Bovine embryo-oviduct interaction *in vitro* reveals an early cross talk mediated by BMP signaling

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Abstract

Signaling components of bone morphogenetic proteins (BMPs) are expressed in an anatomically and temporally regulated fashion in bovine oviduct. However, a local response of this signaling to the presence of the embryo has yet to be elucidated. The aim of the present study was to evaluate if early embryo-oviduct interaction induces changes in the gene expression of BMP signaling components. For this purpose, we used an *in vitro* co-culture system to investigate the local interaction between bovine oviductal epithelial cells (BOEC) from the isthmus region with early embryos during two developmental periods: before (from the 2-cell to 8-cell stage) or during (from the 8-cell to 16-cell stage) the main phase of embryonic genome activation (EGA). Exposure to embryos, irrespective of the period, significantly reduced the relative abundance of *BMPR1B*, *BMPR2*, *SMAD1*, *SMAD6* and *ID2* mRNAs in BOEC. In contrast, embryos that interacted with BOEC before EGA showed a significant increase in the relative abundance of *SMAD1* mRNA at the 8-cell stage compared to embryos cultured without BOEC. Moreover, embryos at the 16-cell stage that interacted with BOEC during EGA showed a significant increase in *BMPR1B*, *BMPR2* and *ID2* mRNA. These results demonstrate that embryo-oviduct interaction *in vitro* induces specific changes in the transcriptional levels of BMP signaling, causing a bidirectional response that reduces the expression levels of this signaling in the oviductal cells while increases them in the early embryo. This suggests that BMP signaling pathway could be involved in an early cross talk between the bovine embryo and the oviduct during the first stages of development.

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Introduction

Maternal-embryo communication during the preimplantation period plays a critical role for the establishment and maintenance of pregnancy. Any disturbance in this reciprocal cross talk can lead to pregnancy failure and can also have long-term consequences in the developmental potential and health of the offspring (Wolf *et al.* 2003, Fazeli 2008, Rizos *et al.* 2016).

In mammals, the first maternal site that makes contact with the embryo is the oviduct. In the particular case of cattle, this contact is established during the first four days after fertilization (Maillo *et al.* 2016). Within this window of time, important developmental events occur in the embryo, including the first cleavage divisions (Lonergan *et al.* 1999) and the epigenetic reprogramming and activation of the embryonic genome (Memili & First 2000, Graf *et al.* 2014). To provide an optimal

environment and to ensure the correct synchronization of these events, the oviduct needs to initiate a molecular dialog with the developing embryo. Studies in mice, pigs and horses have provided evidence for this initial cross talk, demonstrating that the presence of the embryo(s) produces transcriptional changes in the oviductal cells (Lee *et al.* 2002, Almiñana *et al.* 2012, Smits *et al.* 2016). In cattle, although the presence of a single embryo *in vivo* has no detectable effect on the oviductal transcriptome, the transfer of 50 embryos into the oviduct of heifers produces changes in the gene expression pattern of the oviductal cells, suggesting that the effect of the embryo, particularly in mono-ovulatory species, seems to be more local than was expected (Maillo *et al.* 2015).

It is known that epithelial cells lining the lumen of the mammalian oviduct can synthesize and secrete a wide range of proteins, including diverse growth factors and cytokines that can mediate a local interaction with the

embryo through different signaling pathways (Buhi *et al.* 2000, Lee & Yeung 2006, Aviles *et al.* 2010). Among the candidate signaling pathways that could actively participate in this local embryo-maternal cross talk in the oviduct, we focused our attention on the signaling mediated by bone morphogenetic proteins (BMPs). BMPs are a subfamily of growth factors that belongs to the TGF- β superfamily (Bragdon *et al.* 2011). These factors exert their effects as ligand dimers by binding to tetrameric complexes of type I (BMPRI1A, BMPRI1B or ACVR1A, also known as ALK3, ALK6 and ALK2 respectively) and type II (BMPRI2, ACVR2A or ACVR2B) serine/threonine kinase receptors leading to signal transduction through SMAD-dependent pathways which regulates transcriptional responses (Beyer *et al.* 2013). Several BMPs are expressed in the maternal tract (ovary, uterus and placenta) and play important roles as autocrine and paracrine regulators of ovarian follicular development, blastocyst implantation in the uterus, and morphogenesis and organogenesis during embryo development (Shimasaki *et al.* 2004, Kishigami & Mishina 2005, Jones *et al.* 2006, Knight & Glister 2006). In addition, different components of the BMP signaling pathway are expressed by the embryo in a developmentally regulated fashion during the preimplantation development (Roelen *et al.* 1997, Zhang *et al.* 2007, Lee *et al.* 2014). Particularly in mice, knockout experiments and inhibition assays demonstrated that during the preimplantation period BMP signaling is critical for cell cleavage and the normal development of extra-embryonic lineages (Graham *et al.* 2014, de Mochel *et al.* 2015).

Previous studies have revealed that different members of the BMP family are expressed in the epithelial cells of the bovine oviduct in an anatomically and temporally regulated fashion during the estrous cycle (Garcia *et al.* 2014). In particular, BMP5 exhibits differential expression in the isthmus region and high transcriptional levels during the periovulatory phase. The presence of this factor in the oviductal fluid suggests that BMP ligands produced by the oviduct could have a paracrine action on the bovine embryo (Garcia *et al.* 2014). In fact, the addition of BMP5 to the culture medium during the first two days after fertilization produces an increase in the blastocyst rate and the relative mRNA abundance of pluripotency-related genes (Garcia *et al.* 2015). Interestingly, mRNA expression of BMP receptors is greater in early stage embryos (from 2-cell to 8-cell stage), suggesting that the embryo could be target of BMPs during its transit through the oviduct (Garcia *et al.* 2015). Moreover, in a recent study by RNA sequencing, Maillo and coworkers (Maillo *et al.* 2015) observed that the presence of multiple embryos induces changes in the expression levels of several genes in the bovine oviduct *in vivo*, included among them are various genes

associated with the BMP signaling pathway. However, until now the participation of this signaling pathway in an initial cross talk between the embryo and the oviduct during the preimplantation period has not been explored in depth.

