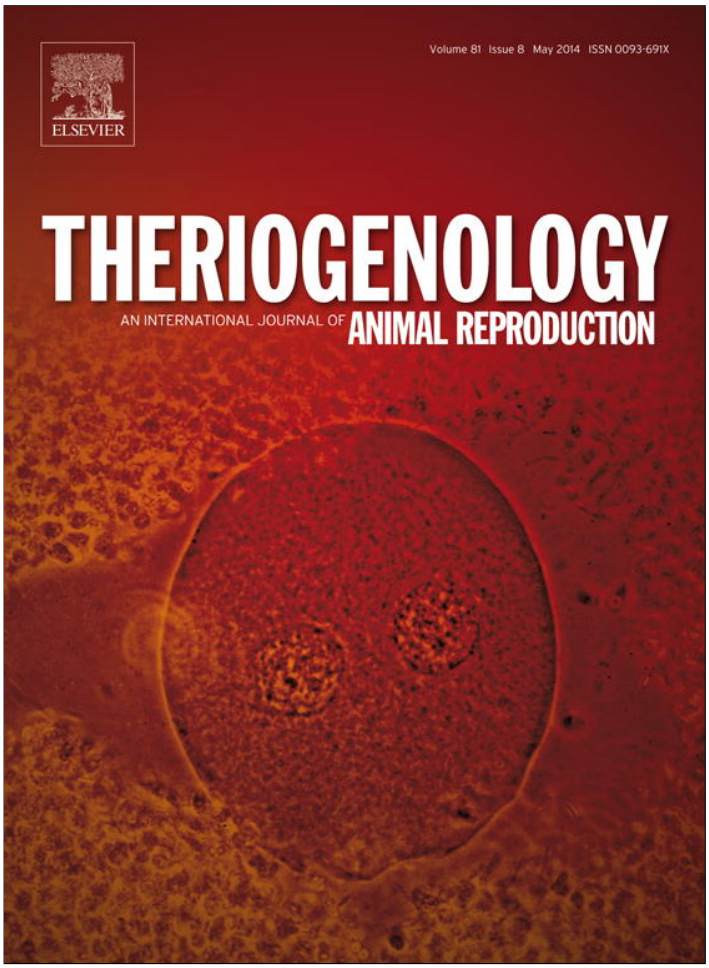

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Bone morphogenetic proteins in the bovine oviduct: Differential expression of BMP-5 in the isthmus during the estrous cycle

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ABSTRACT

Bone morphogenetic proteins (BMPs) play a crucial role in mammalian reproduction, but little is known about their expression and function in the oviduct, where preimplantation events take place. In the present study, messenger RNA (mRNA) expression of BMPs was examined by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) in bovine oviduct epithelial cells obtained from ampulla and isthmus at different stages of the estrous cycle. Expression of BMP-2, -3, -4, -7, -10 and -15 mRNA was detected in epithelial cells of both anatomic regions, whereas BMP-5 mRNA was specifically expressed in isthmus epithelial cells throughout the estrous cycle. High expression levels for BMP-5 and for BMP-2, -4, and -7 mRNA were observed during the preovulatory stage. Considering the region-specific gene expression of BMP-5, its protein localization in the oviduct and its presence in the oviductal fluid were evaluated by immunohistochemistry and Western blot analysis. BMP-5 protein staining was observed in isthmus sections with a more intense signal in the luminal epithelial cell layer. In addition, a 21 kDa protein corresponding to the BMP-5 mature monomeric form was detected in bovine oviductal fluid throughout the estrous cycle. In conclusion, these results demonstrate that different members of the BMP family are expressed in the bovine oviduct during the estrous cycle, and reveal that BMP-5 is differentially expressed in the isthmus. The expression of this factor in the oviduct epithelium and its presence in the luminal fluid suggest a possible action of BMP-5 as a new autocrine and/or paracrine regulator of the reproductive events that occur in the bovine oviductal environment.

1. Introduction

Bone morphogenetic proteins (BMPs) comprise the largest subgroup of ligands within the transforming growth factor-β (TGF-β) superfamily, with more than 20 members described [1]. Like other TGF-βs, BMPs participate during embryonic and adult life regulating growth, differentiation, chemotaxis, and apoptosis of different cell types [2,3]. Bone morphogenetic proteins are active as disulfide-linked dimers, and exert their effects by binding tetrameric complexes of type I and type II transmembrane serine/threonine kinase receptors that lead to signal propagation through Smad-dependent pathways [4–6].

