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EFFECT OF BONE MORPHOGENETIC PROTEIN-15 ON GONADOTROPIN-STIMULATED SYNTHESIS OF HYALURONAN AND PROGESTERONE IN PORCINE OVARIAN FOLLICLE

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Bone morphogenetic protein-15 (BMP-15), an oocyte-derived growth factor, has been shown to play integral roles in regulation of ovarian follicular function in mammals. Despite the recognition of the physiological importance of the BMP system in regulation of gonadotropin action in the ovary, molecular mechanisms of BMP-15 effect on oocyte and somatic follicular cell functions remain poorly understood. The objective of this study was to determine the effect of BMP-15 on the FSH/LH-stimulated synthesis of hyaluronan (HA) by oocyte cumulus complexes (OCC) and progesterone by OCC and granulosa cells (GC) in the presence or absence of serum using primary porcine cultures. In addition, the effect of BMP-15 on oocyte maturation- and steroidogenesis-related transcripts after 4, 8, 16, and 24 hours of cultivation was evaluated using real-time RT-PCR. We demonstrated that the FSH/LH-induced cumulus expansion was accompanied by a significant increase in *CD44, PTGS2, CYP11A1* (at 4 h) and *AREG, HAS2, TNFAIP6, STAR* (at 8 h) mRNAs. While FSH/LH-stimulated total HA synthesis by OCC was not affected by BMP-15 in serum-supplemented medium, its retention within the complex was significantly increased after the action of BMP-15 in comparison to FSH/LH alone (P < 0.001; 65% versus 35%, respectively). Moreover, we detected a significant increase in the expression of *AREG* and *TNFAIP6* (both at 16 h), and *CYP11A1* (at 24 h) in FSH/LH-stimulated OCC due to the action of BMP-15 compared to complexes cultured only with FSH/LH. In the presence of serum, BMP-15 markedly increased FSH/LH-stimulated progesterone secretion by OCC (about 69%) and induced a significant decrease in FSH/LH-induced progesterone release by GC (about 35%) compared to FSH/LH alone. The present results indicate that the addition of BMP-15 to the gonadotropin-stimulated OCC cultured in serum-supplemented medium might improve oocyte-cumulus maturation.

K e y w o r d s : *bone morphogenetic protein-15, cumulus extracellular matrix, granulosa cells, hyaluronic acid, progesterone, ovarian follicle*

INTRODUCTION

Under physiological conditions, ovulation is the process induced by the surge of luteinizing hormone (LH) that involves at least four essential events: 1) the resumption of oocyte meiotic maturation, 2) differentiation of the mural granulosa cells (GC) including modulation of steroidogenic activity, 3) cumulus expansion and 4) rupture of the follicle wall to release the fertilizable ovum (1). In addition to pituitary-secreted gonadotropins, transforming growth factor-β (TGF-β) and related polypeptides, including bone morphogenetic proteins (BMPs), are factors that regulate cellular processes such as proliferation and differentiation in rat GC (2) and cumulus expansion in mice (3). Two members of the TGF-β superfamily, BMP-15 and growth differentiation factor 9 (GDF9), are oocyte-derived growth factors (4) that bind to their type I and type II membrane receptors and trigger serine/threonine protein kinase activity. This kinase activity further phosphorylates specific Smad transcription factors: Smad2/3 in response to GDF9 and Smad 1/5/8 in response to BMP-15. Phosphorylated Smad2/3 or Smad1/5/8 then heterodimerize with a common Smad (Smad4), and translocate into the nucleus, where they modulate the transcription of target genes.

Both growth factors participate in the regulation of specific genes related to cumulus expansion and luteinization in mice (5) and pigs (6). In pigs, Paradis *et al.* (7) demonstrated the expression and localization of mRNA for members of the TGF-β superfamily and their cognate receptors (*BMP2, BMP6, BMP15, GDF9, BMPR1A, BMPR1B, BMPR2* and *TGFBR1*) during preovulatory follicular development. The authors revealed that with the exception of *BMP6* mRNA, which was absent in GC, all the examined genes were expressed in every cell type (oocytes, GC and theca cells (TC)). Although *BMP6, BMP15* and *GDF9* were most abundant in the oocyte, their expression remained relatively constant during follicular development. Finally, Paradis *et al.* (7) found an increase in *BMPR1B* mRNA and protein abundance during the periovulatory period in porcine GC and TC and suggested a role for BMP-15 in the ovulatory process. In mice, BMP-15 was detected in expanded cumulus extracellular matrix (ECM) (8) and the *Bmpr1b* null mice exhibited defects in

cumulus expansion (9). In addition, Yan *et al.* (10) showed that *Gdf9$^{+/-}$ Bmp15$^{-/-}$* eggs lack associated cumulus cells (CC), both within follicles and after ovulation, indicating that cumulus cell-oocyte communication is markedly disrupted in these mice.

It is well accepted that the formation of the expanded structural-stable cumulus ECM rich in hyaluronan (HA) is essential for female fertility (11, 12). Beside HA, other proteoglycans are involved in the formation of expanded cumulus ECM (11), which critically affects basic cellular processes (proliferation, differentiation) (13). A number of studies have shown that oocyte-derived factors including BMPs induced expression of several target genes related to ECM formation and stability (14, 15).

Previously, evidence about the role of *BMP15* and *BMPR1B* during pig preovulatory follicular development has been provided (7). Although it is known that BMP signalling molecules translocate into the nucleus and modulate the transcription of specific genes related to luteinization (16), there is little information about the role of BMP-15 in the regulation of OCC and GC differentiation during gonadotropin-induced meiotic maturation in pigs. Moreover, BMPs are considered important regulators of the organization of the HA-rich ECM (14). Therefore, in the present study, we examined the effect of BMP-15 on gonadotropin-stimulated cumulus expansion of porcine OCC, and HA synthesis and retention by OCC. Effect of BPM-15 on transcript levels of selected genes related to OCC maturation (*AREG, CD44, HAS2, PTGS2, TNFAIP6*), proliferation and differentiation (*CCND2, PCNA, CYP11A1, STAR)* was studied. Finally, we investigated the effect of BMP-15 on gonadotropin-stimulated progesterone production by OCC and GC using primary cultures from porcine follicles.