Considering this evidence, BMP signaling could be a candidate pathway for embryo-maternal communication during the preimplantation period. Within this context, the objective of this study was to evaluate whether early embryo-oviduct interaction induces changes in the gene expression levels of BMP signaling components both in the oviductal cells and in the preimplantation embryos. For this purpose, an *in vitro* co-culture system allowing a local and temporal interaction between early bovine embryos and isthmus epithelial cells was established.

Materials and methods

Chemicals

All reagents were purchased from Sigma Chemical Química S.A Company unless otherwise stated.

Bovine oviduct epithelial cell isolation and *in vitro* culture

Bovine oviducts at the early luteal phase (corresponding to Days 3–5 of the estrous cycle) were obtained from slaughtered heifers and selected based on ovarian morphology according to Ireland and coworkers (Ireland *et al.* 1980). The oviducts were sealed in a plastic bag and transported to the laboratory on ice within 2 h of slaughter. The surrounding fat and connective tissues were carefully removed and each oviduct was washed three times in sterile PBS (pH 7.4). The isthmus region was separated and the oviductal mucosa was collected by squeezing and was washed 2 times with PBS by centrifugation at 300g for 10 min. The cell pellet was then resuspended in 1 mL of synthetic oviductal fluid (SOF) supplemented with 10% (v/v) of fetal calf serum (FCS) and was passed 10 times through a 25 G syringe needle to obtain a single cell suspension. Bovine oviduct epithelial cells (BOEC) were counted in a hemocytometer, diluted to a final concentration of 2×10^6 cells/mL and then cultured in 500 μ L of SOF+10% FCS in four-well plates at 38.5°C, 5% CO₂, 5% O₂, 90% N₂ and saturated humidity until confluence. Half of the media (SOF+10% FCS) was replaced every 48 h. On Day 5 of culture, the medium was changed to SOF with 5% FCS; and 24 h later, half of the media was renewed and the cells were used for co-culture with embryos.

Immunocytochemical analysis with antibodies anti-bovine-pancadherin (C1821), anti-bovine-pancytokeratin (C2931) and anti-bovine-vimentin (V2258), following the protocol described by Lopera-Vásquez and coworkers (Lopera-Vásquez *et al.* 2016), confirmed the epithelial nature of the cultured cells showing positive staining for the epithelial markers cadherin and cytokeratin, and negative staining for the fibroblast marker vimentin (Fig. 1A).

Figure 1 *In vitro* co-culture system. (A) Immunofluorescence analysis of BOEC monolayers showing positive staining for the epithelial markers, cadherin and cytokeratin, and negative staining for the fibroblast marker, vimentin. Cell nuclei were counterstained with Hoechst stain (blue). (B) Representative image of the polyester meshes employed to establish a local area of contact between the embryos and the BOEC monolayer. Thirty embryos/well were transferred to the mesh and placed in a 6×5 openings grid covering an area of 2.1 mm^2 over the monolayer. Mesh dimensions: $170 \mu\text{m}$ opening and $96 \mu\text{m}$ filament diameter.

Oocyte collection and *in vitro* maturation

Immature cumulus–oocyte complexes (COCs) were recovered by aspirating follicles (2–8 mm in diameter) from ovaries of heifers slaughtered at local abattoir and *in vitro* maturation was performed as described by Lopera-Vázquez and coworkers (Lopera-Vázquez *et al.* 2015). Only oocytes with a compact, non-atretic cumulus of at least three layers and a homogeneous ooplasm (Class 1 and 2 COCs) were selected and matured for 24 h in 500 μL of maturation medium (TCM 199 (M4530) supplemented with 10% (v/v) FCS and 10 ng/mL epidermal growth factor (E4127)) in four-well dishes (50 COCs per well) at 38.5°C under an atmosphere of 5% CO_2 , with maximum humidity.

In vitro fertilization

Frozen semen from a single Asturian Valley bull (ASEAVA, Asturias, Spain), previously tested for IVF, was thawed at 37°C in a water bath for 1 min and selected on a gradient of Bovipure (Nidacon Laboratories AB, Gthenborg, Sweden) following the protocol described by Lopera-Vzquez and coworkers (Lopera-Vzquez *et al.* 2016). Sperm concentration was determined and adjusted to a final concentration of 1×10^6 sperm cells/mL. Gametes were coincubated for 18–22 h in 500 μL of fertilization medium (Tyrode's medium with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate and 6 mg/mL fatty acid-free bovine serum albumin (BSA) supplemented with 10 $\mu\text{g/mL}$ heparin sodium salt (Calbiochem)) in a four-well dish, in groups of 50 COCs per well under an atmosphere of 5% CO_2 , with maximum humidity at 38.5°C .

