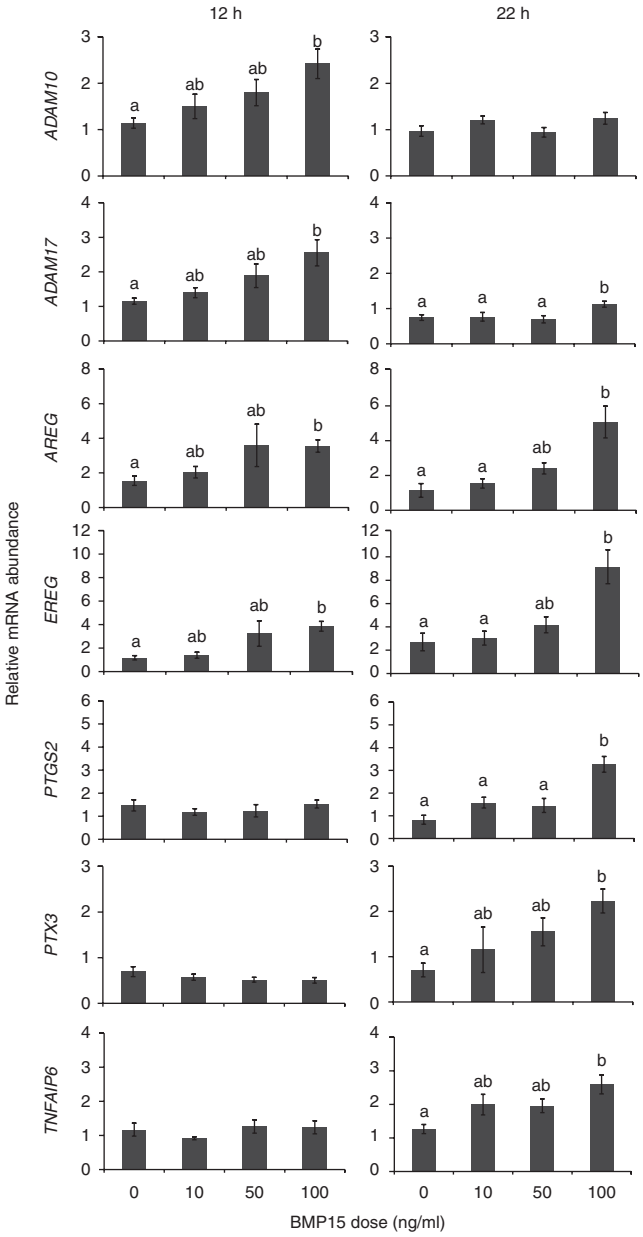

Figure 2 Effects of graded doses of BMP15 on *ADAM10/17*, *AREG*, *EREG*, *PTGS2*, *PTX3*, and *TNFAIP6* mRNA abundance in cumulus cells. COCs were cultured with increasing doses of BMP15 for 12 and 22 h. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔ*C*t method with efficiency correction. Bars with different letters are significantly different ($P<0.05$). Data were derived from four independent replicates for each time point.

FGF10 did not affect abundance of mRNA encoding *ADAM10*, *ADAM17*, *EGF*-like factors, or *EGFR* (data not shown) but stimulated *PTGS2* mRNA levels by 4 h of culture, which was also observed at 12 and 22 of culture (Fig. 3). FGF10 increased *PTX3* mRNA levels at 12 and 22 h and *TNFAIP6* mRNA levels only at 22 h (Fig. 3). BMP15 and FGF10 in combination did not alter abundance of mRNA of any of these genes compared with each growth factor alone at 22 h of culture (data not shown).

Effects of FGF10 and BMP15 on glucose metabolism

FGF10 and BMP15 alone each stimulated COC glucose uptake and the combination of the two did not further increase uptake (Fig. 4). In contrast, neither growth factor alone or in combination increased COC lactate production (Fig. 4). BMP15 increased abundance of mRNA encoding *GLUT1* and *GFPT1* at 22 h of culture, increased *GFPT2* mRNA levels at 12 and 22 h, and increased *HAS2* mRNA levels at 12 h (Fig. 5). Addition of FGF10 increased *GLUT1*, *GLUT4*, and *GFPT1* at 12 h but had no effect on *HAS2* or *GFPT2* mRNA abundance (Fig. 6).

The potential interaction of FGF10 and BMP15 on glucose metabolic enzyme genes was tested at 22 h of culture. BMP15 alone increased *GFPT2* mRNA abundance and there was no further change in the presence of both BMP15 and FGF10. Neither BMP15 nor FGF10, alone or together, altered *GFPT1*, *PFKP*, or *LDHA* mRNA levels (data not shown).