MATERIALS AND METHODS

Isolation and culture of oocyte-cumulus complexes and granulosa cells

Porcine ovaries were collected at a local abattoir and immediately transported to the laboratory. Oocyte-cumulus complexes (OCC) were aspirated from medium-sized antral follicles about 3 – 5 mm in diameter. The culture medium was M199 (Gibco, Invitrogen) supplemented with 20 mM NaHCO$_3$, 6.25 mM HEPES, 0.91 mM sodium pyruvate, 1.62 mM calcium lactate and antibiotics (all from Sigma, Prague, Czech Republic). The incubation of OCC was carried out in 96-well dishes at 38.5°C in an atmosphere of 5% CO$_2$. Recombinant human bone morphogenetic protein-15 (BMP-15) was obtained from R&D Systems (Europe). The stock solution was prepared by the dilution of 5 µg of BMP-15 in 500 µl of sterile 4 mM hydrochloric acid (HCL; Sigma, Prague, Czech Republic) and then diluted in culture medium to the final tested concentrations. Groups of 10 OCC were incubated in 100 µl media/well with or without the tested chemicals (BMP-15; 100 ng/ml). For progesterone assay and PCR experiments, groups of 20 OCC were cultured in 4-well dishes (Nunclon, Roskilde, Denmark). The OCC were incubated for the indicated time intervals in the culture medium supplemented with rhFSH (100 ng/ml; Gonal, recombinant human follicle stimulating hormone, Merck Serono, Modugno, Italy), rhLH (100 ng/ml; Luveris, recombinant human luteinizing hormone; Merck Serono, Modugno, Italy), and 5% fetal bovine serum (FBS; Sigma-Aldrich, Schnelldorf, Germany) or PVP (polyvinyl pyrolidone; 3 mg/ml; Sigma, Prague, Czech Republic).

Table 1. Primers used for real time RT-PCR.

Gene transcript	Sequence 5´-3´	Amplicon length (bp)	T$_{an}$ (ºC)	Gene Accession Number
AREG	F: CTA TTG CTG TTG TTA TTA C R: GTT CTG TCT TCT TAT GAT	221	54	NM_214376
CCND2	F: CAA GTG CGT GCA GAA GGA TA R: TGG CGA ACT TGA AGT CAG TG	456	58	NM_214088
CD44	F: GAG GCG GCC CTG AAC ATA R: AAG GTA TTA GGC AGG TCT GTG AC	217	58	XM_013994409
CYP11A1	F: ATA CCT CGT GAA TGA CTT R: CCT GGA TTT GAG AAG AAG	111	54	NM_214427
HAS2	F: GAA GTC ATG GGC AGG GAC AAT TC R: TGG CAG GCC CTT TCT ATG TTA	408	54	NM_214053
PCNA	F: TAA TGC AGA CAC CTT GGC ACT R: GCA AAT TCA CCA GAA GGC ATC	154	58	NM_001291925.1
PTGS2	F: TCG ACC AGA GCA GAG AGA TGA GAT R: ACC ATA GAG CGC TTC TAA CTC TGC	132	55	NM_214321
STAR	F: CTG GAA GTC CCT CAA AGA R: CAG GTG ATT GGC AAA GTC	122	58	NM_213755
TNFAIP6	F: CAG AAG ACA TCA TTA GTA R: CAG TAG AAG TAG TAG TTG	150	54	NM_001159607
B2M	F: AAA CGG AAA GCC AAA TTA CCT GA R: ATC TCT GTG ATG CCG GTT AGT G	267	56	NM_213978

T$_{an}$, annealing temperature; *AREG*, Sus scrofa amphiregulin; *CCND2*, Sus scrofa cyclin D2; Predicted: : *CD44*, Sus scrofa CD44 molecule (Indian blood group) transcript variant X1; *CYP11A1*, Sus scrofa cytochrome P450, family 11, subfamily A, polypeptide 1; *SHAS2*, Sus scrofa hyaluronan synthase 2; *PCNA*, Sus scrofa proliferating cell nuclear antigen; *PTGS2*, Sus scrofa prostaglandin-endoperoxide synthase 2; *STAR*, Sus scrofa steroidogenic acute regulatory protein; *TNFAIP6*, Sus scrofa tumor necrosis factor, alpha-induced protein 6; *B2M*, Sus scrofa beta-2-microglobulin.

Granulosa cells (GC) were aspirated from medium-sized porcine ovarian follicles (3 – 5 mm in diameter), isolated by centrifugation for 10 min at 200 × g, washed and dispersed in M199 medium with Earl's salt and Hepes buffer supplemented with L-glutamine (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml) (all from Sigma-Aldrich, Munich, Germany). The incubation of GC monolayer was carried out in 24-well plates (TPP AG, Switzerland) at 37°C in an atmosphere of 5% CO_2. The density and cell viability were determined in a haemocytometer by trypan blue exclusion. The cell viability ranged from 80% to 90%. Cells were incubated at a density of $1.0 – 1.2 \times 10^6$ viable cells/0.5 ml of culture medium supplemented with 5% FBS in the presence or absence of gonadotropins (rhFSH and rhLH; both 100 ng/ml; Merck Serono, Modugno, Italy) and BMP-15 (0.1, 1 and 10 ng/ml) for 72 hours.

Progesterone assay

At the end of the incubation period, the cultured media were collected for progesterone determination with a commercial radioimmunoassay kit (Institute of Isotopes, Budapest, Hungary). The cross-reactivity of the progesterone antibody was less than 13% with other progestins, and less than 0.01% with the androgens and estrogens tested. The intra- and inter-assay coefficients of variation were below or equal to 10.2% and 11.8%, respectively.