Numerous studies have reported the importance of BMP signaling in mammalian reproduction and fertility [7].

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Several BMP family members, including BMP-2, -3, -4, -5, -6, -7, and -15 are expressed in ovary [8–11], uterus [12,13], and in the oocyte and granulosa cells [14–18]. They perform important biological activities as regulators of ovarian follicular development, female reproductive tract differentiation, blastocyst implantation in the uterus, and morphogenesis and organogenesis during embryo development [7,19–21]. Additionally, there is evidence that naturally occurring mutations or targeted deletions of certain BMP family genes can alter early embryonic development or cause reproductive defects [7,22–26].

Although these findings underscore the importance of BMPs in female fertility, the presence and function of BMPs in the oviduct have not been extensively explored. Within the mammalian reproductive system, the oviduct and its secretions provide the optimal microenvironment necessary for the reproductive events that precede implantation, including gamete transport, sperm capacitation, fertilization, and early embryonic development [27–29]. Many studies conducted in this organ have focused on growth factors because they can act as autocrine and paracrine regulators during embryo-maternal cross-talk, and stimulate cellular processes such as proliferation, differentiation, and apoptosis that are critical to successful preimplantation embryonic development [30–32]. Several growth factors of the TGF-β superfamily have been detected in the oviduct of mammalian species: TGFα and TGFβ1-3 [33–35], activin βA-B and inhibin α [36–38] and lefty2 [39,40]. Regarding the BMP subgroup, the expression of BMP-2, -4, and -7 has been detected in mouse oviduct [13,41]. In addition, information derived from transcriptomic analysis, and stored in different databases provides clues to the presence of BMP-7 in bovine oviduct [42], BMP-2, -5, -7, and -8a in human oviduct [43] and BMP-4, -5, -7, and -15 in porcine oviduct [44]. Although all these studies report the presence of BMP transcripts in the oviduct of different species, the mechanisms regulating BMP expression and the physiological action of these factors in the mammalian oviduct are still unknown. Studies in chicken oviduct revealed that BMP-7 regulates apoptosis in primary oviductal cells and that estradiol treatment represses its expression [45], but it is not known if the same occurs in mammalian oviduct. Moreover, expression of other BMP family members and involvement of BMPs during early reproductive events in the bovine oviduct are unknown.

Considering that the oviduct undergoes physiological and hormonal changes during the ovarian cycle, and that expression pattern of the oviduct epithelium is modified according to the anatomic region, the ovarian cycle status, and the presence of gametes and embryos, more precise information is needed concerning the expression, presence, and secretion of BMPs in the oviduct to clarify their role. Within this context, the aim of the present study was to characterize messenger RNA (mRNA) expression of BMPs in bovine oviduct epithelial cells during the estrous cycle, analyzing regional and temporal differences in expression levels that depend on the anatomic region of the oviduct, and the stage of the estrous cycle. In addition, after observation of differential expression of BMP-5 mRNA in the isthmus region, the protein localization of this factor in the bovine oviduct and its presence in the oviductal fluid during different stages of the estrous cycle were further analyzed.

2. Materials and methods

2.1. Bovine oviduct collection

Genital tracts of beef cows (*Bos taurus*) obtained from local abattoirs were transported on ice to the laboratory, and processed within 3 hours after animal death. Only samples from nonpregnant animals without anatomic abnormalities or defects in their reproductive tracts were included in the study. The stage of the estrous cycle was determined by visual examination of the ovarian morphology, and the tracts were classified into three groups: (a) Pre-Ov, preovulatory stage (ovary containing a dominant follicle or close to ovulate); (b) Post-Ov, postovulatory stage (ovary containing a corpus hemorrhagicum); and (c) Mid-L, mid-luteal stage (ovary containing a vascularized CL). Once classified, ipsilateral oviducts (n = 39) to the dominant ovary were separated from the tracts, washed in sterile ice-cold PBS, pH 7.4, and transferred to Petri dishes on ice before being dissected to remove blood vessels, connective tissue, and adhering fat. The oviducts selected were then processed for mRNA analysis (n = 15; five oviducts for each estrous cycle stage), immunohistochemical (n = 9; three oviducts for each estrous cycle stage), and Western blot assays (n = 15; five oviducts for each estrous cycle stage) as described below.

