Animal Reproduction Science 185 (2017) 89–96

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Expression of bone morphogenetic protein receptors in bovine oviductal epithelial cells: Evidence of autocrine BMP signaling

Pablo Alberto Valdecantos[a,*], Rocío del Carmen Bravo Miana[a], Elina Vanesa García[a,b], Daniela Celeste García[a,b], Mariela Roldán-Olarte[a,b], Dora Cristina Miceli[a,b]

[a] Instituto de Biología "Dr. Francisco D. Barbieri", Facultad de Bioquímica, Química y Farmacia, UNT. Chacabuco 461, T4000ILI, San Miguel de Tucumán, Argentina

[b] Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT. Chacabuco 461, T4000ILI, San Miguel de Tucumán, Argentina

ARTICLE INFO

Keywords:
BMP
TGF-β
Oviduct
Bovine oviduct epithelial cells

ABSTRACT

Members of the transforming growth factor beta (TGF-β) family, including bone morphogenetic proteins (BMPs), are expressed in the epithelial cells of the mammalian oviduct. These signaling molecules play important roles in development and tissue homeostasis; however, little is known about their function in the mammalian oviduct. In the present study, RT-qPCR was used to analyze the mRNA abundance of BMP type I (BMPR1A, BMPR1B, ACVR1) and type II receptors (BMPR2, ACVR2A, ACVR2B) in the bovine oviduct epithelial cells (BOEC) isolated from ampulla and isthmus at both the follicular (FP) and the luteal (LP) phase of the estrous cycle. Results indicate that mRNAs for all the BMP receptors studied are expressed in the BOEC. Significant mRNA abundance differences were observed for both BMPR1B and ACVR2B when comparing both the ampulla and isthmus regions with the greater abundance at the isthmus. When both FP and LP samples were compared, ACVR2B mRNA showed greater abundance during the LP, with significant differences in the isthmus region. These variations highlight differences between the isthmus and ampulla regions of the oviduct. By means of wound healing assays on BOEC primary cultures, exogenous recombinant human BMP5 induced a significant increase in wound healing at 24 h. The observed changes at the mRNA abundance of components of the signaling pathway and the BMP5 effect on oviductal epithelial cells suggest a possible autocrine role for the BMP pathway that could affect epithelial cell functions necessary for normal physiology and reproductive success in BOEC homeostasis.

1. Introduction

Bone Morphogenetic Proteins (BMPs) are extracellular multifunctional proteins that belong to the Transforming Growth Factor beta (TGF-β) superfamily (Bragdon et al., 2011). Like other TGF-βs, BMPs regulate various processes of a large variety of cell types in embryogenesis, development and adult tissue homeostasis (Wang et al., 2014). Bone Morphogenetic Proteins mediate their cellular effects by binding to type I (ACVRL1, ACVR1, BMPR1A, and BMPR1B) and type II (BMPR2, ACVR2A, and ACVR2B) serine/threonine kinase receptors, forming heterotetrameric complexes of two dimers of type I and type II receptors (Miyazono et al., 2005). Upon receptors activation, a signal transduction cascade is initiated by phosphorylation of downstream BMP-Smad effector proteins; non-

* Corresponding author.
E-mail addresses: pvaldecantos@fbqf.unt.edu.ar, valdecantospablo@gmail.com (P.A. Valdecantos), rocibm@hotmail.com (R.d.C. Bravo Miana), evgarcia@fbqf.unt.edu.ar (E.V. García), dani.c.garcia@gmail.com (D.C. García), emroldanolarte@fbqf.unt.edu.ar (M. Roldán-Olarte), micelidora75@gmail.com (D.C. Miceli).

http://dx.doi.org/10.1016/j.anireprosci.2017.08.006
Received 13 February 2017; Received in revised form 20 July 2017; Accepted 9 August 2017
Available online 14 August 2017
0378-4320/

Smad specific signal transduction pathways such as MAPK/PI3K/Akt can also be activated by BMPs (Wang et al., 2014).

A number of studies have reported the importance of BMPs as regulators of mammalian reproduction and fertility events (Shimasaki et al., 2004). Bone Morphogenetic Proteins 2, 4, and 7 mRNAs were detected in mouse oviduct (Tanwar and McFarlane, 2011); Bone Morphogenetic Proteins 2, 3, 4, 7, 10, and 15 mRNAs were found to be expressed in bovine oviduct epithelial cells (BOEC) throughout the estrous cycle at both ampulla and isthmus, and BMP5 mRNA is specifically expressed in isthmus BOEC (E.V. García et al., 2014). Large-scale transcriptional analyses in bovine, porcine and human oviducts also contributed to show the presence of BMPs and BMP signaling transcripts (Almiñana et al., 2014; Bauersachs et al., 2004; Hess et al., 2013; Mondéjar et al., 2012). These reports suggest physiological roles for BMPs in the mammalian oviduct that have not been extensively explored.

As BMP pathways are active in oocytes and embryos, studies showing the effects of BMPs on *in vitro* embryo development suggest paracrine functions in the regulation of embryo development for oviduct-secreted BMPs (García et al., 2015; La Rosa et al., 2011). On the other hand, transgenic and knockout mice generated to study TGF-β members display reproductive pathologies, highlighting the importance of TGF-β in maintaining reproductive homeostasis (Li et al., 2011; Pangas et al., 2008; Rodriguez et al., 2016; Tian et al., 2010); therefore, oviduct-produced BMPs might act as both paracrine and autocrine factors in regulating oviduct reproductive events.

The mammalian oviduct plays an important role in gamete transport, fertilization and early embryo development (Ghersevich et al., 2015; Maillo et al., 2016). The extent to which TGF-β/BMP signaling in the mammalian oviduct contributes to fertility and organ homeostasis needs to be explored thoroughly. The aim of the present work was to evaluate if BMPs produced by oviductal epithelial cells can act as autocrine factors that could affect cellular functions necessary for normal physiology and reproductive success.