Hyaluronic acid synthesis

The synthesis of hyaluronic acid was measured as described previously (17). Briefly, groups of 10 porcine OCC were cultured in the FSH/LH- and/or BMP-15-supplemented medium with or without serum in the presence of 2.5 μCi of D-[6-3H] glucosamine hydrochloride (MP Biomedicals, Santa Ana CA) for 24 hours. The cultures were terminated by adding 10 μl of a solution containing 50 mg/ml pronase and 10% Triton X-100 in 0.2 M Tris buffer, pH 7.8 (all from Sigma, Prague, Czech Republic). The samples were incubated for 1 h at 38.5°C and then transferred to Whatman 3MM filter paper circles. The circles were air-dried and then washed with three changes of solution containing 0.5% cetylpyridinium chloride and 10 mM nonradioactive glucosamine hydrochloride (Sigma, Prague, Czech Republic) for 45 min each. The circles were dried once again and radioactivity was measured using a liquid scintillation counter. The synthesis of HA was measured either in medium plus OCC (total HA) or within the complexes alone (retained HA).

RNA isolation and quantitative real-time PCR analysis

Total RNA from 20 OCC was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA was eluted in 50 μl of RNase-free H_2O and stored at –80°C.

The quantitative reverse transcription-polymerase chain reaction was carried out using a One-Step RT-PCR kit (Qiagen) using specific oligonucleotide primers (*Table 1*).

The total RNA of the samples was reverse-transcribed and subsequently amplified in a 25 μl reaction mixture containing 5 μl of reaction buffer, dNTP (each 400 μM), SybrGreenI (0.5 μl of 1:1000 stock solution, Molecular Probes, Eugene, Oregon), primers (each 400 μM), RNasine inhibitor (5 IU; Promega,
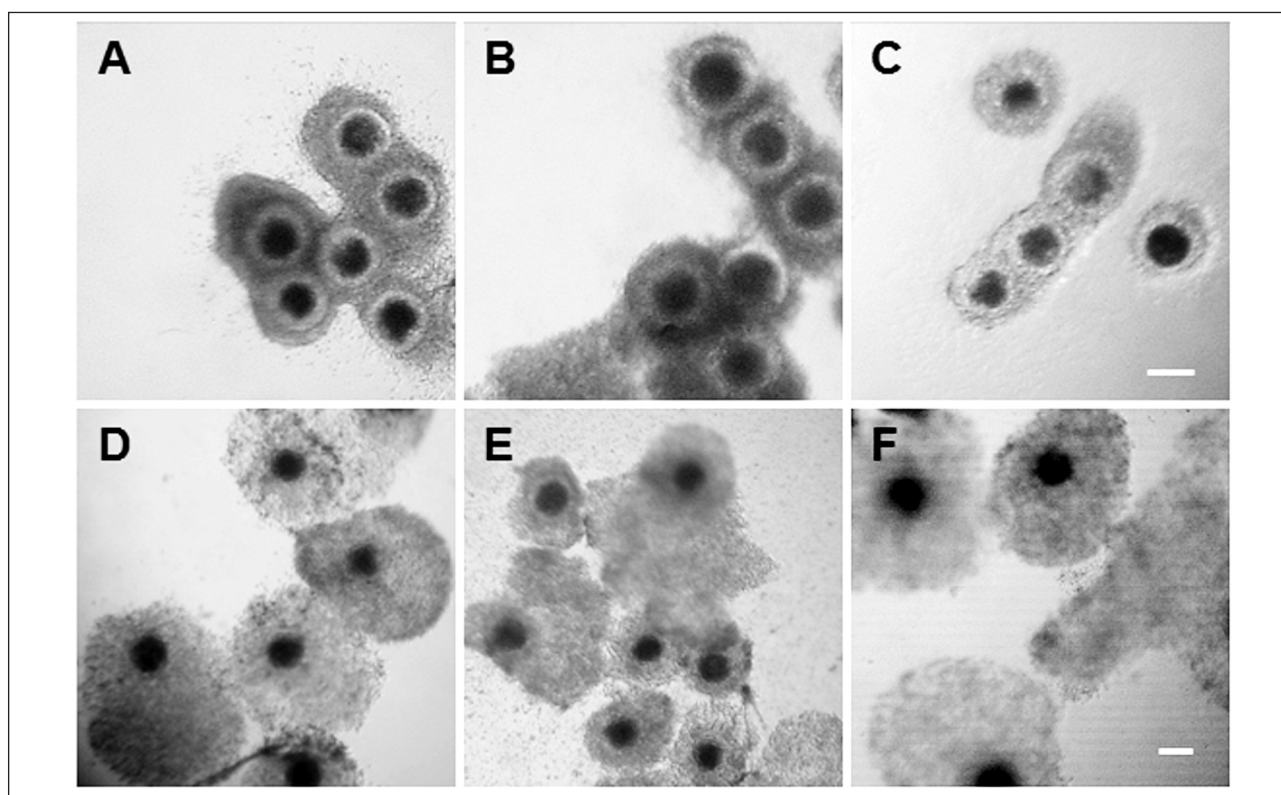

Fig. 1. Effect of BMP-15 (100 ng/ml) on expansion of porcine oocyte-cumulus complexes (OCC) after 24 hours of culture. (*A*) Unstimulated OCC in serum-supplemented medium; (*B*) unstimulated OCC in PVP-supplemented medium with BMP-15; (*C*) unstimulated OCC in serum-supplemented medium with BMP-15; (*D*) FSH/LH-stimulated OCC in serum-supplemented medium; (*E*) FSH/LH-stimulated OCC in PVP-supplemented medium with BMP-15; (*F*) FSH/LH-stimulated OCC in serum-supplemented medium with BMP-15. Scale bars represent 100 μm.