2.2. RNA isolation from bovine oviduct epithelial cells

Isthmus and ampulla regions of bovine oviducts (n = 5 per stage of the estrous cycle) were separated and opened longitudinally under sterile conditions. Bovine oviduct epithelial cells from each anatomic region were separately collected by gently scraping the mucosal epithelial layer with the blunt side of a sterile scalpel [46]. Scraped epithelial cells were transferred with PBS to a 2 mL tube, washed twice, and centrifuged at 200 × *g* for 5 minutes to remove any blood contamination, and immediately processed for RNA extraction. Total RNA from ampulla and isthmus epithelial cells was extracted using TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, USA), according to the manufacturer's instructions. The resultant RNA pellet was resuspended in 15 to 20 μL of RNase-free water. The quality and quantity of RNA samples was assessed spectrophotometrically by measuring the optical density of each sample at 260 and 280 nm, and integrity was examined 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.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Reverse transcription was performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA) and Oligo(dT)$_{17}$ primer. The reaction mixture (25 μL) consisted of 2 μg of total heat-denatured RNA, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM of each dNTP, 25 pmol of Oligo (dT)$_{17}$, 200 units of reverse transcriptase and RNase-free water. Reactions were performed by incubating the mixture in a

thermal cycler at 42 °C for 90 minutes followed by a reverse-transcriptase inactivation at 94 °C for 5 minutes.

Polymerase chain reactions were performed in a final volume of 20 μL containing 1 μL complementary DNA (cDNA), 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 2.0 units of *Taq* DNA polymerase (Invitrogen) and 1 μM of each specific primer for the transcript of interest (see Table 1). The reactions were carried out in a thermal cycler under the following conditions: initial denaturation at 94 °C for 2 minutes 30 seconds, 30 cycles at 94 °C for 30 seconds, 58 °C for 30 seconds, 72 °C for 50 seconds and a final extension step at 72 °C for 7 minutes. Polymerase chain reaction (PCR) conditions were previously assayed to determine the appropriate number of cycles for amplification of each fragment within the linear range for each primer set, and 30 cycles proved to be optimal for the detection of quantitative differences between the samples, and ensured that PCR amplifications did not reach a plateau. All PCR reactions were performed twice for each cDNA sample. To exclude false-positive results derived from contaminations or genomic DNA, two negative controls without template (water instead of cDNA) and containing non–reverse transcribed RNA were included in all PCR reactions. β-Actin cDNA was amplified as internal control. The PCR products were analyzed electrophoretically on 1.5% agarose gels, and visualized with SYBR Safe DNA Gel Stain. To confirm the identity of PCR products, the amplified fragments were purified with a PureLink Quick Gel Extraction Kit (Invitrogen), cloned into the pGEM-T Easy Vector (Promega), and sequenced in both directions (Macrogen Genomics). The nucleotide sequences of PCR products confirmed to be 100% identical with their bovine sequences.

For semiquantitative analysis of BMPs mRNA expression levels, gel images were captured on a Gel Doc 1000 imaging system (Bio-Rad), and the optical densities of PCR products were quantified using Molecular Analyst 1.4.1 software (Bio-Rad). The relative abundance of each specific gene was normalized against that of β-actin (reference gene), and the BMP-to-β-actin ratio was calculated for each analyzed factor.