2. Materials and methods

2.1. Isolation of bovine oviductal epithelial cells

Genital tracts from young beef cows (*Bos taurus*) were collected at a commercial abattoir and transported to the laboratory for processing within 3 h of slaughter. Only samples from non-pregnant animals with no anatomical abnormalities or defects in their reproductive tracts were included in the study. The stage of the estrous cycle was identified according to previous reports based on the direct examination of ovarian morphology and the presence and viscosity of the mucus in the uterine horns (D.C. Garcia et al., 2014; Ulbrich et al., 2004). Once classified, ipsilateral oviducts to the active ovary (corpus luteum/dominant follicle) were separated. Six oviducts from animals at either the follicular (FP) or the luteal phase (LP) were used to study the mRNA abundance of BMP receptors, ID2, and SMAD6 (n = 3 oviducts per stage). The oviducts were dissected free from surrounding tissues and washed with PBS containing 100 IU/mL of penicillin, 100 mg/mL of streptomycin and 0.25 μg/mL of fungizone (Gibco, Life Technology, Burlington, ON, Canada). The ampulla and isthmus regions were isolated from each oviduct and the BOEC were mechanically dislodged by gently squeezing each region separately with forceps, as described elsewhere (D.C. García et al., 2014; Rottmayer et al., 2006). Then, the collected sheets of BOEC from each region of the oviducts were separated from the rest of the contaminating cells by three rounds of simple sedimentation in 15 mL conical tubes with PBS. Epithelial sheets were obtained and controlled under microscope observation to ensure their being free of cells from the oviductal stroma. Cell viability testing was performed with Trypan blue (Sigma) staining. BOEC were pelleted and immediately used for total RNA isolation or primary cultures.

2.2. Bovine oviductal epithelial cell culture

For primary BOEC cultures, istmus portions of ipsilateral oviducts to the ovary with a dominant follicle were used. Epithelial sheets, obtained as described above, from four different istmus were pooled and cultured for each *in vitro* BMP5 assay (ID2 and SMAD6 mRNAs abundance, and wound healing assay) that consisted of four biological replicates performed in duplicated. The collected sheets of BOEC were transferred to Tissue Culture Medium 199 with Earle's salts (TCM-199) (Gibco, Life Technology, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) (Internegocios, Buenos Aires, Argentina), 25 mM Hepes (Gibco, Life Technology, Burlington, ON, Canada), 0.2 mM sodium pyruvate, 100 IU/mL of penicillin, 100 μg/mL of streptomycin and 0.25 μg/mL of fungizone (Gibco, Life Technology, Burlington, ON, Canada). The cell suspension was pipetted 5 times through a 21-gauge syringe needle; then, three steps of washing were performed, each followed by 25 min sedimentation in culture medium in the cell culture incubator. Cell viability at seeding was analyzed by Trypan blue (Sigma) staining and microscopic observation of beating cilia. Cells were grown in 25 mm^2 culture flasks (Nunclon, Roskilde, Denmark) in 5 mL TCM-199 supplemented as described earlier (D.C. García et al., 2014). Culture took place at 38.5 °C in an atmosphere of 5% CO_2 in air in 100% humidity. Culture media was replaced every 48 h until the cells reached 70–80% confluency. Isolated epithelial sheets spontaneously form vesicles after 24 h of culture. During media changes vesicles were maintained in culture to allow attachment to the bottom of the plate after they formed a monolayer. After that, BOEC cultures were washed with PBS and trypsinized (0.05% trypsin–EDTA; GIBCO) until single cells appeared. These cells were then plated into 24-well culture plates (Nunclon, Roskilde, Denmark) at a density of 10^4 cells per well and incubated at 38.5 °C in 5% CO_2 in culture medium supplemented with 10% FBS, until cells reached 90% confluency.

For BMP5 assays, the culture medium was replaced by serum-free medium and cultured overnight. BOEC monolayer cultures were treated with 100 ng/mL BMP5 for 24 h. The selected concentration was used previously by other authors (García et al., 2015; Romagnoli et al., 2012). Untreated cells, used as control, were incubated for the same time period as the treated group with an equivalent volume of vehicle, 4 mmol/L HCl in serum-free medium, as described by García et al. (2015). At the specified time, total RNA was isolated from control and treated cells to study the expression pattern of *ID2* and *SMAD6* genes.

2.3. Immunocytochemical analysis

To confirm the epithelial nature of the cultured cells, BOEC were cultured on microscope slides and ethanol fixed for immunocytochemical analysis that was performed with a Ventana BenchMark automated immunostainer (Ventana, Tucson, USA) using the Ventana ultraView Universal Diaminobenzidine (DAB) Detection Kit (Ventana Medical Systems). Ready to use anti-pan Keratin (AE1/AE3/PCK26) primary antibody (Ventana Medical Systems, Inc., 760–2595) and anti-Vimentin (V9) monoclonal primary antibody (Ventana Medical Systems, Inc., 790–2917) were employed. For keratin detection, the protocol involved a 2-min protease digestion-based antigen retrieval; for vimentin detection, antigen retrieval was performed by incubating slides for 8-min at 95 °C; both according to manufacturer's recommendations. Finally, samples were counterstain with hematoxylin II for 32 min followed by Bluing Reagent for 8 min (Ventana). After staining, images were acquired with a Leica DM500 microscope, digital camera (Leica ICC50 HD) and the LAS EZ 3.2.1 Software.

2.4. Total RNA isolation

Total RNA from ampulla and isthmus BOEC corresponding to the follicular (FP) and luteal (LP) phases of the estrous cycle and from BOEC cultures was isolated using TRI-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA), according to the manufactureŕs instructions. The quantity and quality of RNA samples was assessed spectrophotometrically at 260 and 280 nm, and electrophoretically on 1.5% (wt/vol) agarose gels stained with SYBR Safe DNA Gel Stain (Invitrogen, Burlington, ON, Canada). All RNA samples were stored at −80 °C until further use.