Madison, WI, USA), Qiagen One-Step RT-PCR enzyme mix (1 μl) and total RNA (2 μl). The amplification was performed in a RotorGene 2000 cycler (Cobett Research, Sydney, Australia). The reaction conditions were as follows: cDNA synthesis at 50°C for 30 min, initial activation at 95°C for 15 min, cycling: denaturation (95°C for 15 s), annealing (at a specific temperature for each set of primers for 15 s), extension (72°C for 20 s) and final extension (72°C for 5 min). Fluorescence data were acquired during an addition step at approximately 3°C below the melting temperature of the product. After the cycling, melting curves were generated to verify the amplification of one specific target in each tube. In addition, the specificity of RT-PCR products was assessed by gel electrophoresis on 1.5% agarose gel with ethidium bromide staining. The relative concentration of templates in different samples was determined using comparative analysis software (Corbett Research). The ratio of the target gene concentration to reference gene (*B2M*, beta-2-microglobulin) mRNA concentration was calculated in each sample.

Statistical analysis

Three independent sets of total RNA samples were analyzed. RT-PCR reactions were performed in duplicate. The quantifications of qRT-PCR results between OCC cultivated in FSH/LH alone or supplemented with BMP-15 at the set time intervals were compared using Student's t-test. The differences
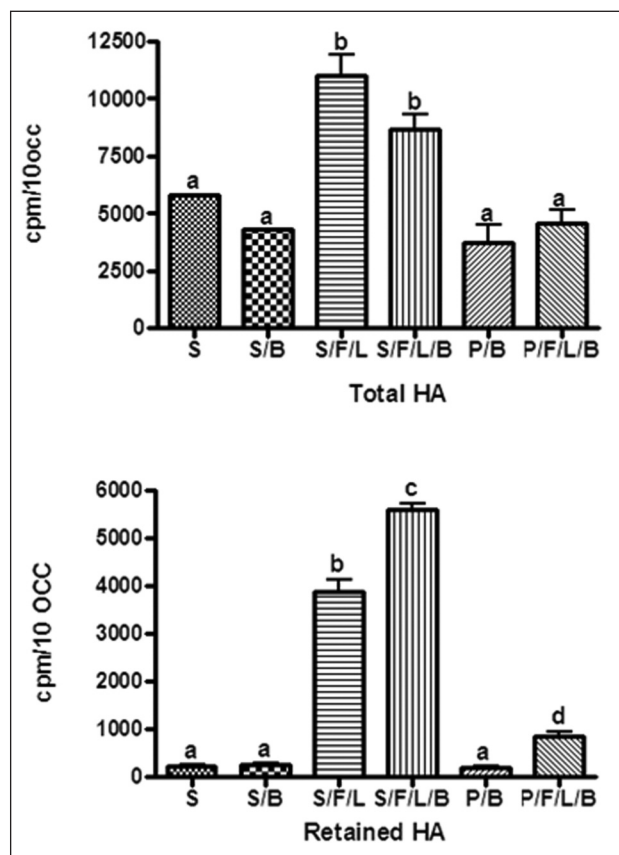
in the relative concentration of templates during cultivation in the presence or absence of BMP-15 were analyzed by ANOVA followed by Tukey's post-test using the SigmaStat software package (Jandel Scientific, CA).

RESULTS

Effect of bone morphogenetic protein-15 on cumulus expansion

The effects of BMP-15 (100 ng/ml) on the cumulus expansion of porcine OCC cultured for 24 h in serum- or PVP-supplemented medium in the absence/presence of gonadotropins (FSH, LH) were examined. According to a subjective evaluation by light stereomicroscope, we did not observe any differences among the groups of OCC cultured in the presence of serum or PVP without the addition of gonadotropins, even though the medium was supplemented with BMP-15 (*Fig. 1A* - FBS; *Fig. 1B* - PVP + BMP15; *Fig. 1C* - FBS + BMP15). The complexes were unable to

Fig. 2. FSH/LH-stimulated (F/L) total production (*A*) and retention (*B*) of hyaluronan (HA) in porcine oocyte-cumulus complexes (OCC) cultured for 24 h in the presence of BMP-15 (B; 100 ng/ml) in serum (S)- or PVP (P)-supplemented medium. Data represent the mean ± S.E.M. from three replicates for each treatment, prepared in duplicate. The columns with various letters are significantly different (P < 0.05).
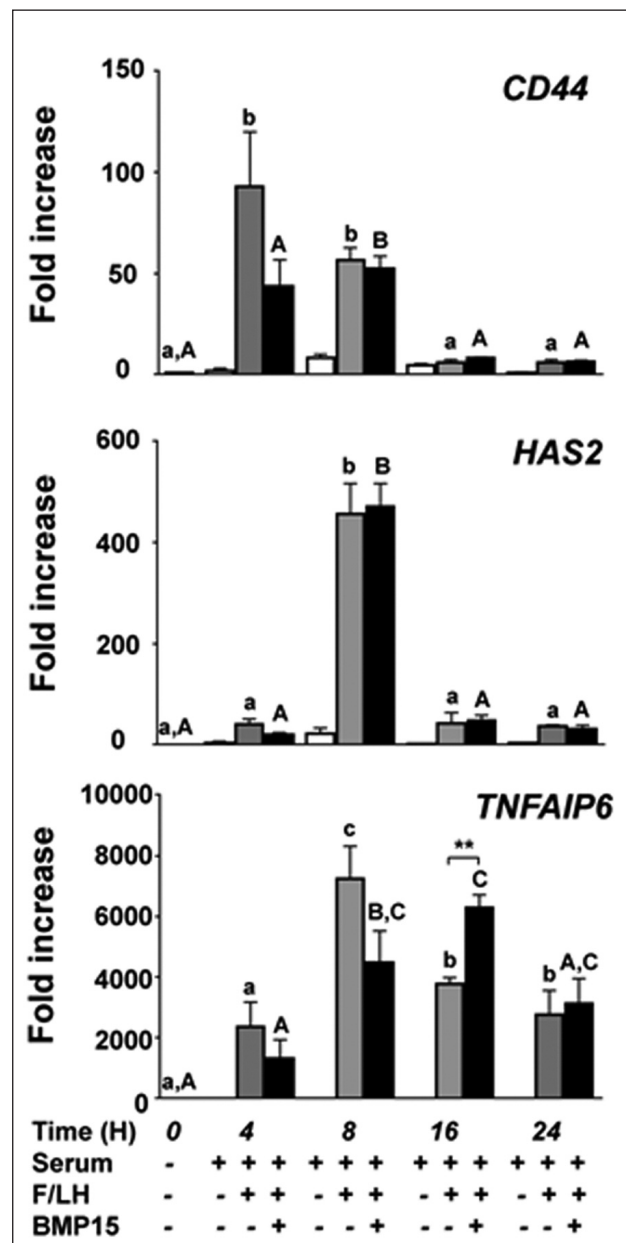