Discussion

Expansion of the cumulus is enhanced by OSFs, including BMP15 and FGF10 (Yoshino *et al.* 2006, Zhang *et al.* 2010), but the mechanisms of action of these growth factors have not been explored. There is also evidence of synergy between BMPs and FGFs, as BMP15 and FGF8 synergize to increase glucose metabolism in mouse cumulus cells (Sugiura *et al.* 2007). In the present study, we provide evidence that BMP15 and FGF10 enhance cumulus expansion *in vitro* through modulation of *PTGS2* mRNA abundance and/or upstream genes and that both factors enhance glucose metabolism through the hexosamine pathway. However, we found no evidence for synergy between BMP15 and FGF10 in the regulation of expansion, gene expression, or glucose metabolism of cumulus cells from *in vitro*-matured bovine COCs.

BMP15 enhanced cumulus expansion *in vitro* in cattle in the present study, in agreement with a previous study in mice (Yoshino *et al.* 2006); this is consistent with data showing that BMP15 improves oocyte competence and early embryo development in cattle (Hussein *et al.* 2006, 2011), as increased cumulus expansion has been previously associated with improved blastocyst rates (Furnus *et al.* 1998). The relatively low proportion of

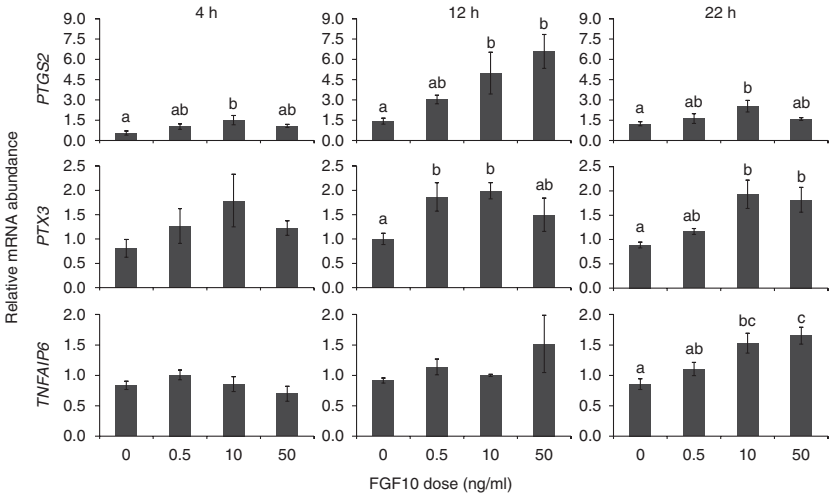

Figure 3 Effects of graded doses of FGF10 on *PTGS2*, *PTX3*, and *TNFAIP6* mRNA abundance in cumulus cells. COCs were cultured with increasing doses of FGF10 for 4, 12, and 22 h. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the $\Delta\Delta Ct$ method with efficiency correction. Bars with different letters are significantly different ($P<0.05$). Data were derived from four independent replicates for each time point.

COCs reaching full expansion in the control group in the present study is probably due to the absence of serum in the culture medium, as we aimed to assess the effects of OSFs under defined culture conditions. In association with this enhancement in cumulus expansion, BMP15 stimulated the expression of genes critical for this process. BMP15 increased abundance of mRNA encoding *AREG*, *EREG*, *PTGS2*, *PTX3*, and *TNFAIP6* as previously observed in the mouse (Yoshino *et al*. 2006, Li *et al*. 2009*a*), although BMP15 had no effect on *BTC* mRNA levels in cattle unlike the situation in mice (Yoshino *et al*. 2006); this is probably because of the marked species differences in the regulation of *BTC* expression (Caixeta *et al*. 2012). Most interesting however, is the stimulatory action of BMP15 on mRNA expression of *ADAM10* and *ADAM17* in the present study. To our knowledge, this has not been previously described in any species and suggests that BMP15 acts at a major upstream event that influences not only EGF-like growth factor shedding but also proteolytic processing of matrix components such as versican that is required for ovulation (Ben-Ami *et al*. 2006, Russell & Robker 2007).

In vitro cumulus expansion of bovine COCs was also enhanced by FGF10, confirming a previous report (Zhang *et al*. 2010). However, in contrast to BMP15, FGF10 did not alter levels of RNA encoding ADAM10, ADAM17, or EGF-like factors but did affect the expression of downstream genes in our study. *PTGS2* expression was the first stimulated by FGF10, which occurred at 4 h of culture. Next was *PTX3*, which was increased by FGF10 at 12 h, followed by *TNFAIP6* expression, which was upregulated at 22 h of culture. *TNFAIP6* was reported to be a target of PG action in mice and pigs, and either silencing of *PTGS2* or disruption of PG signaling severely impaired *TNFAIP6* expression (Ochsner *et al*. 2003, Takahashi *et al*. 2006, Yamashita *et al*. 2011). Therefore, the effects of FGF10 on *TNFAIP6* expression are probably mediated through increased PTGS2.