2.5. Real-time RT-PCR analysis

The mRNA abundance of BMP type I (ACVR1, BMPR1A, and BMPR1B) and type II (BMPR2, ACVR2A, and ACVR2B) serine/threonine kinase receptors, and ID2 and SMAD6 in each RNA sample of follicular and luteal phase bovine oviduct epithelial cells of the estrous cycle were determined by quantitative Real-time RT-PCR (RT-qPCR) in a CFX96 Real-Time PCR detection System (Bio-Rad, Hercules, CA, USA). The mRNA abundance of ID2 and SMAD6 of BOEC cultures were also analyzed. Specific primers (Table 1) were previously validated for adequate primer efficiency, and specificity of their PCR products was confirmed by agarose gel electrophoresis and sequencing. Briefly, cDNA was synthesized using Moloney murine leukemia virus (M-MLV) enzyme (Promega, Madison, WI, USA), oligo(dT)17 and random primers (D.C. García et al., 2014), with 1.0 μg of total RNA from each sample. A 25 μL final volume of the reaction mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM of each dNTP, 25 pM oligo(dT) and random primers, 10 mM dithiothreitol and 200 units of reverse transcriptase was incubated at 42 °C for 1 h followed by a reverse transcriptase inactivation at 94 °C for 5 min. Real-time RT-qPCR was performed with a total volume of 20 μL per reaction, with each reaction containing 5 μL of cDNA (diluted 1:10 as determined by a standard curve), 5 μL of a 2 μM of primer pairs stock solution (forward and reverse), and 10 μL of 2x Fast EvaGreen qPCR Master Mix (Biotium, Hayward, CA, USA). The PCR program consisted of an initial step of 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C for annealing and extension. At the end of each qPCR amplification, melt curve analysis was performed for all genes; typical dissociation curves confirmed the specificity of each primer set and the absence of primer dimers. As negative controls, reactions containing no template and no-reverse transcribed RNA were included to verify that the PCR products obtained were not derived from contamination or genomic DN. Cq value was noted for each transcript and normalized to GAPDH. The relative quantitation of each mRNA was performed using the $2^{-\Delta\Delta Cq}$ comparative method (Schmittgen and Livak, 2008). Cq values for GAPDH were stable in all samples and with no significant

Table 1
Sequences and accession numbers of the primers used for RT-qPCR.

Gene name	Genbank accession number		Primers (5′ − 3′)	Fragment size (bp)
ACVR1	NM_176663.3	Forward	CAACGTTGGAGACAGCACTTTAGCA	140
		Reverse	CATACCTGCCCTTCCCAACACACT	
BMPR1A	NM_001076800.1	Forward	GATTGCCCTTACTGGTTCAGCGA	102
		Reverse	CCACGCCATTTACCCATCCACA	
BMPR1B	NM_001105328.1	Forward	GGCGTGGCGAAAAGGTAGCT	141
		Reverse	CCGTCCCTTTGATATCTGCAGCAA	
BMPR2	NM_001304285.1	Forward	CCACCTCCAGACACAACACCAC	144
		Reverse	GCGGTCTCCTGTCAGCATCCTA	
ACVR2A	NM_174227.3	Forward	CCACAAACCCGCCATATCTCACA	111
		Reverse	CCAGCCTCAAACTTTAACGCCAA	
ACVR2B	NM_174495.2	Forward	AGCCATCTATTGCCCACAGGGA	99
		Reverse	CAAACCGAACAGCCAGGCCAAA	
ID2	NM_001034231.2	Forward	CCTCAACACCGACATCAGCA	105
		Reverse	TGAACACCATTTATTCAGCCACAG	
SMAD6	NM_001206145.1	Forward	GCCACTGGATCTATCTGATTCCACA	101
		Reverse	GAGACATGCTGGCGTCTGAGAA	
GAPDH	NM_001034034	Forward	AGATGGTGAAGGTCGGAGTG	117
		Reverse	GAAGGTCAATGAAGGGGTCA	

differences between treatments.

2.6. In vitro wound healing assay

For each experiment (n = 4), primary BOEC from isthmus were cultured in duplicate in 12-well plates until 80–90% confluence; then they were cultured without serum overnight. On the following day, a scratch was made approximately in the center of each monolayer using a sterile 200 μL pipette tip. The medium and cell debris were aspirated away and replaced with fresh serum-free TCM-199 medium supplemented with or without recombinant human BMP5 (615-BMC-020/CF; R & D Systems, Inc., Minneapolis, MN, USA) at 100 ng/mL. Images of three different areas of the scratches were photographed at 0 h, 12 h, and 24 h using an inverted light microscopy (Zeiss Axiovert 25). In order to obtain the same field of interest during the observations, etches were made on the bottom of each well and used as microscopic reference points to make sure that measurements were always carried out at the same spot (Jordaens et al., 2015). Measures of each scratch area were obtained with the Image J software at every time point. The wound area at 0 h was assigned as 100% and used to calculate percent wound closure at 12 h and 24 h.

2.7. Nuclear Hoëchst staining of BOEC cultures

Monolayer cells treated for 24 h with BMP5 and controls (n = 4) were fixed in 4% paraformaldehyde for 30 min at room temperature, washed five times with PBS and then stained for 15 min in the dark with a Hoëchst 33342 solution (1 μg/mL in PBS). The cells were washed three times with PBS, mounted in 5 μL of a mounting medium and covered with a coverslip. Samples were examined immediately at 400X in an epifluorescence microscope (Olympus BX40). Stained culture cells were photographed in three different fields of each well culture plate. Microphotographs were analyzed using the ImageJ software. The total number of cells was determined by counting stained nuclei. Also, mitotic cells were recorded and their percentages were determined for each image analyzed.