In vitro embryo culture and co-culture conditions

After the fertilization period, presumptive zygotes were completely denuded of cumulus cells by vortexing and groups of 50 were initially cultured in 500 μL of SOF supplemented with 4.2 mM sodium lactate (L4263), 0.73 mM sodium pyruvate (P4562), 30 $\mu\text{L/mL}$ basal medium eagle (BME) amino acids (B6766), 10 $\mu\text{L/mL}$ minimum essential medium (MEM) amino acids (M7145), 1 $\mu\text{g/mL}$ phenol red (P0290) and 3 mg/mL bovine serum albumin (BSA; A9647) in a four-well dish, under an atmosphere of 5% CO_2 , 5% O_2 and 90% N_2 at 38.5°C . At 33 and 54 h post-insemination (hpi); those embryos that reach the 2-cell stage and the 8-cell stage respectively were selected and randomly cultured in groups of 30 embryos in SOF supplemented with 3 mg/mL of BSA or in SOF supplemented with 5% (v/v) of FCS in the presence or absence of a BOEC monolayer, depending on the experimental group to which they were assigned (see 'Experimental design' described below for more details). In order to limit and establish a local area of contact between the embryos and the BOEC monolayer, a nontoxic woven polyester mesh (Sefar Petex; Sefar, Bury, Lancashire, UK) was used with a grid size of 41×41 openings, covering an area of 121 mm^2 that fitted perfectly inside the well of a NuncTM 4-well dish. First of all, one mesh square containing delimited marked areas of 6×5 openings was placed outside at the bottom of the wells and was used as a guide to localize the co-culture area (Fig. 1B). Secondly, another mesh square of the same size was washed once with 70% ethanol, thrice with PBS and twice with SOF media, and then was introduced into the well so that the external and internal mesh were overlapped. Then, 30 embryos/well were

transferred to the mesh and placed in a 6×5 grid, covering an area of 2.1 mm² over the monolayer (Fig. 1B). Preferably, the central grid was selected. Following culture, embryos were transferred and cultured in 25 µL droplets of SOF with BSA until Day 9pi.

RNA isolation and reverse transcription

Five independent samples of BOEC cultured with and without embryo under each experimental condition, and also three independent groups of 10 embryos per stage (8-cell, 16-cell and blastocyst stage) and per experimental group were collected and processed for poly(A) RNA extraction. Poly(A) RNA was extracted using the Dynabeads mRNA Direct Extraction Kit (Dynal Biotech, Oslo, Norway) following the manufacturer's instructions and with minor modifications as it was described previously by Bermejo-Alvarez and coworkers (Bermejo-Alvarez *et al.* 2008). After 10 min of incubation in lysis buffer with Dynabeads, poly(A) RNA attached to the Dynabeads was extracted with a magnet and washed twice in washing buffer A and washing buffer B. RNA was then eluted with Tris–HCl. Immediately after extraction, the reverse transcription (RT) reaction was performed as recommended by the manufacturer (Epicentre Technologies Corp, Madison, WI, USA). Briefly, oligo-dT (0.2 µM) and random primers (0.5 µM) were added to each RNA sample and were heated for 5 min at 70°C to denature the secondary RNA structure. Next, the tubes were incubated at 25°C for 10 min to promote the annealing of the primers. Then, the RNA was reverse-transcribed for 60 min at 37°C in a final volume of 50 µL containing 0.375 mM dNTPs (Biotools, Madrid, Spain), 6.25 U RNasin RNase inhibitor (Promega), 10× MMLV-RT buffer with 8 mM dithiothreitol

and 5U MMLV high performance reverse transcriptase (Epicentre Technologies Corp, Madison, WI, USA), followed by incubation at 85°C for 5 min to inactivate the RT enzyme.

Gene expression analysis

The mRNA expression levels of the selected genes were determined by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) using specific primers designed with Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to span exon-exon boundaries when possible. Primers (Table 1) were previously validated for adequate primer efficiency; and specificity of their PCR products was confirmed by electrophoresis on a 2% agarose gel. All target genes showed efficiencies between 97 and 100% and correlation coefficients close to 1.0.

All PCR reactions were performed in a final volume of 20 µL, containing 0.25 mM of forward and reverse primers, 10 µL of GoTaq qPCR Master Mix (Promega) and 2 µL of each cDNA sample derived from BOEC (≈60 ng/µL), 8-cell embryos (≈4 ng/µL), 16-cell embryos (≈20 ng/µL) or blastocysts (≈60 ng/µL), using a Rotorgene 6000 Real Time Cycler (Corbett Research, Sydney, Australia) and SYBR Green as double-stranded DNA-specific fluorescent dye. For each experimental group, five different cDNA samples from BOEC and three different cDNA samples from each embryonic stage were used in two repetitions for all genes of interest. The PCR program consisted of an initial denaturalization step at 94°C for 2 min, followed by 35 cycles of denaturalization at 94°C for 10s, annealing at 56°C for 30s, extension at 72°C for 15s and 10s of fluorescence acquisition defined for each primer. At the end of each PCR run, melt curve analyses were performed for all

Table 1 Set of primers used for qRT-PCR assays.

Gene		Primer sequences (5'–3') ^a	Amplicon length (bp)	GenBank accession number
BMP5	Forward	GATGTGGGTTGGCTTGCTT	271	NM_001305016.1
	Reverse	CCTGATGAGAGCCGGATTTA		
BMP7	Forward	AACCATGCCATCGTGCAGACGC	250	NM_001206015.1
	Reverse	AAGCCCGGACAACCATGTTTGC		
BMPR1A	Forward	TTGGGAAATGGCTCGTCGTT	142	NM_001076800.1
	Reverse	AGACACAATTGGCCGCAAAC		
BMPR1B	Forward	TTTGGGAGGTCGCTAGGAGA	132	NM_001105328.1
	Reverse	GCCGACGCTTCTTGATACAC		
BMPR2	Forward	TCTTTCAGCCACCAATGTCCT	158	NM_001304285.1
	Reverse	GTTCAAGTGGAAATGACCCAGG		
SMAD1	Forward	TGTGAACCACGGCTTCGAGACG	99	NM_001076223.2
	Reverse	TCCTGGCGGTGGTATTCTGCTC		
SMAD5	Forward	TTGCTCAGCTTCTGGCTCAGTC	119	NM_001077107.3
	Reverse	TTGCCGGTGATACTCTGCTCC		
SMAD6	Forward	GGAGAAATTCGCTCCAAGTGC	242	NM_001206145.1
	Reverse	CCCTGCCTTTAAACCCAAGC		
ID2	Forward	CGACATCAGCATCCTGTCCTT	145	NM_001034231.2
	Reverse	AGAGCCTGTGGATTGTTGT		
H2AFZ	Forward	AGGACGACTAGCCATGGACGTGTG	209	NM_174809.2
	Reverse	CCACCACGACCAATTGTAGCCTTG		
ACTB	Forward	GAGAAGCTCTGCTACGTCG	263	NM_173979.3
	Reverse	CCAGACAGCACCGTGTGG		

^aAll the primers were designed with NCBI Primer-BLAST online tool of the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

genes to ensure single product amplification and exclude the possible interference of dimers.