Fig. 3A

expand, their cumuli were not sticky and after taking them into/out of a micropipette, the oocytes quickly became denuded. Porcine OCC stimulated with gonadotropins in serum-supplemented medium underwent complete expansion in a manner independent of the addition of BMP-15 (*Fig. 1D* - F/L + FBS; *Fig. 1F* - F/L + FBS + BMP15). The complexes had all cumulus layers expanded, their cumuli were sticky with a tendency to make elastic structure-chains, and it was not easy to remove cumulus cells from the oocytes without Streptomyces hyaluronidase enzymatic digestion. In contrast, when the FSH/LH-stimulated complexes were cultured in PVP-supplemented medium in the presence of BMP-15 (*Fig. 1E* - F/L + PVP + BMP15), the OCC lost their elastic structure and

many cells appeared on the bottom of Petri dishes, showing clearly that most of the HA synthesized by cumuli in the absence of serum, was released into the medium. If these complexes were taken into/out of a micropipette, their oocytes were denuded quickly without any enzymatic digestion.

Effect of bone morphogenetic protein-15 on hyaluronan synthesis

To clarify the molecular mechanisms of the BMP15 regulation of FSH/LH signalling during cumulus expansion in OCC and its consequences on the organization of HA-rich cumulus ECM, we measured total HA synthesis by porcine OCC
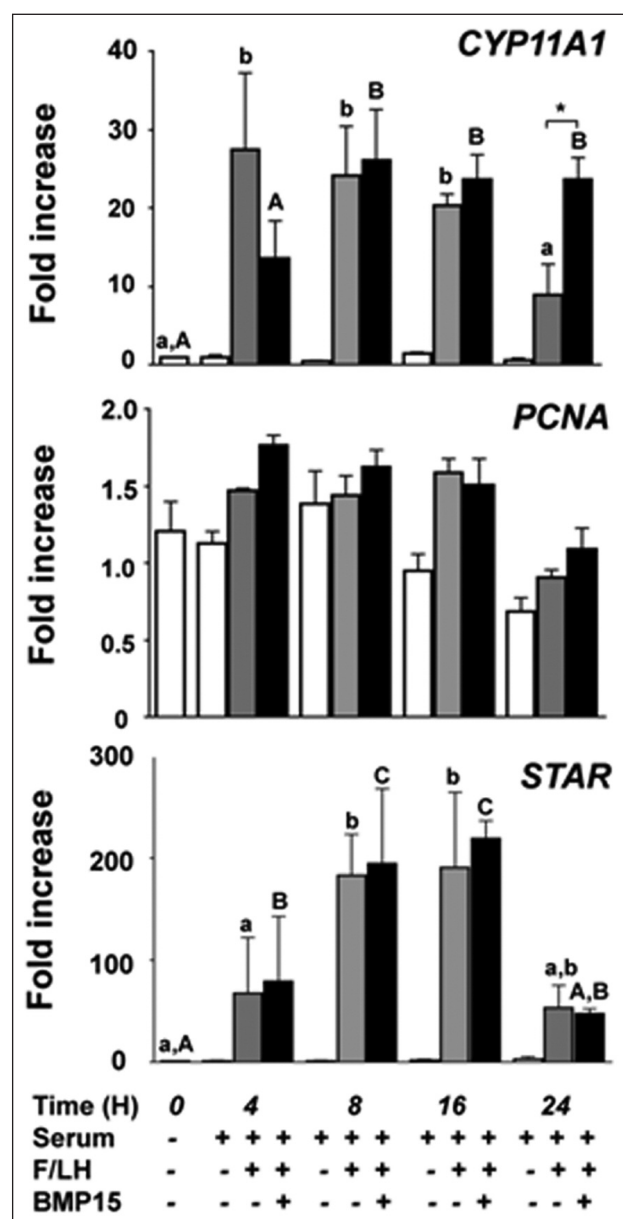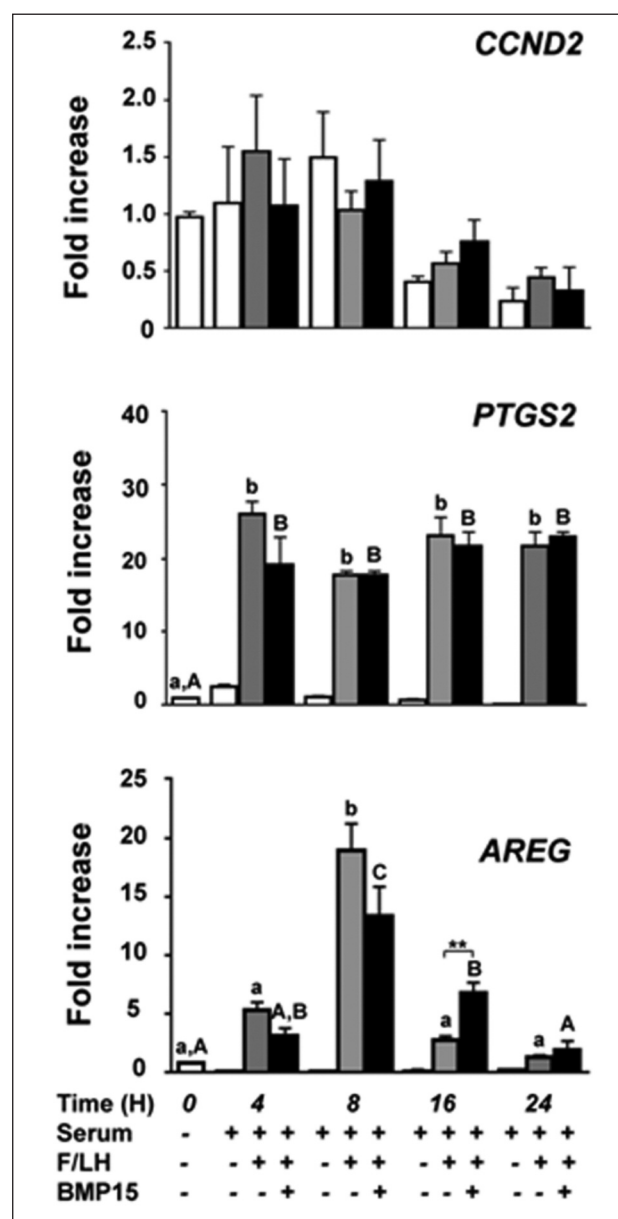