2.4. BMP-5 immunohistochemistry

For immunohistochemistry analysis, pieces of 10 mm from ampulla and isthmus regions of bovine oviducts in Pre-Ov (n = 3), Post-Ov (n = 3), and Mid-L (n = 3) stages were fixed overnight in 4% formaldehyde in PBS (pH 7.4), embedded in paraffin, and processed according to Roldán-Olarte, et al. [47]. Briefly, paraffin wax–embedded blocks of oviduct segments were transversally sliced into 5 μm sections, and mounted on poly-L-lysine–coated microscope slides. After deparaffinization and rehydration, sections were rinsed with PBS, pH 7.4, and endogenous peroxidase activity was eliminated by incubation with 3% (vol/vol) H_2O_2 in PBS, pH 7.4 for 30 minutes. After washing with PBS, sections were blocked with 1 mg/mL BSA-PBS solution for 1 hour at room temperature. Then, sections were incubated overnight with 8 μg/mL goat anti-BMP-5 polyclonal antibody (B3805; Sigma) at 4 °C in a humidified chamber. Afterward, sections were rinsed three times with PBS, and incubated at room temperature for 1 hour with rabbit anti-goat immunoglobulin G (IgG) biotin-conjugated antibody (B7024; Sigma) at a dilution of 1:50. Next, samples were incubated at room temperature for 1 hour with streptavidin–peroxidase conjugate (E2886, 1:100 dilution; Sigma), and then incubated for 10 minutes with 3,3′-diaminobenzidine (B8001; Sigma) substrate for color development. Sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. A minimum of five sections for each sample were examined under an Olympus

Table 1
Primers design for reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Gene	Oligonucleotides (5′–3′) (upstream/downstream)[a]		Product size (bp)	GenBank accesion number
BMP-2	Forward	GTCCTGAGCGAGTTCGAGTT	478	NM_001099141
	Reverse	CGAAGCTCTCCCACCTACTG		
BMP-3	Forward	CTTTGTGTCCTGGCTGTCAA	348	XM_587912
	Reverse	TGATATTCTGCCCCAGGAAG		
BMP-4	Forward	AGTCTGGGGAGGAGGAAGAG	394	NM_001045877
	Reverse	GCAGGGCTCACATCAAAAGT		
BMP-5	Forward	GATGTGGGTTGGCTTGTCTT	271	XM_584340
	Reverse	CCTGATGAGAGCCGGATTTA		
BMP-6	Forward	TGGCTGGAGTTTGACATCAC	298	XM_869844
	Reverse	TGTAGTCTGAGGCGCTGGAG		
BMP-7	Forward	GGCAGGACTGGATCATCG	191	XM_612246
	Reverse	GAGCACAGAGATGGCATTGA		
BMP-10	Forward	TGAAGGGGAAAGAAACATGC	318	XM_583418
	Reverse	TCCATCTCTGGGAATTGCTC		
BMP-12	Forward	ACGTGGCAGACGCCTTAC	215	XM_616701
	Reverse	CTCTCGGAACAGGCTCTCCT		
BMP-13	Forward	GAAGCAGCTGTGCTTGGAG	177	XM_867875
	Reverse	GACCTGGAGAACACGACGAG		
BMP-15	Forward	TCAGGAAGAGGCTCCTCAAA	168	NM_001031752
	Reverse	CCACCAGAACTCACGAACCT		
β-actin	Forward	GAGCTACGAGCTTCCTGACG	245	NM_173979
	Reverse	GGGCAGTGATCTCTTTCTGC		

Abbreviation: BMP, bone morphogenetic protein.
[a] All the primers were designed with Primer-BLAST online tool of the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

BX40 light microscope and photographed with an Olympus C-5060 wide zoom digital camera. For control sections, either primary or secondary antibodies were omitted and replaced by buffer. In addition, an immunohistochemistry analysis (following the same methodology described above) with an anti-BMP-2 goat polyclonal antibody (sc-6895, 1:50 dilution; Santa Cruz Biotechnology) was performed to compare the cellular distribution of BMP-5 in the oviduct with other member of the BMP family.

2.5. BMP-5 detection in oviductal fluid by Western blot

Bovine oviductal fluid was collected from 15 oviducts in Pre-Ov (n = 5), Post-Ov (n = 5), and Mid-L (n = 5) stages, flushing from the isthmus end 70 μL of a 10 mM Tris–HCl and 150 mM NaCl (pH 7.4) solution at 4 °C. The fluid collected from the five oviducts of each stage of the estrous cycle was combined into three single pools (Pre-Ov, Post-Ov, and Mid-L oviductal fluid pool). Each pool was centrifuged at 7000 × *g* for 10 minutes at 4 °C to remove cellular debris, fractionated, and stored at −80 °C until use. Total protein concentration of the oviductal fluid pools was measured with a Micro BCA Protein Assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The protein concentration of the samples was 27.65 μg/μL, 26.83 μg/μL, and 15.45 μg/μL for the Pre-Ov, Post-Ov, and Mid-L pool of oviductal fluid, respectively.