Relative expression levels were quantified by the comparative cycle threshold ($\Delta\Delta CT$) method (Schmittgen & Livak 2008). Values were normalized using two housekeeping genes (*H2AFZ* and *ACTB*) that were tested in previous studies (Maillo *et al.* 2015). Fluorescence was acquired in each cycle to determine the threshold cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. According to the comparative CT method, the ΔCT value was determined by subtracting the mean CT value of the two housekeeping genes (*H2AFZ* and *ACTB*) for each sample from each gene CT value of the sample. Calculation of $\Delta\Delta CT$ involved using the highest sample ΔCT value (i.e. the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$ (Livak & Schmittgen 2001). All the study were carried out following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments recommendations (Bustin *et al.* 2009).

Experimental design

Experiment 1: Effect of BOEC co-culture on early embryo development *in vitro*

In the first experiment, an *in vitro* co-culture system was established to provide a local and temporal interaction between a BOEC monolayer and early bovine embryos during two developmental periods: (a) Experimental Group 1 (G1): from 2-cell to 8-cell stage, before the main phase of embryonic

genome activation (EGA); and (b) Experimental Group 2 (G2): from 8-cell to 16-cell stage, during the main phase of EGA (Fig. 2). To determine if this *in vitro* co-culture system had an effect on early embryonic development, cleavage rate and blastocyst yield were evaluated.

For this purpose, isthmus epithelial cells obtained from a pool of three post-ovulatory stage oviducts ($n=21$, 3 oviducts per experimental replicate) were cultured in a monolayer as described previously. On Day 6 of culture, the BOEC monolayer was cultured in the absence or presence of *in vitro*-produced embryos from 2- to 8-cell stage (G1 BOEC; 33–54 hpi) or from 8- to 16-cell stage (G2 BOEC; 54–98 hpi) in SOF medium supplemented with 5% FCS, under an atmosphere of 5% CO_2 , 5% O_2 and 90% N_2 at 38.5°C. As mentioned above, 30 embryos/well were placed in a 6 × 5 grid over the monolayer. In addition, as control without BOEC, groups of 30 embryos in both developmental stages were cultured either in SOF + 5% FCS (G1 FCS and G2 FCS) or in SOF + 3 mg/mL BSA (G1 BSA and G2 BSA). At 54 hpi (G1 BOEC/FCS/BSA) or 98 hpi (G2 BOEC/FCS/BSA), embryos that reached the 8- or 16-cell stage respectively were transferred to SOF + BSA and cultured until Day 9, maintaining the different experimental groups separately (Fig. 2).

Considering that during the experiment it was necessary to preselect the embryos at different stages of development, the developmental parameters were calculated as follows: (I) developmental rate at 33 hpi: percentage of presumptive zygotes that developed to the 2-cell stage at 33 hpi; (II) developmental rate at 54 hpi: percentage of selected 2-cell embryos that developed to the 8-cell stage at 54 hpi; (III) developmental rate at 98 hpi: percentage of selected 8-cell embryos that developed to the 16-cell stage at 98 hpi; and

Figure 2 Experimental design. Experimental group 1: Two-cell IVF bovine embryos were selected at 33 hpi and cultured before the main phase of EGA (from 2-cell to 8-cell stage: 33–54 hpi) in medium SOF + BSA (G1 BSA), SOF + FCS (G1 FCS) or co-cultured with BOEC monolayer (G1 BOEC). At 54 hpi, embryos that reached the 8-cell stage were transferred to SOF + BSA and cultured until Day 9. Experimental group 2: Eight-cell IVF bovine embryos were selected at 54 hpi and cultured during the main phase of EGA (from 8-cell to 16-cell stage: 54–98 hpi) in medium SOF + BSA (G2 BSA), SOF + FCS (G2 FCS) or co-cultured with BOEC monolayer (G2 BOEC). At 98 hpi, embryos that reached the 16-cell stage were transferred to SOF + BSA and cultured until Day 9. Samples of BOEC cultured with (BOEC G1+/G2+) or without embryos (BOEC G1–/G2–) before or during EGA, and embryos at 8-cell (54 hpi), 16-cell (98 hpi) and blastocyst stage (Day 7–8) were collected from the different experimental groups to evaluate the mRNA expression levels of BMP signaling components by qRT-PCR. EGA, embryonic genome activation; hpi, hours post insemination.

(IV) blastocyst yield: percentage of selected 8-cell embryos or 16-cell embryos that continued in culture and developed to the blastocyst stage at Days 7, 8 and 9 after insemination.

Seven experimental replicates were performed under the same assay conditions and a total of 2550 presumptive zygotes were *in vitro* cultured: 1192 were used for experimental groups 1 (G1 BOEC/FCS/BSA) and 1358 for groups 2 (G2 BOEC/FCS/BSA).

Experiment 2: Effect of early embryo-oviduct interaction in vitro on mRNA expression of BMP signaling components in oviductal epithelial cells

To evaluate whether *in vitro* interaction with early embryos induces changes in the gene expression levels of BMP signaling components in the oviductal cells, an expression analysis was performed by qRT-PCR in 5 samples of BOEC cultured with or without embryos before (G1 BOEC) or during EGA (G2 BOEC), obtained from 5 different experimental replicates of Experiment 1.