Fig. 3B

Fig. 3C

Fig. 3. Expression of mRNAs in OCC cultured in control or FSH/LH-supplemented medium in the absence/presence of BMP-15 (100 ng/ml). Genes are grouped according to their biological function. (*A*) Cumulus expansion (*CD44, HAS2,* and *TNFAIP6)*; (*B*) Proliferation and differentiation (*CYP11A1, PCNA* and *StAR*); (*C*) Cell cycle, prostaglandin biosynthesis and signal transduction (*CCND2, PTGS2* and *AREG)*. Data are expressed in arbitrary units as fold strength increases over the level found in OCC at time 0 h. Data represent the mean ± S.E.M. from three replicates for each treatment, prepared in duplicate. The different superscripts above the columns indicate significant differences among samples during cultivation with FSH/LH alone (a, b, c) or supplemented with BMP-15 (A, B, C). Asterisks above the columns indicate significant differences (*P < 0.05; **P < 0.01) between untreated and treated samples.

(measured as HA production by OCC and HA released in culture medium) and its retention within the complexes. In the absence of gonadotropins, both HA production and retention by OCC, was not improved by the action of BMP-15 (*Fig. 2A* and *2B*). The addition of gonadotropins to serum-supplemented medium significantly increased the total HA synthesis as well as its retention within OCC (*Fig. 2A* and *2B*). While the presence of BMP-15 did not affect FSH/LH-stimulated total HA synthesis by OCC (*Fig. 2A*), its retention within the complexes was significantly increased after the action of BMP-15 in comparison to FSH/LH alone (about 65% versus 35 %, respectively, P < 0.001; *Fig. 2B*). In PVP-supplemented medium, addition of gonadotropins induced small increase of the retention of HA within the complexes in the presence of BMP-15 (*Fig. 2B*).

It seems that BMP-15 added to the FSH/LH-supplemented medium may significantly increase the retention of HA within the complexes and thus improve the formation and stability of HA-rich cumulus ECM.

Effect of bone morphogenetic protein-15 on cumulus transcript levels

To elucidate the possible molecular mechanisms of the BMP-15 action on cumulus expansion of OCC, we investigated the effect of BMP-15 (100 ng/ml) on expression of OCC maturation-related transcripts (*AREG, CD44, HAS2, PTGS2, TNFAIP6*), and proliferation- and differentiation-related transcripts (*CCND2, PCNA, CYP11A1, STAR)* at different time points (4, 8, 16 and 24 hours) in porcine OCC cultured in serum-supplemented medium using real-time RT-PCR (*Fig. 3A-3C*). We demonstrated that FSH/LH-induced expansion of the cumulus is accompanied by a substantial enhancement of *AREG, CD44, CYP11A1, HAS2, PTGS2, STAR,* and *TNFAIP6* mRNA levels. A significant increase in the expression was observed after as little as 4 h (*CD44, CYP11A1,* and *PTGS2*) or 8 h (*AREG, HAS2, TNFAIP6,* and *STAR*) of culture. *PCNA* and *CCND2* transcript levels were not affected by the gonadotropin stimulation, although they tended to decline at later time points (16 h) for *CCND2*. For most of the genes studied, BMP-15 did not affect their expression. However, BMP-15 enhanced the FSH/LH-induced expression of *TNFAIP6* (*Fig. 3A*) and *AREG* (*Fig. 3C*) (both after 16 h), and *CYP11A1* (after 24 h; *Fig. 3B*).

These results indicate that recombinant BMP-15 may exhibit specific biological activities on porcine OCC cultured *in vitro*, since BMP-15 affected mRNA levels of *AREG* - implicated in gonadotropin signal transduction, *TNFAIP6* - implicated in the stability of HA-rich cumulus ECM and *CYP11A1* - responsible for the conversion of pregnenolone to progesterone.

Effect of bone morphogenetic protein-15 on progesterone secretion by oocyte cumulus complexes and granulosa cells

We investigated the effect of BMP-15 (100 ng/ml) on basal and FSH/LH-induced progesterone secretion by porcine OCC cultured for 44 hours. In serum-supplemented medium, BMP-15 alone did not affect basal progesterone production by OCC (*Fig. 4A*). As expected, FSH/LH significantly increased progesterone secretion by OCC compared to unstimulated complexes (P < 0.001). The presence of BMP-15 additionally increased (about 69%) FSH/LH-stimulated progesterone production by OCC compared to FSH/LH alone (P < 0.01; *Fig. 4A*). In the PVP-supplemented medium, BMP-15 did not exert a similar augmenting effect on FSH/LH-stimulated progesterone production by OCC (*Fig. 4A*).