A volume equivalent to 90 μg of total protein from each oviductal fluid pool (Pre-Ov, Post-Ov, and Mid-L) was mixed with sample buffer containing 10% (vol/vol) 2-β-mercaptoethanol, boiled at 95 °C for 5 minutes, and then subjected to SDS-PAGE on a 12% (wt/vol) acrylamidegel, using a Mini-Protean II apparatus (Bio-Rad). After electrophoresis, proteins were electroblotted onto a nitrocellulose membrane in a Mini Trans-Blot electrophoretic transfer system (Bio-Rad) at constant voltage (40 V for 12 hours). Membranes were incubated overnight with 2% (wt/vol) BSA in PBS, pH 7.4, at 4 °C to block nonspecific binding sites. Then, membranes were incubated with 4 μg/mL goat anti-BMP-5 polyclonal antibody (B3805; Sigma) at room temperature for 3 hours. After extensive washing with PBS containing 0.02% Tween-20, membranes were incubated with 1:500 diluted biotin-conjugated anti-goat immunoglobulin G (IgG) as a secondary antibody at room temperature for 2 hours. Specific binding of anti-BMP-5 was revealed by incubation with 1:2000 streptavidin–peroxidase conjugate (Sigma) for 20 minutes, and visualized with 0.1 mg/mL 3,3′-diaminobenzidine (3,3′,4,4′-tetra-aminobiphenyl) and 0.01% H_2O_2 in 0.1 M Tris–HCl, pH 7.2.

Densitometric analysis was performed using ImageJ 1.44p software (Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA; http://imagej.nih.gov/ij). For statistical analysis, a density profile plot was generated for each protein band in three different blots. For comparative purposes, the relative intensity of each band was expressed with reference to the preovulatory samples.

2.6. Statistical analyses

Statistical analyses were performed with SigmaStat 3.5 and SigmaPlot 10.0 statistical software (Systat Software). The relative levels of expression of BMPs (BMP-to-β-actin ratio) obtained by semiquantitative RT-PCR were analyzed by two-way ANOVA to evaluate the effects of the oviduct section (isthmus and ampulla) and the stage of the estrous cycle (Pre-Ov, Post-Ov, and Mid-L) on the gene expression levels. For the densitometric analysis of Western blots one-way ANOVA was used to test for differences. When ANOVA showed differences, Tukey test was used to test for significance. Data are presented as means ± standard deviation. Results were considered statistically significant when P-value was less than 0.05.

3. Results

3.1. Expression of BMPs in bovine oviduct epithelial cells

As a first approach to identify which BMPs were expressed in the bovine oviduct, the expression of 10 BMP family members (see Table 1) was analyzed by RT-PCR in bovine oviduct epithelial cells obtained from ampulla and isthmus regions. For this purpose, cDNA samples of epithelial cells obtained from ipsilateral oviducts at different stages of the estrous cycle were pooled for each anatomic region (ampulla or isthmus). BMP-2, -3, -4, -7, -10, and -15 transcripts were detected in epithelial cells of both anatomic regions, whereas BMP-5 mRNA was only expressed in epithelial cells of the isthmus (Fig. 1). As shown in Figure 1, no expression of BMP-6, -12, and -13 mRNAs was observed in bovine oviduct epithelial cells of either of the two anatomic regions.

3.2. Expression of BMP-2, -4, -5, and -7 mRNA during the estrous cycle

To evaluate whether the differential expression of BMP-5 mRNA in the isthmus was associated with a specific phase of the bovine estrous cycle, the expression level of this transcript was evaluated with semiquantitative RT-PCR in bovine oviduct epithelial cells obtained from ampulla and isthmus during Pre-Ov, Post-Ov, and Mid-L stages. For comparison purposes, expression of BMP-2, -4, and -7 mRNA was also analyzed considering that, together with BMP-5, they belong to two closely-related BMP subclasses: BMP-2/4 and BMP-5/6/7.