After the co-culture period, the polyester mesh and the embryos were removed and the BOEC monolayer was quickly washed twice with PBS. The cell area directly beneath the embryos was recovered from G1 BOEC at 54 hpi (BOEC G1+, $n=5$) and from G2 BOEC at 98 hpi (BOEC G2+, $n=5$) by mechanical scraping with a micropipette tip. At the same time, an area of the same size but from a different well of BOEC, cultured without embryos, was collected as control (BOEC G1– and BOEC G2–; $n=5$). The BOEC samples collected were immediately snap-frozen in liquid nitrogen and kept at -80°C until RNA extraction.

The mRNA expression levels of two BMP ligands (*BMP5/7*), three BMP receptors (*BMPRI1A/1B/2*), two signaling proteins (*SMAD1/5*), one inhibitor (*SMAD6*) and one target gene (*ID2*) were analyzed by qRT-PCR according to the procedures described above.

Experiment 3: Effect of early embryo-oviduct interaction in vitro on mRNA expression of BMP signaling components in preimplantation embryos

The objective of the third experiment was to evaluate if the *in vitro* interaction with the oviductal cells induces changes in the expression levels of BMP signaling components in the embryos at specific developmental stages. For this purpose, gene expression analysis by qRT-PCR was performed in three independent groups of 10 embryos per stage (8-cell, 16-cell and blastocyst stage) obtained from each experimental group that was cultured with or without BOEC, before or during EGA (corresponding to groups 1 (G1 BOEC/BSA/FCS) and groups 2 (G2 BOEC/BSA/FCS) respectively) in Experiment 1.

After the co-culture period, at 54 hpi (G1 BOEC/FCS/BSA) or 98 hpi (G2 BOEC/FCS/BSA), embryos that reached the 8- or 16-cell stage respectively were removed, quickly washed in PBS; and 10 embryos per stage and experimental group were immediately snap-frozen in liquid nitrogen and kept at -80°C until RNA extraction. The remaining embryos were transferred to 25 μL droplets of SOF+BSA under mineral oil and cultured until Day 9 pi. At Day 7–8 pi, pools of 10 expanding blastocysts per treatment were collected, snap-frozen and stored at -80°C until use for gene expression analysis.

The mRNA expression levels of two BMP ligands (*BMP5/7*), three BMP receptors (*BMPRI1A/1B/2*), two signaling proteins (*SMAD1/5*), one inhibitor (*SMAD6*) and one target gene (*ID2*) were analyzed by qRT-PCR according to the procedures described above.

Statistical analysis

Data were analyzed using SigmaStat 3.5 and SigmaPlot 10.0 statistical software (Systat Software, Richmond, CA, USA). Cleavage rate, blastocyst yield and relative mRNA abundance levels were analyzed using one-way analysis of variance (ANOVA), followed by Holm–Sidak *post hoc* test, when applicable, to determine statistical differences between the experimental groups. Probability values less than 0.05 ($P \leq 0.05$) were considered statistically significant.

Results

Experiment 1: Effect of BOEC co-culture on early embryo development in vitro

For all the experimental groups, only embryos that reached the 2-cell stage at 33 hpi were selected for the study. As shown in Fig. 3A cleavage rates at 33 hpi were similar for G1 and G2 (G1: $66.8 \pm 2.0\%$ vs G2: $67.6 \pm 1.2\%$; $P > 0.05$). In general, most of the embryos reached the 2-cell stage at 33 hpi (G1: $53.9 \pm 3.8\%$ and G2: $58.8 \pm 3.5\%$) and a low percentage of embryos were at a more advanced stage (G1: $12.8 \pm 3.5\%$ and G2: $8.8 \pm 2.7\%$).

At 54 hpi in the G1 BOEC group, a lower proportion of embryos reached the 8-cell stage than control groups (G1 BOEC: $57.1 \pm 4.9\%$ vs G1 BSA: $78.6 \pm 4.0\%$ and G1 FCS: $79.0 \pm 7.4\%$ $P < 0.05$) (Fig. 3B). As a consequence, a significantly higher proportion of embryos with a delayed development (<8 cells) was observed in G1 BOEC than control groups (G1 BOEC: $42.9 \pm 4.9\%$ vs G1 BSA: $21.4 \pm 4.0\%$ and G1 FCS: $21.0 \pm 7.4\%$, $P < 0.05$) (Fig. 3B).

Similarly, at 98 hpi, the G2 BOEC group showed a lower but not significant proportion of embryos reaching the 16-cell stage than control groups (G2 BOEC: $52.4 \pm 8.4\%$, G2 BSA: $66.7 \pm 5.9\%$ and G2 FCS: $65.2 \pm 6.8\%$) (Fig. 3C). In this case, a significantly higher proportion of embryos with a delayed development (<16 cells) was observed in the G2 BOEC group in comparison with the control groups (G2 BOEC: $47.6 \pm 8.4\%$ vs G2 BSA: $33.3 \pm 5.9\%$ and G2 FCS: $34.8 \pm 6.8\%$, $P < 0.05$) (Fig. 3C).

Despite this initial delay observed in the developmental kinetics of the embryos, no significant differences were observed in the blastocyst rate between the control groups (G1 BSA/FCS and G2 BSA/FCS) and the groups that were co-cultured with BOEC before or during EGA (G1 BOEC or G2 BOEC) (Fig. 3D and E).