Furthermore, we investigated the effect of different concentrations of BMP-15 (0.1, 1, 10 ng/ml) on basal and FSH/LH-stimulated progesterone production by primary culture of porcine GC. BMP-15 alone did not affect basal progesterone production by GC (*Fig. 4B*). Gonadotropins induced a significant increase of progesterone production by GC (P < 0.001; *Fig. 4B*). The addition of BMP-15 to the cell culture caused a significant dose-independent decrease (about 35%; P < 0.001) in the FSH/LH-induced progesterone production (*Fig. 4B*).

DISCUSSION

It is well known that bidirectional communication between cumulus cells and the oocyte is necessary for oocyte cytoplasmic maturation and subsequent developmental competence (18). Factors secreted by the oocyte, including BMP-15, a member of TGF-β superfamily, have been shown to be implicated in the regulation of follicular cell function. However, the complex role of BMP-15 within the OCC is not fully understood. The signalling through the BMP pathway is regulated by molecules that bind BMP ligands in the extracellular space (15). To elucidate the mechanisms of BMP-15 action on the regulation of the FSH/LH signalling pathway in porcine ovarian follicles, we investigated the effect of BMP-15 on FSH/LH-induced 1) cumulus expansion; 2) HA synthesis; 3) expression of oocyte maturation-related transcripts (*AREG, CCND2, CD44, CYP11A1, HAS2, PCNA, PTGS2, STAR,* and *TNFAIP6*) at different time points in their cultivation; and P4 production either by porcine OCC or GC.

The results presented here show that differences in culture conditions, such as the absence/presence of serum, strongly affect the structural organization of cumulus ECM and consequently the functionality of CC surrounding the oocyte. We observed that gonadotropin-stimulated OCC cultured in serum-supplemented medium with BMP-15 were expanded and sticky with a tendency to form elastic structure chains (*Fig. 1D* and *1F*). In contrast, gonadotropin-stimulated OCC cultured in serum-free medium, although they were expanded over a larger area than unstimulated complexes, were not sticky (*Fig. 1E*). These OCC lost elastic structure and many cumulus cells, which that appeared at the bottom of the Petri dishes. An important relationship has been shown between the serum and HA retention within the expanded OCC in mice (19) and pigs (17). Thus, HA is retained in the expanded cumulus to act as a major component of cumulus cell function (20, 21). In the present study, we found that HA retained within the complexes was significantly increased after the action of BMP-15 compared to FSH/LH alone (65% versus 35%, respectively). The enzyme HAS2 is responsible for HA synthesis in porcine follicles (22). Sugiura *et al.* (23) showed that *Has2* short-hairpin RNA expression suppressed cumulus expansion and *Has2* mRNA levels in mouse OCC by more than 70% without affecting the expression levels of cumulus expansion-related transcripts, such as *Ptgs2, Ptx3, and Tnfaip6* mRNAs. These results provide additional evidence that HA is critical for maintaining the correct balance and structural organization of other extracellular components. Importantly, during cumulus expansion in mouse (24) and porcine OCC (20), a covalent binding is formed between HA and heavy chains of inter-alpha-trypsin inhibitor (the serum-derived factor), which is responsible for the formation of stabile HA-rich cumulus ECM (11, 12).

Paradis *et al.* (7) investigating BMP signalling in porcine follicles found an increase in the receptor *BMPR1B* mRNA and protein during the periovulatory period in porcine GC and TC suggesting an important role of BMP-15 in the ovulatory process. In addition, *BMP-15* mRNA and protein expression levels were low in immature porcine oocytes and increased to
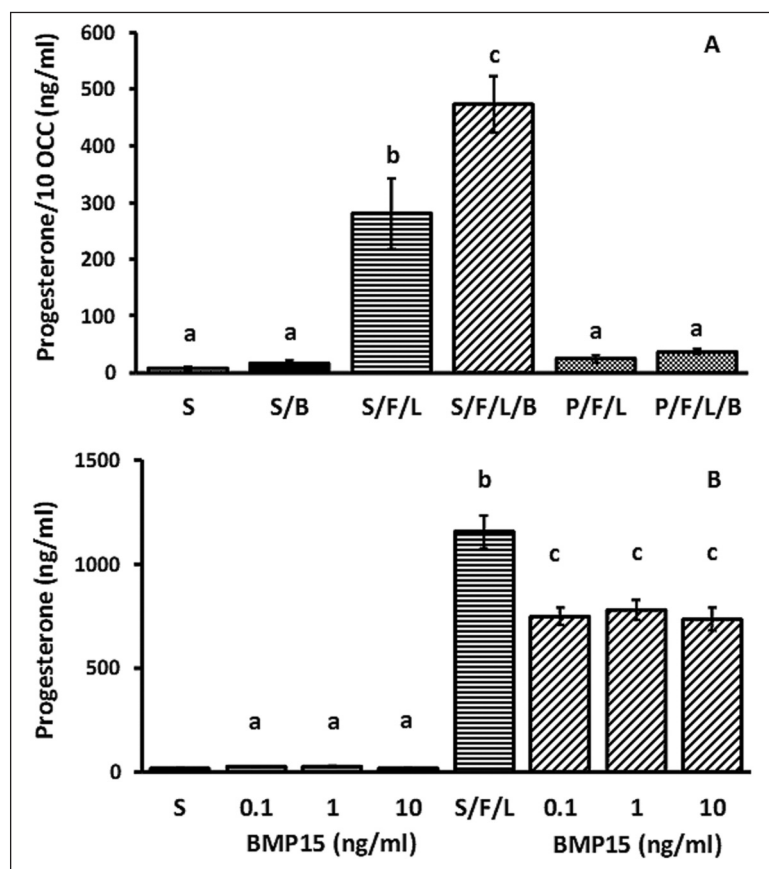
Fig. 4. (*A*) Effect of BMP-15 (B; 100 ng/ml) on basal and FSH/LH-induced (F/L) progesterone production by porcine oocyte-cumulus complexes (OCC) cultured for 44 h in serum (S)- or PVP (P)-supplemented medium. Data represent the mean ± S.E.M. from three replicates for each treatment, prepared in duplicate. Effect of different concentrations of BMP-15 (B) on basal and FSH/LH-induced (F/L) progesterone production by porcine granulosa cells cultured for 72 h in serum-supplemented (S) medium. Data represent the mean ± S.E.M. from three replicates for each treatment, prepared in quadruplicate. The columns with various superscripts are significantly different (P < 0.05).