2.8. Statistical analysis

Real Time RT-qPCR, wound closure and percentage of mitotic cells data are expressed as mean ± standard error of the mean (SEM). The statistical analysis of the data was performed using the InfoStat Statistical Package (InfoStat 2015, http://www.infostat.com.ar). Model parameters for uncultured BOEC included oviduct sections (ampulla and isthmus) and the different stages of the estrous cycle (follicular and luteal phases); ANOVA analysis with LSD Fischer's post hoc test was used to study the differences among the means. *In vitro* BMP5 assays included two kinds of experiments: a) determination of ID2 and SMAD6 mRNAs abundance at 12 h and 24 h after BMP5 treatment, and b) determination of the wound healing percentage after 12 h and 24 h of BMP5 treatment. In (a), ANOVA analysis with LSD Fischer's test was done to determine significant differences; in (b), the Student's *t* test was used to determine significant differences between control and BMP5-treated groups at 12 h or 24 h. Comparisons of mitotic cell percentages after 24 h of BMP5 treatment were also analyzed by the *t* test. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Bovine oviductal epithelial cells express BMP type I and type II receptors

Previous studies demonstrated that BOEC express various BMP genes through the bovine estrous cycle. In order to determine if the oviductal epithelium expresses the BMP receptors necessary for signaling, the mRNA abundance of three BMP type I (ACVR1, BMPR1A, BMPR1B) and the three type II (BMPR2, ACVR2A, ACVR2B) serine/threonine kinase receptors was determined by RT-qPCR. Total RNA isolated from the bovine oviduct epithelial cells obtained from the ampulla and isthmus oviducts of animals at either the follicular (FP) or luteal (LP) phases were used. Fig. 1 shows that ACVR1, BMPR1A, BMPR1B, BMPR2, ACVR2A, and ACVR2B mRNAs are present in the BOEC of both ampulla and isthmus throughout the estrous cycle. Significant differences for mRNA abundances were observed for both BMPR1B and ACVR2 B when comparing both the ampulla and isthmus regions with the greater abundance at the isthmus region ($p < 0.05$). Similar behaviors were observed for ACVR1, BMPR1A, and BMPR2 mRNAs, although no significant differences were found. When comparing both FP and LP samples, ACVR2B mRNA showed greater abundance during the LP, with significant differences in the isthmus region ($p < 0.05$) (Fig. 1).

3.2. BMP5 enhances wound healing of BOEC monolayers

In order to test if BMP5 was able to affect the migration capacity of *in vitro* cultured BOEC monolayers, cells from the isthmus were cultured for 24 h in the presence of BMP5 (100 ng/mL) after a scratch was made in the monolayers; the scratched area was measured at 12 and 24 h after the addition of BMP5 and compared with non-treated control groups. Previously, BMP receptors expression was checked in the BOEC cultured cells by conventional RT-PCR to determine if they were also expressed in the *in vitro* conditions (data not shown). Immunocytochemical analyses also confirmed the epithelial nature of the cultured cells, as evidenced by immunostainig for keratin and vimentin (Fig. 2). As shown in Fig. 3A, BMP5 significantly increased the ability of the cells to close the scratched area at 24 h. Hoëchst nuclear staining was used to determine the mitotic index as a measure to verify that treatment did not induce cell proliferation. Total cells per field were counted and the percentages of mitotic cells were determined; low mitotic indexes were

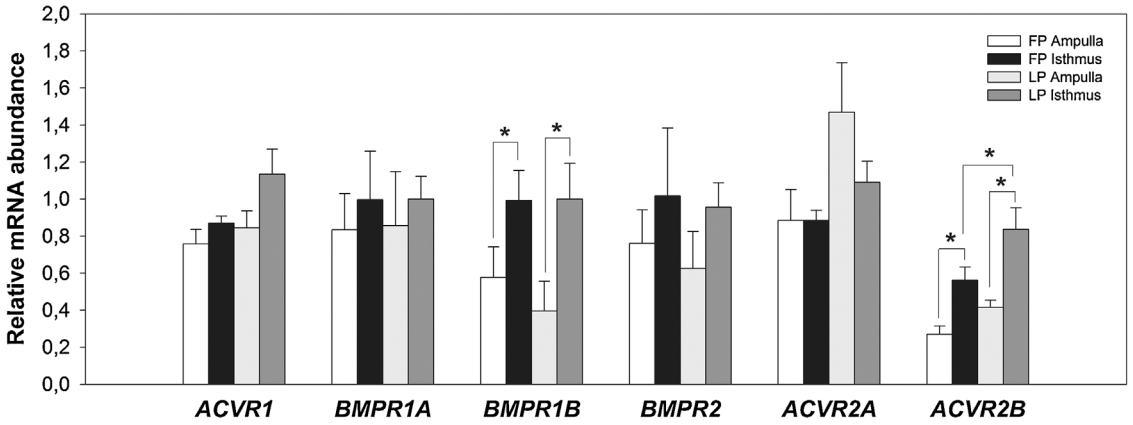

Fig. 1. mRNA abundance of BMP type I (ACVR1, BMPR1A, BMPR1B) and type II (BMPR2, ACVR2A, and ACVR2B) receptors on BOEC at the follicular (FP) and luteal phase (LP) of the estrus cycle. Bars represent the mRNA abundance normalized for GAPDH mRNA. Values are mean ± SEM of BOEC from ipsilateral oviducts of three animals at each stage of the mentioned estrous cycle. * indicates significant differences between samples ($p < 0.05$).

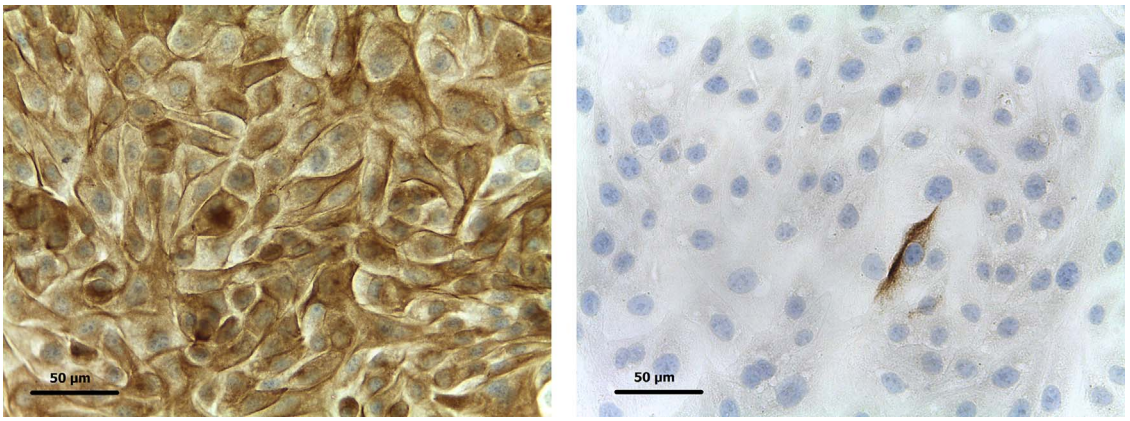

Fig. 2. Immunocytochemistry staining of *in vitro* cultured BOEC for (A) keratin and (B) vimentin using the Ventana ultraView Universal DAB Detection Kit (Ventana Medical Systems). Hematoxylin counterstained images are at 40X magnification. Scale bar = 50 µm.