Figure 3 Developmental rates of *in vitro*-produced bovine embryos cultured before or during embryonic genome activation with or without BOEC. (A) Cleavage rate at 33 h post-insemination (hpi) and percentages of IVF embryos that reached the 2-cell stage (2c) or more (>2c), from G1 and G2 experimental groups. (B) Developmental rate at 54 hpi for experimental groups 1 (G1 BSA/FCS/BOEC). (C) Developmental rate at 98 hpi for experimental groups 2 (G2 BSA/FCS/BOEC). (D) Blastocyst rate at Days 7–9 pi (*in vitro* fertilization = Day 0) derived from experimental groups 1 (G1 BSA/FCS/BOEC). (E) Blastocyst rate at Days 7–9 pi derived from experimental groups 2 (G2 BSA/FCS/BOEC). Results are expressed as mean $\pm$ S.E.M. Significant differences ($P < 0.05$) are indicated with different letters. G1: embryos cultured from 2-cell stage to 8-cell stage in medium SOF+BSA (G1 BSA), SOF+FCS (G1 FCS) or co-cultured with a BOEC monolayer (G1 BOEC). G2: embryos cultured from 8-cell stage to 16-cell stage in medium SOF+BSA (G2 BSA), SOF+FCS (G2 FCS) or co-cultured with a BOEC monolayer (G2 BOEC).

Experiment 2: Effect of early embryo-oviduct interaction *in vitro* on mRNA expression of BMP signaling components in oviductal epithelial cells

Gene expression analysis in BOEC showed that the interaction with the embryos, before and during the main phase of EGA, produced a decrease in the mRNA expression levels of *BMP7*, *BMPR1B*, *BMPR2*, *SMAD1*, *SMAD6* and *ID2* compared with the significant higher levels detected in those transcripts when BOEC were cultured without embryos (Fig. 4). The embryonic presence produced a similar transcriptional response in the oviductal cells, irrespective of the embryonic stage.

On the other hand, expression levels of *BMPR1A* and *SMAD5* mRNA tended to be similar to those levels observed for *BMP7* mRNA. However, the relative abundance of *BMPR1A* and *SMAD5* transcripts did not show significant differences between BOEC cultured with or without embryos (Fig. 4). In the particular case

of *BMP5*, the expression of its mRNA was not detected in the oviductal cells cultured *in vitro*. In this case, to confirm that *BMP5* primers work efficiently, samples of bovine oviduct epithelial cells freshly obtained from the isthmus region (BOEC *in vivo*) were used as positive control (data not shown).

Experiment 3: Effect of early embryo-oviduct interaction *in vitro* on mRNA expression of BMP signaling components in preimplantation embryos

Gene expression analysis in early stage embryos revealed a different transcriptional response to the interaction with BOEC, depending on the embryonic stage in which they interacted. At the 8-cell stage, embryos from G1 BOEC group showed a significant increase in the relative abundance of *SMAD1* mRNA, compared to the levels detected in embryos derived from control groups (G1 BSA and G1 FCS) (Fig. 5A). Moreover, at the 16-cell stage, embryos from G2 BOEC group showed a significant increase in the relative abundance of *BMPR1B*, *BMPR2* and *ID2* mRNA compared to controls (G2 BSA and G2 FCS) (Fig. 5B). Despite these transcriptional changes detected in early stage embryos, in general, no differences were observed in the mRNA expression levels of BMP signaling components between the blastocysts obtained from the different experimental groups cultured in the presence or absence of BOEC (Fig. 6).

Discussion

The *in vitro* culture of oviductal cells has provided a suitable model for the study of the mechanisms involved in the interaction of gametes and embryos with the mammalian oviduct. Recent advances in *in vitro* modeling of the oviductal epithelium of different species, including bovine, porcine and human, have enabled the development of new culture techniques that better mimic the morphological and physiological characteristics of the oviduct (Gualtieri *et al.* 2012, Chen *et al.* 2013, Lawrenson *et al.* 2013, Simintiras *et al.* 2017). However, considering the increasing complexity of studies aimed at understanding the regulatory mechanisms that control embryo-maternal communication at a molecular level, it is necessary to establish novel strategies for *in vitro* culture that allow a more controlled interaction, in terms of area and contact time, between the embryo and the oviductal cells.

The main objective of the present study was to evaluate if the interaction of the embryo with the oviduct can induce changes in the gene expression of BMP signaling components. Considering the limitation of carrying out this study *in vivo*, an *in vitro* co-culture system was established to study the interaction between isthmus epithelial cells and *in vitro*-produced bovine embryos at developmental stages during which, under

Figure 4 Relative mRNA abundance of BMP signaling-related genes in BOEC cultured in the presence or absence of early embryos, before or during embryonic genome activation. BOEC G1+: bovine oviductal epithelial cells cultured with embryos from 2-cell to 8-cell stage (33–54 hpi). BOEC G1–: bovine oviductal epithelial cells cultured in the absence of embryos from 33 hpi to 54 hpi. BOEC G2+: bovine oviductal epithelial cells cultured with embryos from 8-cell to 16-cell stages (54–98 hpi). BOEC G2–: bovine oviductal epithelial cells cultured in the absence of embryos from 54 hpi to 98 hpi. Bars represent the relative abundance of the transcripts analyzed and normalized to *H2AFZ* and *ACTB* as housekeeping genes. The experimental groups are represented by columns. Results are expressed as means $\pm$ S.E.M. Different superscript letters indicate significant differences ($P < 0.05$) between treatments. Data were obtained from five replicates of independent BOEC samples per experimental group.

physiological conditions, the embryo makes contact with the oviduct. To provide a local interaction, the area of contact between the embryo and the monolayer of oviductal cells was controlled and delimited with a polyester mesh. Moreover, a temporal interaction was established by analyzing two periods of embryonic development: before (from the 2-cell to 8-cell stage) and during (from the 8-cell to 16-cell stage) the main phase of embryonic genome activation (EGA). These two time windows were established taking into account

that bovine embryonic genome is gradually activated, starting with minor activation, presumably initiated at the 2-cell stage and followed by a major activation at the 8- to 16-cell stage (Gad et al. 2012, Graf et al. 2014).