In our study, both BMP15 and FGF10 increased glucose uptake by COCs but did not alter lactate production or the expression of the glycolytic enzymes PFKP and LDHA, suggesting that, unlike the combination of BMP15 plus FGF8 in the mouse (Sugiura *et al*. 2007), BMP15 plus FGF10 do not increase glycolysis in the

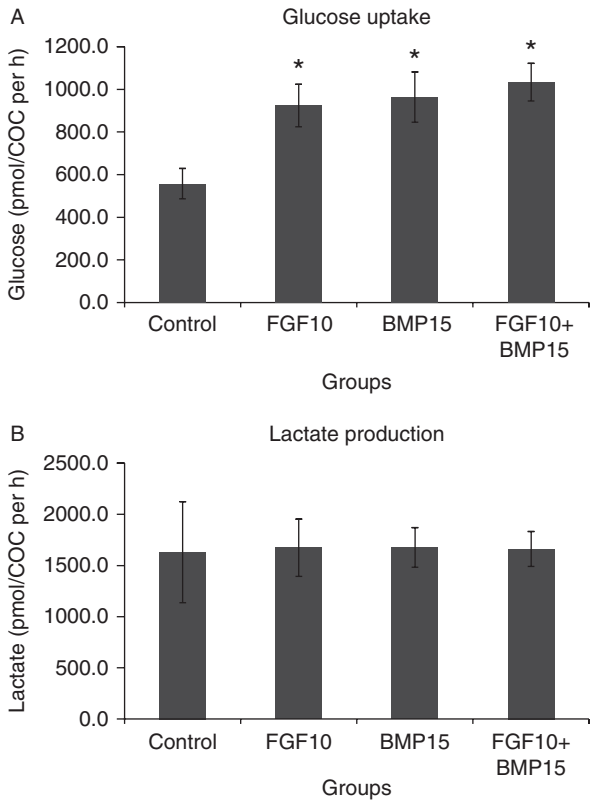

Figure 4 Effects of BMP15 and FGF10 on glucose metabolism in cumulus cells. (A and B) COCs were cultured for 22 h with BMP15 (100 ng/ml), FGF10 (10 ng/ml), or both together, and glucose uptake and lactate production were measured at the end of culture. Asterisks denote means that were significantly different from control values ($P<0.05$). Data were derived from three independent replicates for each treatment.

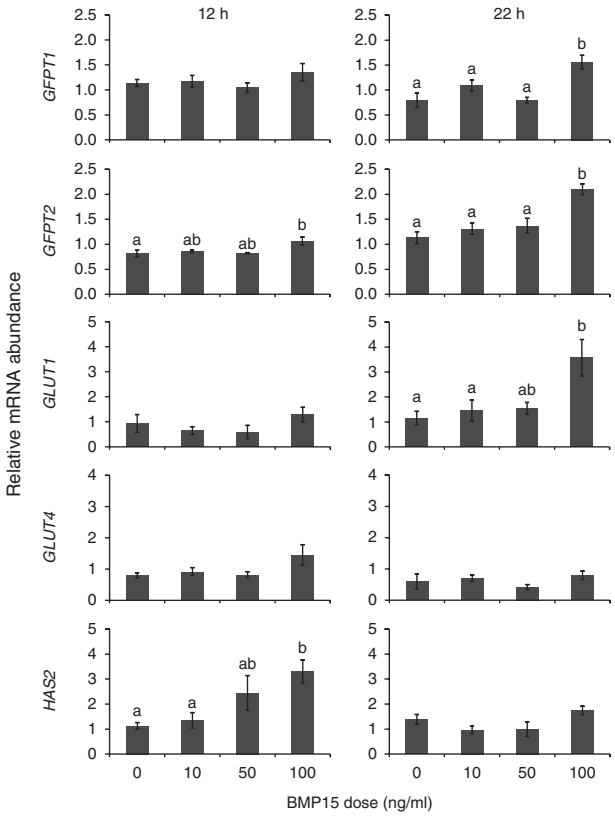

Figure 5 Effects of graded doses of BMP15 on *GFPT1*, *GFPT2*, *GLUT1*, *GLUT4*, and *HAS2* mRNA abundance in cumulus cells. COCs were cultured with increasing doses of BMP15 for 12 and 22 h. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to 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 for each time point.