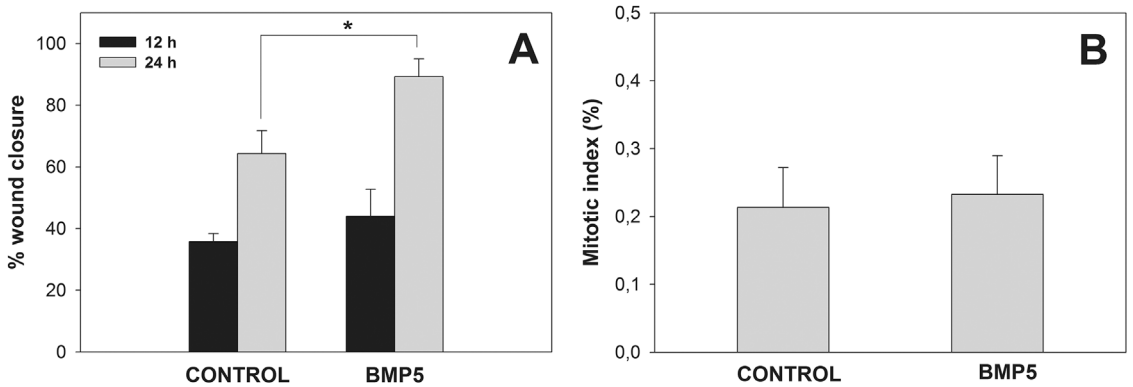

Fig. 3. BMP5 enhances BOEC migration. (A) Wounded BOEC monolayers were allowed to migrate in the presence or absence of rhBMP5 (100 ng/mL) for 24 h, wound closure was measured at 12 h and 24 h in three specific fields from four independent experiments (mean ± SEM; *$p < 0.05$). (B) Percentage of mitotic cells of BOEC primary cultures after 24 h of rhBMP5 treatment determined by nuclear Hoëchst staining. Values are the mean ± SEM (n = 4).

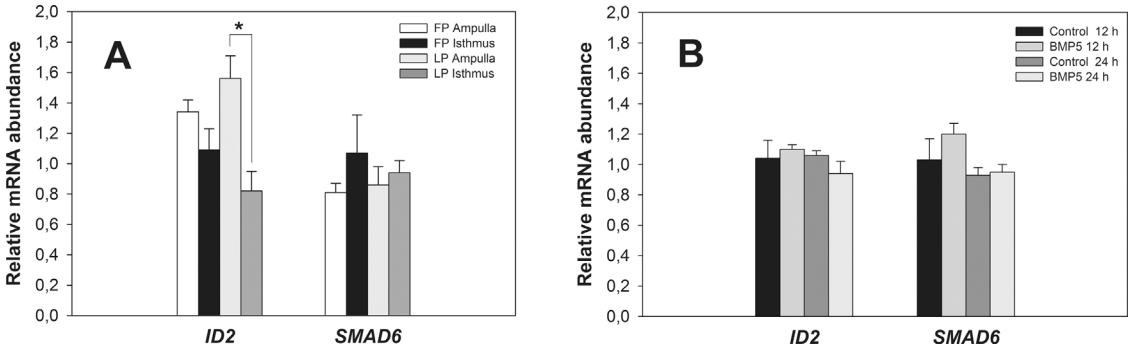

Fig. 4. mRNA abundance of ID2 and SMAD6 (A) on BOEC at the follicular (FP) and luteal phase (LP) of the estrus cycle of BOEC from ipsilateral oviducts of three animals at each mentioned stage; and (B) on primary BOEC cultures at 12 h and 24 h after rhBMP5 treatment (100 ng/mL) from four independent experiments. Bars represent the mRNA abundance normalized for GAPDH mRNA. Values are mean ± SEM. * indicates significant differences between samples ($p < 0.05$).

observed, with no changes between the controls and BMP5-treated cultures (Fig. 3B), indicating that BMP5 induced cell migration instead of cell proliferation.

3.3. ID2 and SMAD6 mRNA expression analysis in bovine epithelial cells

Canonical BMP signaling pathway through the SMAD activates the transcription of a variety of genes including *ID2* and *SMAD6* (Miyazono et al., 2005). As different BMPs and their receptors are expressed in the BOEC, we studied the mRNA abundance of these selected BMP-target genes at the follicular (FP) and luteal (LP) phases. Results showed greater abundance of ID2 mRNA in ampulla BOEC with respect to the isthmus region for both stages, with significant differences between ampulla and isthmus during the luteal phase; no significant differences being observed for SMAD6 mRNA (Fig. 4A). Due to the observed biological effect of BMP5 on cell migration, we decided to determine if the mRNA abundance of the above genes was affected *in vitro* by BMP5. Results showed similar mRNA abundance for both mRNAs in BOEC cultures as those observed in the freshly obtained BOEC. After 24 h of BMP5 treatment, no significant differences in mRNA abundance for either mRNA were observed (Fig. 4B).

4. Discussion

In the present work, we showed that the epithelial cells of the bovine oviduct express type I (ACVR1, BMPR1A, and BMPR1B) and type II (BMPR2, ACVR2A, and ACVR2B) serine/threonine kinase receptors responsible for BMP signaling. We also found that BMP5, which is specifically secreted by the epithelial cells of the isthmus region of the bovine oviduct (E.V. García et al., 2014), has the ability to induce a cell response measured by an *in vitro* wound healing assay. The oviduct is an important reproductive organ that provides an optimal microenvironment necessary for the reproductive events that precede implantation, including gamete transport, sperm capacitation, fertilization, and early embryonic development, in part by the secretion of a large number of protein components (Avilés et al., 2010; Ghersevich et al., 2015; Maillo et al., 2016). García et al. (2014) have clearly shown that several BMP coding genes are expressed by the epithelial cells of the bovine oviduct throughout the estrous cycle.