Embryos derived from the different experimental groups that were co-cultured with BOEC showed an early delay in developmental kinetics compared to embryos cultured without oviductal cells. However, this initial delay had no impact on the final production rate of blastocysts. A negative effect of the polyester mesh

Figure 5 Relative mRNA abundance of BMP signaling-related genes in *in vitro*-produced bovine embryos cultured in the presence or absence of BOEC, before or during embryonic genome activation. (A) Bars represent the relative abundance of BMP signaling transcripts measured in 8-cell stage embryos obtained from experimental groups that were cultured from 2-cell stage to 8-cell stage in medium SOF + BSA (G1 BSA), SOF + FCS (G1 FCS) or co-cultured with a BOEC monolayer (G1 BOEC). (B) Bars represent the relative abundance of BMP signaling transcripts measured in 16-cell stage embryos obtained from experimental groups that were cultured from 8-cell stage to 16-cell stage in medium SOF + BSA (G2 BSA), SOF + FCS (G2 FCS) or co-cultured with a BOEC monolayer (G2 BOEC). The relative abundance of the transcripts was normalized to *H2AFZ* and *ACTB* as housekeeping genes. The control groups (G1/G2 BSA and G1/G2 FCS) are represented by black and white columns, while embryos cultured with oviductal cells are represented by grey columns. Results are expressed as mean $\pm$ S.E.M. Different superscript letters indicate significant differences ($P < 0.05$) between experimental groups. Data were obtained from three replicates of independent groups of 10 embryos per stage and experimental group.

Figure 6 Relative mRNA abundance of BMP signaling-related genes in bovine blastocysts obtained from embryos co-cultured *in vitro* before or during embryonic genome activation in the presence or absence of BOEC. (A) Bars represent the relative abundance of BMP signaling transcripts measured in blastocysts derived from embryos cultured from the 2-cell stage to the 8-cell stage in the absence (G1 BSA and G1 FCS) or presence of a BOEC monolayer (G1 BOEC). (B) Bars represent the relative abundance of BMP signaling transcripts measured in blastocysts derived from embryos cultured from the 8-cell stage to the 16-cell stage in the absence (G2 BSA and G2 FCS) or presence of a BOEC monolayer (G2 BOEC). The relative abundance of the transcripts was normalized to *H2AFZ* and *ACTB* as housekeeping genes. The control groups (G1/G2 BSA and G1/G2 FCS) are represented by black and white columns, while groups cultured with oviductal cells are represented by grey columns. Results are expressed as mean $\pm$ S.E.M. Different superscripts letters indicate significant differences ($P < 0.05$) between groups. Data were obtained from three replicates of independent groups of 10 blastocysts per experimental group.

was ruled out as control embryos cultured without BOEC showed normal development in the presence of the mesh. In concordance with this fact, Matoba and coworkers (Matoba *et al.* 2010) showed that the same type of mesh has no effect on embryonic development *in vitro*.

Other studies reported that the *in vitro* co-culture of BOEC with bovine embryos during the first 4 days (Rief *et al.* 2002, Cordova *et al.* 2014) or the entire 8 days of embryo development (Schmaltz-Panneau *et al.* 2014, 2015) can improve the early cleavage rate and/or the development to the blastocyst stage. Undoubtedly, the time of co-culture and the culture conditions are variables that have to be considered before evaluating and comparing these results. In particular, in the present study, the time of co-culture evaluated was shorter than that used in those studies. This allows to infer that an extended co-culture time between the embryo and the oviductal cells seems to be necessary to improve embryo development rate. In fact, other authors have shown that the embryotrophic effect of human oviductal cells cultured with mouse embryos is enhanced when the time of co-culture is longer and it lasts for at least four days (Xu *et al.* 2001).

On the other hand, gene expression analysis showed that the interaction with the embryos, before and during EGA, induced a significant decrease in the transcriptional levels of BMP signaling components in oviductal cells cultured *in vitro*. In particular, this reduction was observed in the mRNA levels of one

ligand (*BMP7*), two receptors (*BMPR1B* and *BMPR2*), one signaling mediator (*SMAD1*) and two BMP target genes (*SMAD6* and *ID2*). As can be seen, the effect is observed in all the genes encoding factors required for the signal transduction, suggesting a possible decrease in the activity of BMP pathway in the oviduct when the embryo is present.

It has been reported that *BMP7* has a critical role in the oviduct, by acting as an inducer of apoptosis during tissue remodeling (Monroe *et al.* 2000). In fact, estrogen exerts a protective role by reducing the expression of this factor in the oviductal epithelium (Monroe *et al.* 2002, Kusumegi *et al.* 2004). Considering this, it would be interesting to explore if the downregulation of *BMP7* expression, induced by the presence of the embryo, could be associated with a protective effect in order to maintain the integrity of the oviduct as the embryo passes.

In reference to BMP receptors, previous studies demonstrated the expression of *BMPR1B* and *BMPR2* in the bovine embryo, showing high abundance of their transcripts at the early embryonic stages (from 2-cell stage to 8-cell stage) (Garcia *et al.* 2015). This temporal gene expression pattern of the receptors suggests that the embryo could respond to BMP ligands during the first developmental stages during transit through the oviduct. Based on the present study, the mRNA expression of the same receptors was detected in the oviductal cells and the presence of the embryo downregulated their expression. In this sense, the embryo could be sending

specific signals to reduce the expression levels of these receptors in the oviduct to avoid competence. If so, BMP ligands present in the oviductal microenvironment could act preferably on the embryo. However, further studies are required to confirm this possibility.