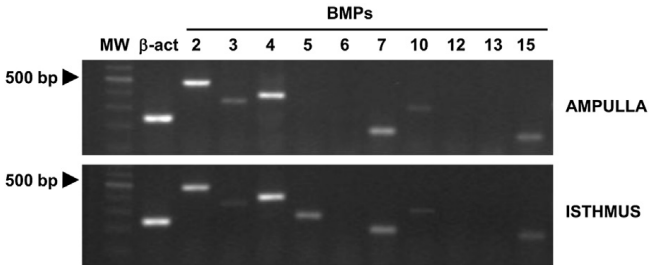

Fig. 1. Bone morphogenetic proteins (BMPs) mRNA expression in bovine oviduct epithelial cells from ampulla and isthmus regions. Electrophoresis on 1.5% agarose gel showing specific reverse transcription-polymerase chain reaction (RT-PCR) products for β-actin (245 bp), BMP-2 (478 bp), BMP-3 (348 bp), BMP-4 (394 bp), BMP-5 (271 bp), BMP-7 (191 bp), BMP-10 (318 bp), and BMP-15 (168 bp) mRNA. MW: 100 bp molecular weight marker. mRNA, messenger RNA; MW, molecular weight.

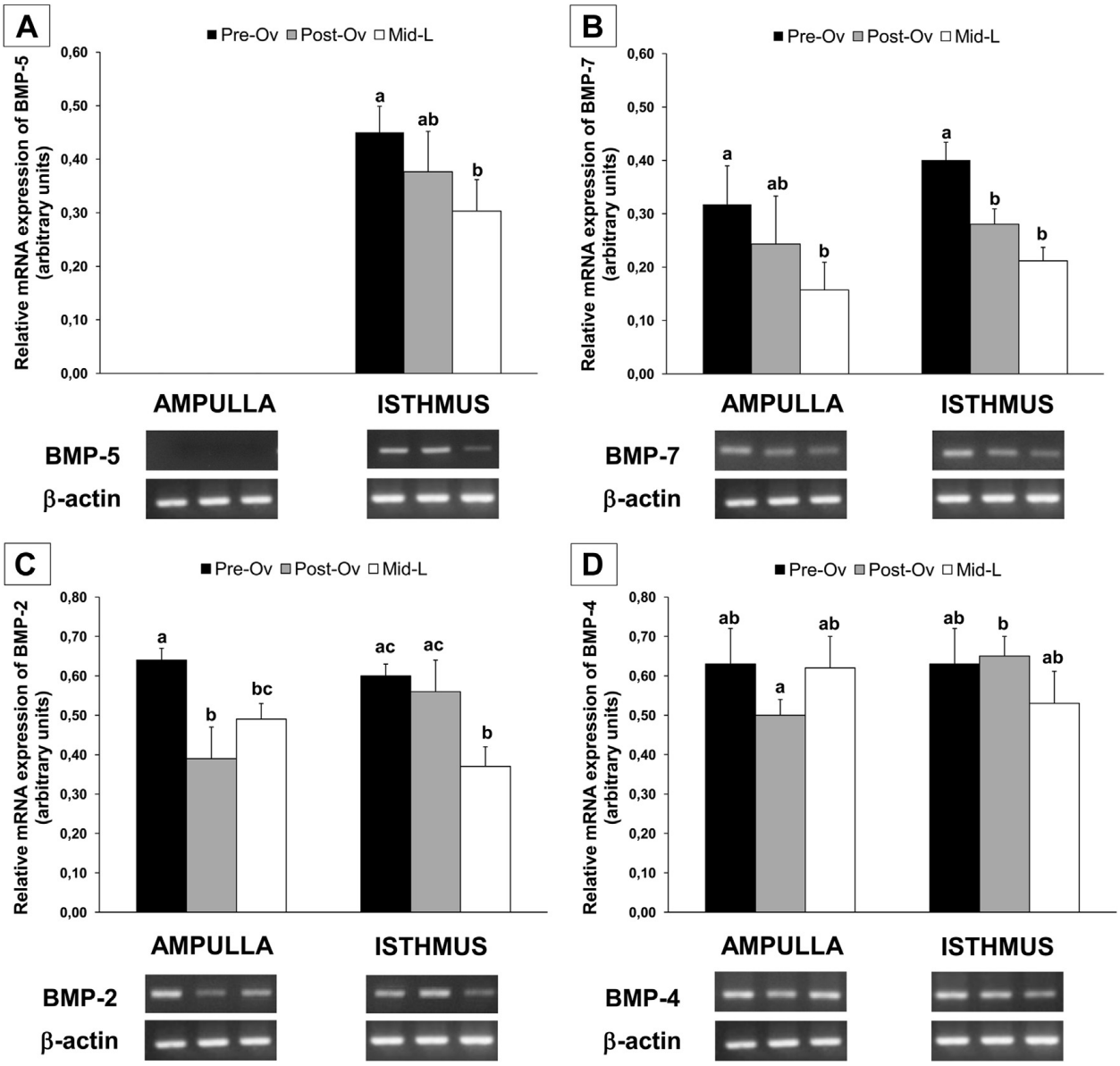

Fig. 2. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of BMPs mRNA expression in bovine oviductal epithelial cells from ampulla and isthmus during the estrous cycle. Bar densities representing BMP-5 (A), BMP-7 (B), BMP-2 (C), and BMP-4 (D) mRNA levels normalized to β-actin mRNA levels are shown. Results are expressed as means ± SD of five different animals for each estrous cycle stage (one ipsilateral oviduct per animal). Significant differences ($P < 0.05$) are indicated with different letters. BMP, bone morphogenetic protein; Mid-L, mid-luteal stage (white bars); mRNA, messenger RNA; Post-Ov, postovulatory stage (gray bars); Pre-Ov, preovulatory stage (black bars); SD, standard deviation.