It is known that BMP signaling is required for early embryo development (Graham et al., 2014; Reyes de Mochel et al., 2015); therefore, a physiological paracrine role of oviduct secreted BMPs in embryo development can be inferred. This hypothesis is supported by the *in vitro* experimental evidence showing that BMP4, BMP5, and Noggin affect the development of *in vitro* bovine cultured embryos (García et al., 2015; La Rosa et al., 2011). Although specific roles for BMPs at the bovine oviduct have not yet been established, it is important to note that BOEC express BMP genes (E.V. García et al., 2014), whose products are secreted into the lumen. These products probably contribute to create an oviductal microenvironment with controlled BMP activities that might be necessary for the signaling required for successful embryo preimplantation development by a direct action on the embryo (paracrine) and on the epithelial lining of the oviduct (autocrine). In fact, there are reports showing that the TGF-β/BMP signaling pathways are essential for reproductive success (Shimasaki et al., 2004). Recently, Rodriguez et al. (2016), using conditional knockout for Smads in Amhr2cre KO females, showed the importance of this signaling pathway in oviductal development, since it affects the expression of multiple genes essential for oviduct and smooth muscle development. Consequently, it seems possible that this group of ligands plays a role in the regulation of the cyclic adaptation of the mammalian oviduct and in the maintenance of epithelial integrity.

BMP signaling is regulated by both extra- and intracellular events, including the availability of BMP receptors, BMP antagonists preventing ligand receptor interaction, proteasome-mediated degradation via Smurf proteins, inhibition via the inhibitory Smads proteins, and protein phosphorylation/dephosphorylation (Wang et al., 2014). In this work, in the BOEC of both ampulla and isthmus, we detected the mRNAs of three type I receptors (ACVR1, BMPR1A, BMPR1B), and the three type II receptors (BMPR2, ACVR2A, and ACVR2B) that are known to interact with BMPs. In this set of receptors, BMPR1A, BMPR1B, and BMPR2 have been described as specific to BMPs, but due to the high number of TGF-β/BMP ligands, the activin receptors ACVR1, ACVR2A and ACVR2B can function as receptors for both activins and BMPs (Mueller and Nickel, 2012; Olsen et al., 2015). The analysis of the spatio-temporal mRNA abundance of these BMP receptors by RT-qPCR in the BOEC obtained from ampulla and isthmus at both FP and LP

showed changes occurring at both ampulla and isthmus during FP and LP. The major findings were related to the greater mRNA abundance for most BMP receptor mRNAs at the isthmus region with significant differences observed for both BMPR1B and ACVR2B. These differences are probably related to differences between these anatomical regions. When comparing both FP and LP samples, ACVR2B mRNA also showed greater abundance during LP, with significant differences in the isthmus region that could depend on the hormonal differences between the follicular and luteal phases of the estrous cycle. Although the protein level of these receptors was not analyzed, the changes observed might affect BMP receptors at the protein level and are suggestive of a role in the complex regulatory processes of TGF-β/BMP signaling in the epithelial oviduct. It is also important to note that Activins are also produced by oviduct epithelial cells (Refaat and Ledger, 2011); Activins could compete with some BMPs signaling through the BMP-shared A-CVR2A and ACVR2B receptors located in the epithelial cells.

BMP ligands induce phosphorylation of the BMP receptors, which then activate R-SMAD 1, 5, and 8 in the canonical pathway and/or BMP-mitogen activated protein kinase (MAPK) in the non-canonical pathway (Miyazono et al., 2005). Tian et al. (2010) showed that p-Smad1/5/8 are located in the nucleus of the epithelial lining of mice oviduct, suggesting that BMP signaling is active in these cells; therefore, oviductal produced BMPs might act as both paracrine and autocrine factors in regulating oviduct reproductive events. Several genes are targets of the BMP signaling pathways, including the inhibitors of DNA binding/differentiation (*ID*) and the *SMADs* genes, harboring SMAD binding elements in their promoters (Hartung et al., 2006; Miyazono et al., 2005). As *ID2* and *SMAD6* are known BMP-target genes (Miyazono et al., 2005), their mRNA abundance was examined by RT-qPCR in BOEC from ampulla and isthmus of FP and LP. Greater abundance of ID2 mRNA were observed in ampulla than in isthmus, this difference being significant during the LP. We also found that although no significant differences were observed for ID2 mRNA abundance when comparing both the follicular and luteal phases, a different behavior was observed in ampulla compared to isthmus. In ampulla, greater abundance was observed during LP compared with ampulla during FP; in isthmus, the greatest abundance of ID2 mRNA were found during the FP. This behavior might be the consequence of differences between these anatomical regions and could reveal different mechanisms of signaling regulation that might also depend on the hormonal state. Differential expression patterns for other genes between the ampulla and isthmus regions have been described in the bovine oviduct (Maillo et al., 2016); knowledge of this type of differences should be useful to study the function of these parts of the oviduct for reproductive success and in normal and pathological states. No significant differences were found for SMAD6 mRNA, but the presence of this mRNA indicates that the BMP signaling pathway is active in the BOEC oviduct. The mRNA abundance of ID2 and SMAD6 was also examined in *in vitro* cultured BOEC after 24 h of BMP5 treatment. The mRNA abundance for both mRNAs were at the same level as those observed in the freshly obtained BOEC. After 24 h of BMP5 treatment, no changes were observed at the mRNA abundance either for ID2 or SMAD6. These genes have been proposed as direct targets of BMP signaling but we were unable to detect changes at the mRNA abundance after BMP5 treatment; this could be due to the fact that fold changes may occur in a lapse of minutes earlier than the 12 h analyzed. Moreover, as the primary BOEC culture expresses BMPs, except for BMP5 (data not shown), their signaling effects could also impair the BMP5 effect; indeed, mRNA abundance for both target genes were possibly high enough to make changes at the mRNA abundance that are hard to detect.