bovine COCs. The increase in glucose uptake observed here differs from our recent results suggesting that BMP15 (alone and in the presence of FSH) did not significantly alter uptake (Sutton-McDowall *et al.* 2012); however, culture conditions differed with respect to the FSH and BMP15 used. Our previous study used a pro-mature form of the BMP15 protein, consisting of the ~42 kDa BMP15 proregion complexed with the ~17 kDa mature region, whereas the current study uses the smaller mature region only of BMP15 (~17 kDa monomer). Also previously, we used 0.1 IU FSH whereas in the present study we used 0.002 IU, and FSH has been shown to increase glucose uptake (Sutton-McDowall *et al.* 2012). Therefore, it is likely that the substantially higher levels of FSH may mask the potentially beneficial effects of BMP15 on cumulus metabolism. Interestingly, in our study, BMP15 and FGF10 increased expression of mRNA encoding GFPT1 and GFPT2, key enzymes involved in directing glucose metabolism down the hexosamine pathway. In a previous study, the inhibition of GFPT activity attenuated cumulus expansion in bovine COCs (Gutnisky *et al.* 2007). Conversely, in our study, increased GFPT expression could probably result in greater amounts of precursor for hyaluronic acid synthesis, especially in the presence of increased HAS2 and therefore enhanced cumulus expansion. An increase in glucose uptake without increased lactate production has been observed in the latter stages of IVM in cattle, which is consistent with increased hyaluronic acid synthesis by cumulus cells at this time (Sutton-McDowall *et al.* 2010), although it is also possible that part of the increased metabolism of glucose may be directed toward oxidative phosphorylation (Sutton-McDowall *et al.* 2012).

In summary, this study presents novel findings from functional and gene expression studies that allow a better understanding of the actions of OSFs in the regulation of COC maturation in cattle, reinforcing previous evidence that BMP15 and FGF10 may be useful to improve IVM protocols (Hussein *et al.* 2006, 2011, Zhang *et al.* 2010). For the first time, we have demonstrated that BMP15 enhances cumulus expansion

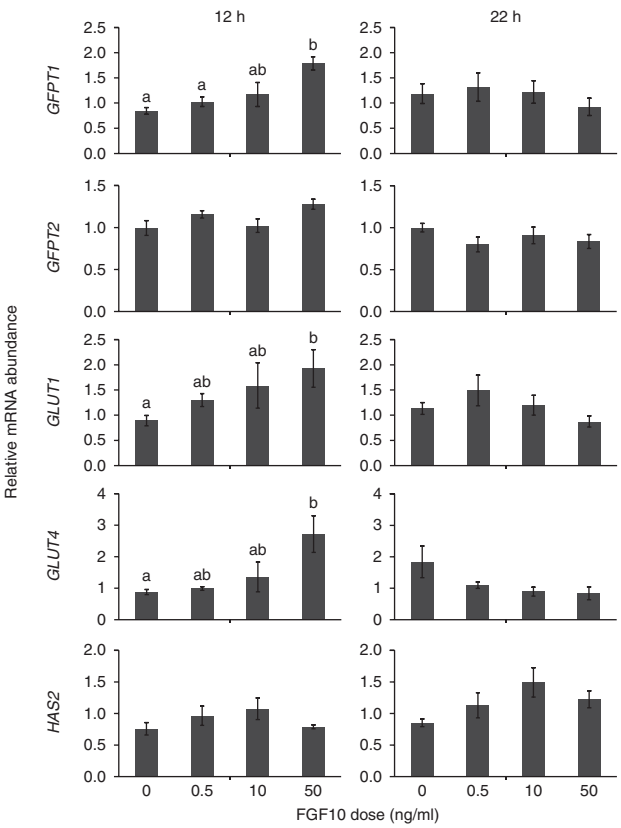

Figure 6 Effects of graded doses of FGF10 on *GFPT1*, *GFPT2*, *GLUT1*, *GLUT4*, and *HAS2* mRNA abundance in cumulus cells. COCs were cultured with increasing doses of BMP15 for 12 and 22 h. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to 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 for each time point.

in cattle and provided evidence that the mechanisms by which BMP15 and FGF10 enhance cumulus expansion involve an increase in glucose uptake by cumulus cells associated with upregulation of the expression of key genes involved in hyaluronic acid production. Both growth factors also increased abundance of mRNA encoding another enzyme critical for ovulation/fertilization, PTGS2, although they seem to act at different steps in the ovulatory cascade; whereas BMP15 appears to act upon *ADAM10*, *ADAM17*, *AREG*, and *EREG*, FGF10 appears to act more directly on *PTGS2* and downstream genes of the cumulus expansion cascade.

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), and National Health and Medical Research Council of Australia (Grant 1008137).

Acknowledgements

The authors thank Drs Rubia B da Silva, Anthony C S Castilho, and Felipe M Dalanezi for their technical assistance.

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Received 27 February 2013
First decision 10 April 2013
Revised manuscript received 29 April 2013
Accepted 2 May 2013