BMP-5 mRNA expression was detected throughout the estrous cycle, but only in isthmus epithelial cells and not in ampulla (Fig. 2 A; $P < 0.001$). The highest expression was observed during the Pre-Ov stage, and decreased during the Mid-L phase (Fig. 2 A; $P < 0.05$). In contrast to BMP-5, BMP-2, -4, and -7 mRNAs were detected in epithelial cells from both anatomic regions during all three phases of the estrous cycle examined (Fig. 2 B, C and D). In general, high expression levels of these transcripts were observed during the Pre-Ov phase as it was shown for BMP-5. During the Post-Ov stage, gene expression of BMP-2 and -4 decreased significantly in the ampulla, but remained high in the isthmus (Fig. 2 C, D; $P < 0.05$). In contrast, BMP-5 and -7 mRNA expression in the isthmus gradually decreased from the preovulatory until the luteal stage (Fig. 2 A, B). Two-way ANOVA indicated a statistically significant interaction between the estrous cycle and the anatomic region for BMP-5 ($P = 0.029$), BMP-2 ($P = 0.002$), and BMP-4 ($P = 0.044$) mRNA expression, but not for BMP-7 ($P = 0.833$) mRNA expression.

3.3. Immunohistochemical localization of BMP-5 in the bovine oviduct

Considering the differential expression of BMP-5 mRNA in the isthmus during the estrous cycle, the next aim was to evaluate the protein distribution and localization of this factor in the oviduct by immunohistochemistry. As shown in Figure 3 C, positive immunostaining for BMP-5 was localized primarily in the oviductal epithelium of isthmus with less immunoreactivity in lamina propria and smooth muscular layer. This specific reaction was observed in both