Essential roles in organogenesis and tissue repair have been described for BMP signaling that can affect proliferation, apoptosis and differentiation of stem cells (Lewis et al., 2014; Li et al., 2015; Tadokoro et al., 2016). In mammals, no specific roles for the BMP expressed genes at the epithelial lining of the oviduct have been described yet, except for chicken oviductal epithelial cells, where Monroe et al. (2000) showed that BMP7 induced apoptosis. The presence of BMP ligands, BMP receptors, and BMP signaling components in the BOEC suggests that they may play a role in the oviductal function. As an approach to test the role of BMP in BOEC, wound healing assays were performed on *in vitro* primary BOEC cultures. As these cells lack BMP5 expression, this BMP ligand was selected to evaluate the closure of scratches made in confluent isthmus BOEC primary cultures. Results showed that exogenous BMP5 is capable of enhancing migration of BOEC. Since this effect was observed in isthmus epithelial cells, it can be postulated that BMP5 would exert an autocrine action on oviductal epithelial cells. The wound healing assays were performed in confluent BOEC monolayers with serum-free medium to prevent the effect of serum components on the assay, and maintaining a very low mitotic index by the absence of growth factors and the confluent monolayer. Increased cell migration was evident at 12 h and significant at 24 h; no differences were observed for the mitotic index between the controls and BMP5-treated cultures, indicating that BMP5 does not induce cell proliferation.

5. Conclusions

The changes observed in the mRNA components of the signaling pathway and the BMP5 effect on oviductal epithelial cells allowed us to suggest a possible autocrine role for the BMP pathway in BOEC homeostasis. This work also highlights the need for more studies of BMPs and their antagonists that could affect epithelial cell functions necessary for normal physiology and reproductive success. Notch and Wnt signaling pathways have been described as responsible for the maintenance of epithelial integrity; as there is crosstalk with the TGF-β/BMP pathways, interactions between these signaling pathways should also be considered in these studies. Efforts to link their activities are also important for the understanding of the physiological processes and functions of this organ during the reproductive cycle.

Funding

This study was supported by research grants from Secretaría de Ciencia, Arte e Innovación Tecnológica of Universidad Nacional de Tucumán, Argentina (PIUNT 26/D551) and from Agencia Nacional de Promoción Científica y Tecnológica of Argentina (BID PICT 2013 No 0770, and BID PICT 2006 No 00502).

Acknowledgments

The authors would like to thank Calchaquí slaughterhouse, Tucumán, Argentina, for providing the biological material from their slaughtered cows.

R.C. Bravo Miana is grateful to Consejo Interuniversitario Nacional (CIN) for her research scholarship.

References

Almiñana, C., Caballero, I., Heath, P.R., Maleki-Dizaji, S., Parrilla, I., Cuello, C., Gil, M.A., Vazquez, J.L., Vazquez, J.M., Roca, J., Martinez, E.A., Holt, W.V., Fazeli, A., 2014. The battle of the sexes starts in the oviduct: modulation of oviductal transcriptome by X and Y-bearing spermatozoa. BMC Genomics 15, 293.

Avilés, M., Gutiérrez-Adán, A., Coy, P., 2010. Oviductal secretions: will they be key factors for the future ARTs? Mol. Hum. Reprod. 16, 896–906.

Bauersachs, S., Rehfeld, S., Ulbrich, S.E., Mallok, S., Prelle, K., Wenigerkind, H., Einspanier, R., Blum, H., Wolf, E., 2004. Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle. J. Mol. Endocrinol. 32, 449–466.

Bragdon, B., Moseychuk, O., Saldanha, S., King, D., Julian, J., Nohe, A., 2011. Bone morphogenetic proteins: a critical review. Cell. Signal. 23, 609–620.

García, D.C., Miceli, D.C., Valdecantos, P.A., García, E.V., Roldán-Olarte, M., 2014. Expression of urokinase type plasminogen activator receptor (uPAR) in the bovine oviduct: relationship with uPA effect on oviductal epithelial cells. Res. Vet. Sci. 97, 118–123.

García, E.V., Valdecantos, P.A., Barrera, D., Roldán-Olarte, M., Miceli, D.C., 2014. Bone morphogenetic proteins in the bovine oviduct: differential expression of BMP-5 in the isthmus during the estrous cycle. Theriogenology 81, 1032–1041.

García, E.V., Miceli, D.C., Rizo, G., Valdecantos, P.A., Barrera, A.D., 2015. Effect of early addition of bone morphogenetic protein 5 (BMP5) to embryo culture medium on in vitro development and expression of developmentally important genes in bovine preimplantation embryos. Theriogenology 84, 589–599.

Ghersevich, S., Massa, E., Zumoffen, C., 2015. Oviductal secretion and gamete interaction. Reproduction 149, R1–R14.

Graham, S.J.L., Wicher, K.B., Jedrusik, A., Guo, G., Herath, W., Robson, P., Zernicka-Goetz, M., 2014. BMP signalling regulates the pre-implantation development of extra-embryonic cell lineages in the mouse embryo. Nat. Commun. 5, 5667.

Hartung, A., Sieber, C., Knaus, P., 2006. Yin and Yang in BMP signaling: impact on the pathology of diseases and potential for tissue regeneration. Signal Transduct. 6, 314–328.

Hess, A.P., Talbi, S., Hamilton, A.E., Baston-Buest, D.M., Nyegaard, M., Irwin, J.C., Barragan, F., Kruessel, J.S., Germeyer, A., Giudice, L.C., 2013. The human oviduct transcriptome reveals an anti-inflammatory, anti-angiogenic, secretory and matrix-stable environment during embryo transit. Reprod. Biomed. Online 27, 423–435.

Jordaens, L., Arias-Alvarez, M., Pintelon, I., Thys, S., Valckx, S., Dezhkam, Y., Bols, P.E.J., Leroy, J.L.M.R., 2015. Elevated non-esterified fatty acid concentrations hamper bovine oviductal epithelial cell physiology in three different in vitro culture systems. Theriogenology 84, 899–910.