the highest level at 18 h of *in vitro* maturation, which coincides with the time of cumulus expansion (25). In contrast, Zhu *et al.* (26) showed that mRNAs of *BMPRIA, BMPRIB* and *BMPRII* were constantly expressed in porcine CC, but in oocytes, the transcription of *BMP-6, BMP-15, GDF9* and *BMPRII* were down regulated during *in vitro* maturation. In the present study, we found that BMP-15 augmented HA retained within the cumulus ECM of FSH/LH-stimulated OCC. Moreover, BMP-15 significantly increased the FSH/LH-stimulated production of progesterone by OCC compared to FSH/LH alone. Sutton-McDowall *et al.* (27) observed a beneficial effect of BMP-15 on increased bovine blastocyst development (without BMP-15 and FSH, 28.4% ± 7.4%; with FSH and BMP-15, 51.5% ± 5.4%). Gueripel *et al.* (8) showed that in mice after hCG injection, during cumulus expansion, BMP-15 was released from the oocyte to the cumulus ECM. BMP-15 immunostaining was only observed in those follicles that exhibited an expanded cumulus. These authors (8) found that oocytes in atretic follicles no longer synthesize any *Bmp-15* mRNA.

Previously, using an interspecies testing system described by Vanderhyden (28), we provided evidence that porcine oocytes produce at least two paracrine factors during *in vitro* meiotic maturation that affect the cumulus expansion of mouse oocytectomized (OOX) complexes, and that the production of these factors is regulated developmentally (29). The secretion of these factors only occurred in germinal vesicle (GV) stage oocytes and during the GV to metaphase I (MI) transition. Oocytes that progressed to and beyond MI appeared to produce a factor or factors that enabled the production of HA after the stimulation of OOX with FSH, but not its retention within expanded cumuli (29). Although the identity of these factors is currently unknown, previous evidence would suggest that GDF9 might be a candidate for the cumulus expansion-enabling factor

(30, 31). In this report, we suggest that BMP-15 also plays an important role in cumulus expansion through the retention of HA within the complex, formation of cumulus HA-rich ECM, and differentiation of CC in pigs. Gilchrist and Ritter (32) showed that porcine oocytes secrete factors that activate SMAD2/3 (in response to GDF9) and SMAD1/5/8 (in response to BMP-15) in GC. While the treatment of OCC with SMAD2/3 phosphorylation inhibitor (SB431542) partially inhibited porcine cumulus expansion and the expression of *TNFAIP6* and *HAS2* (6), SMAD1/5/8 phosphorylation inhibitor (dorsomorphim) had no effect on porcine cumulus function (32). In addition, Zhu *et al.* (26) found that in porcine oocytes matured *in vitro*, the transcription of *BMP15* and *GDF9* were down regulated. Taking into account the results of Paradis *et al.* (7) that demonstrated *in vivo* complete BMP signalling in the porcine preovulatory follicle, we conclude that the addition of BMP-15 to *in vitro* culture of porcine oocytes in FSH/LH-supplemented medium profoundly improves the structural formation of an HA-rich cumulus ECM. We suggest that CC alter their function and differentiate at higher rate, and produce significantly higher progesterone levels with the well-organized cumulus ECM.

Finally, we demonstrated that in the absence of oocytes, BMP-15 induced a significant decrease (about 35%) in the FSH/LH-increased level of progesterone by porcine GC (cultured as a monolayer) compared to FSH/LH alone. Our results are in agreement with the findings on rat primary GC; BMP-15 stimulated GC proliferation and markedly decreased FSH-induced progesterone production (2). Moore *et al.* (33) found that the addition of U0126, an inhibitor of ERK1/2 phosphorylation, suppressed BMP-15 activity on GC mitosis. Chang *et al.* (34) demonstrated that BMP-15 decreased progesterone production in immortalized human GC (SVOG) by down-regulating steroidogenic acute regulatory protein (StAR) expression.

Interestingly, oxidative stress affects female reproduction (35). Surprisingly, an antioxidant (embelin) did not influence either oocyte maturation or steroidogenesis of porcine OCC (36). In contrast, steroidogenesis was changed in ovarian follicles by reproductive factors such as ghrelin (37) or leptin (38). In the present study, the effect of BMP-15 on progesterone production by porcine GC was distinct from its action on CC. In porcine OCC, BMP-15 altered the behaviour of CC involved in expanded cumulus ECM after gonadotropin stimulation, showing that the presence of oocytes is important for the characterization of the BMP-15 signalling pathway in porcine ovarian follicle.

To the best of our knowledge, this is the first study that demonstrates the involvement of the action of BMP-15 in the formation of the HA-rich cumulus ECM during the culture of porcine OCC in FSH/LH-supplemented medium. We suppose, together with Paradis *et al.* (7) and Gueripel *et al.* (8) that complete BMP signalling actively works in preovulatory follicles. Together with the results of Sutton-McDowall *et al.* (27), we suggest that the addition of BMP-15 to the FSH/LH-stimulated OCC cultured in serum-supplemented medium improve oocyte and embryonal development.

Conflict of interest: None declared.

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