Downregulation of mRNA expression of *SMAD1* and the target genes *ID2* and *SMAD6* is also indicative of a suppressed BMP pathway as a response of the oviduct to the embryo. This coincides with a previous study performed *in vivo* in which the transfer of 50 embryos to the bovine oviduct produced changes in the gene expression pattern of the oviductal cells; *SMAD6* was among the genes downregulated by the embryo presence (Maillo *et al.* 2015). This gene is induced via *SMAD1/5* pathway, and at protein level, it acts as an inhibitor self-regulated by the BMP signaling pathway (Ishida *et al.* 2000, Li 2015). A recent study in mice demonstrated that *SMAD* signaling is required for structural integrity of the female reproductive tract and uterine function during early pregnancy (Rodriguez *et al.* 2016). However, until now, little is known about its role in the oviduct, in particular during the early stages of embryo development.

Similarly to other studies (Cordova *et al.* 2014, Schmaltz-Panneau *et al.* 2014, Maillo *et al.* 2015) these results confirm that bovine embryos can have a direct effect on the transcriptome of the oviductal cells. However, it is important to note that this study, in contrast to others, demonstrates that a short period of interaction with the early embryo is sufficient to induce specific transcriptional changes in the oviductal cells. Furthermore, a similar transcriptional response was observed irrespective of the embryonic stage, suggesting that oviductal cells do not show a temporal sensitivity to the different stages of embryo development, at least in the conditions assayed and for the genes analyzed.

On the other hand, gene expression analysis of the embryos revealed significant changes in the expression profile of BMP genes depending on whether they interact with oviductal cells before or during EGA. Embryos that interacted with BOEC before the main phase of EGA (G1 BOEC) showed higher levels of transcripts associated with BMP signaling. Particularly, a significant increase in the relative abundance of *SMAD1* mRNA was observed at the 8-cell stage, compared to control groups cultured without BOEC. *SMAD1* is a key component of the BMP signaling pathway that allows the transduction of the signal from membrane receptors to the nucleus (Bragdon *et al.* 2011). Depending on cell response, *SMAD1* or *SMAD5* can be activated (Li 2015). Thus, the significant increase in the mRNA levels for *SMAD1* in the embryos suggests that the interaction with oviductal cells may induce the activation of BMP signaling in the embryos through the *SMAD1* pathway. In mouse embryos, *Smad1* coordinates the growth of extra-embryonic structures necessary to support embryo development within the uterine environment. As a

consequence, the failure of *Smad1* signal induces defects in extra-embryonic tissues and knockout embryos die due to problems in placentation (Tremblay *et al.* 2001, Arnold *et al.* 2006, Li 2015). Transcriptional analysis of mouse and bovine embryos reveals high abundance of *SMAD1* mRNA at early stages before the main phase of EGA and a significant reduction after EGA up to the blastocyst stage (Wang *et al.* 2004, Lee *et al.* 2014) suggesting that this factor is necessary during the early stages in which the embryo transits through the oviduct. In this sense, our results suggest that early embryo-oviduct interaction could have a critical role in maintaining high transcriptional levels of this factor before EGA, during the early stages in which the embryo interacts with the oviduct.

In the case of the embryos that interacted with BOEC during EGA (G2 BOEC), a significant increase in the relative abundance of *BMPR1B*, *BMPR2* and *ID2* mRNA was detected at the 16-cell stage. This suggests that contact with the oviductal cells might induce the activation of the BMP signaling pathway in the embryo during the early stages of development. However, the precise mechanisms that control this upregulation are still unknown. It is well known that both BMP receptors, *BMPR1B* and *BMPR2*, play a critical role in preimplantation development and female fertility (Soyun *et al.* 2001, Nagashima *et al.* 2013, Graham *et al.* 2014, de Mochel *et al.* 2015). In addition, their transcript levels in the embryo are specifically elevated at early stages before EGA (Lee *et al.* 2014, Garcia *et al.* 2015). In the case of *ID2*, it is a target gene of BMP signaling and it acts as a transcription factor required for maintenance and differentiation of trophoblast cells (Janatpour *et al.* 2000, Roberts *et al.* 2004, Schiffmacher & Keefer 2013). In agreement with our results, transcriptome analysis by microarray in *in vivo*-produced bovine embryos reported that *ID2* increases its transcription during the activation of the bovine embryonic genome (Kues *et al.* 2008). The fact that the transcriptional levels of BMP receptors and the *ID2* gene were higher during the early stages and stimulated by the interaction with the oviduct allows us to infer that BMP signaling pathway could be mediating an active cross talk between the embryo and the mother during the early stages of development.

Coincident with other studies (Wang *et al.* 2004, Zhang *et al.* 2007, Kues *et al.* 2008, Lee *et al.* 2014), our results show that during early embryogenesis, the transcript abundance of BMP signaling components is differentially regulated and that early maternal-embryo interaction can have a critical impact on the transcriptional regulation of this signaling. Moreover, the low levels detected at the blastocyst stage and the fact that at this stage there were no differences in the mRNA expression levels of BMP signaling components supports the hypothesis that BMP signaling is more important during the early stages of development. In fact, the inhibition of this signaling at the beginning

of embryo culture produces a significant reduction in the cleavage and developmental rates of mouse (de Mochel *et al.* 2015) and bovine embryos (La Rosa *et al.* 2011), suggesting that a correct balance of BMP signaling, during early stages, is needed for a proper preimplantation development. Thus, considering our results, the interaction with the oviduct could play a critical role to regulate this balance.

In conclusion, this study provides a new strategy for the study of the maternal-embryonic dialog and gives evidence about possible signaling pathways involved in the communication between the embryo and the bovine oviduct during the preimplantation period. The data presented demonstrate that local embryo-oviduct interaction *in vitro* induces changes in the transcriptional levels of BMP signaling, causing a bidirectional response that reduces the expression levels of this signaling in the oviductal cells while increasing them in the embryo at the early stages. These findings suggest that BMP signaling pathway could be involved in an early cross talk between the bovine embryo and the oviduct during first stages of development. Increased understanding of this intrinsic signaling for maternal-embryo communication is important for future improvements in ART and further understanding of the signaling pathways that potentially contribute to infertility in cattle and humans.

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.

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