La Rosa, I., Camargo, L.S.A., Pereira, M.M., Fernandez-Martin, R., Paz, D.A., Salamone, D.F., 2011. Effects of bone morphogenic protein 4 (BMP4) and its inhibitor, Noggin, on in vitro maturation and culture of bovine preimplantation embryos. Reprod. Biol. Endocrinol. 9, 18.

Lewis, C.J., Mardaryev, A.N., Poterlowicz, K., Sharova, T.Y., Aziz, A., Sharpe, D.T., Botchkareva, N.V., Sharov, A.A., 2014. Bone morphogenetic protein signaling suppresses wound-induced skin repair by inhibiting keratinocyte proliferation and migration. J. Invest. Dermatol. 134, 827–837.

Li, Q., Agno, J.E., Edson, M.A., Nagaraja, A.K., Nagashima, T., Matzuk, M.M., 2011. Transforming growth factor β receptor type 1 is essential for female reproductive tract integrity and function. PLoS Genet. 7, e1002320.

Li, R.X., Yiu, W.H., Tang, S.C.W., 2015. Role of bone morphogenetic protein-7 in renal fibrosis. Front. Physiol. 6, 114.

Maillo, V., Sánchez-Calabuig, M.J., Lopera-Vasquez, R., Hamdi, M., Gutierrez-Adan, A., Lonergan, P., Rizos, D., 2016. Oviductal response to gametes and early embryos in mammals. Reproduction 152, R127–R141.

Miyazono, K., Maeda, S., Imamura, T., 2005. BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev. 16, 251–263.

Mondéjar, I., Acuña, O.S., Izquierdo-Rico, M.J., Coy, P., Avilés, M., 2012. The oviduct: functional genomic and proteomic approach. Reprod. Domest. Anim. 47 (Suppl. 3), 22–29.

Monroe, D.G., Jin, D.F., Sanders, M.M., 2000. Estrogen opposes the apoptotic effects of bone morphogenetic protein 7 on tissue remodeling. Mol. Cell. Biol. 20, 4626–4634.

Mueller, T.D., Nickel, J., 2012. Promiscuity and specificity in BMP receptor activation. FEBS Lett. 586, 1846–1859.

Olsen, O.E., Wader, K.F., Hella, H., Mylin, A.K., Turesson, I., Nesthus, I., Waage, A., Sundan, A., Holien, T., 2015. Activin a inhibits BMP-signaling by binding ACVR2A and ACVR2B. Cell Commun. Signal. 13, 27.

Pangas, S.A., Li, X., Umans, L., Zwijsen, A., Huylebroeck, D., Gutierrez, C., Wang, D., Martin, J.F., Jamin, S.P., Behringer, R.R., Robertson, E.J., Matzuk, M.M., 2008. Conditional deletion of Smad1 and Smad5 in somatic cells of male and female gonads leads to metastatic tumor development in mice. Mol. Cell Biol. 28, 248–257.

Refaat, B., Ledger, W., 2011. The expression of activins, their type II receptors and follistatin in human Fallopian tube during the menstrual cycle and in pseudo-pregnancy. Hum. Reprod. 26, 3346–3354.

Reyes de Mochel, N.S., Luong, M., Chiang, M., Javier, A.L., Luu, E., Toshihiko, F., MacGregor, G.R., Cinquin, O., Cho, K.W.Y., 2015. BMP signaling is required for cell cleavage in preimplantation-mouse embryos. Dev. Biol. 397, 45–55.

Rodriguez, A., Tripurani, S.K., Burton, J.C., Clementi, C., Larina, I., Pangas, S.A., 2016. SMAD signaling is required for structural integrity of the female reproductive tract and uterine function during early pregnancy in mice. Biol. Reprod. 95, 1–16.

Romagnoli, M., Belguise, K., Yu, Z., Wang, X., Landesman-Bollag, E., Seldin, D.C., Chalbos, D., Barillé-Nion, S., Jézéquel, P., Seldin, M.L., Sonenshein, G.E., 2012. Epithelial-to-mesenchymal transition induced by TGF-β1 is mediated by Blimp-1-dependent repression of BMP-5. Cancer Res. 72, 6268–6278.

Rottmayer, R., Ulbrich, S.E., Kölle, S., Prelle, K., Neumueller, C., Sinowatz, F., Meyer, H.H.D., Wolf, E., Hiendleder, S., 2006. A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. Reproduction 132, 637–648.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3, 1101–1108.

Shimasaki, S., Moore, R.K., Otsuka, F., Erickson, G.F., 2004. The bone morphogenetic protein system in mammalian reproduction. Endocr. Rev. 25, 72–101.

Tadokoro, T., Gao, X., Hong, C.C., Hotten, D., Hogan, B.L.M., 2016. BMP signaling and cellular dynamics during regeneration of airway epithelium from basal progenitors. Development 143, 764–773.

Tanwar, P.S., McFarlane, J.R., 2011. Dynamic expression of bone morphogenetic protein 4 in reproductive organs of female mice. Reproduction 142, 573–579.

Tian, X., Halfhill, A.N., Diaz, F.J., 2010. Localization of phosphorylated SMAD proteins in granulosa cells, oocytes and oviduct of female mice. Gene Expr. Patterns 10, 105–112.

Ulbrich, S.E., Schoenfelder, M., Thoene, S., Einspanier, R., 2004. Hyaluronan in the bovine oviduct − modulation of synthases and receptors during the estrous cycle. Mol. Cell. Endocrinol. 214, 9–18.

Wang, R.N., Green, J., Wang, Z., Deng, Y., Qiao, M., Peabody, M., Zhang, Q., Ye, J., Yan, Z., Denduluri, S., Idowu, O., Li, M., Shen, C., Hu, A., Haydon, R.C., Kang, R., Mok, J., Lee, M.J., Luu, H.L., Shi, L.L., 2014. Bone Morphogenetic Protein (BMP) signaling in development and human diseases. Genes Dis. 1, 87–105.