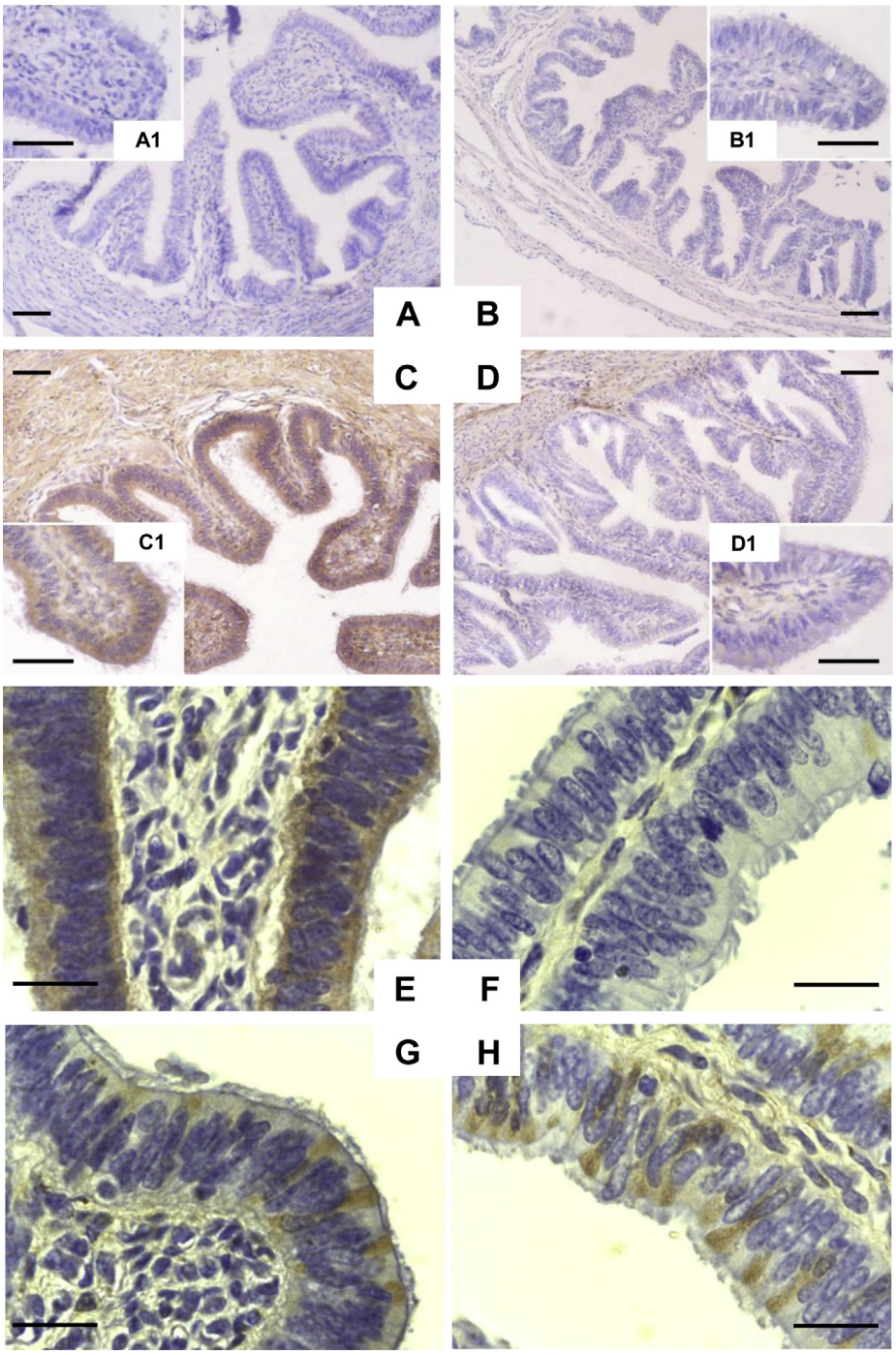

Fig. 3. Localization of BMP-5 in the bovine oviduct. (A, B) Negative controls (without primary antibody) for isthmus (A, A1) and ampulla (B, B1) sections. (C–F) Immunohistochemical staining for BMP-5 in sections of isthmus (C, C1, E) and ampulla (D, D1, F). (G–H) Immunohistochemical staining for BMP-2 in sections of isthmus and ampulla. Representative images during the preovulatory phase are shown. Sections were counterstained with Mayer's hematoxylin. Scale bars: A to D = 50 μm; A1 to D1 = 40 μm; E to H = 20 μm. BMP, bone morphogenetic protein.

ciliated and secretory epithelial cells (Fig. 3 E). In contrast, ampulla sections only showed a weak signal in the lamina propria and muscular layer, and no signal was observed in the oviductal epithelium (Fig. 3 D, D1, and F). Subjectively, the intensity of immunostaining for BMP-5 did not differ appreciably between the three different stages of the estrous cycle analyzed (data not shown). BMP-2, other member of the BMP family, was also immunolocalized in the oviductal epithelium. In contrast to BMP-5, positive immunostaining for BMP-2 was observed in both ampulla and isthmus and primarily in epithelial cells morphologically suggestive to be ciliated cells (Fig. 3 G, H). Control slides incubated without primary or the secondary antibodies were negative for the immunostaining (Fig. 3 A, B).

3.4. Western blot analysis of BMP-5 in bovine oviductal fluid

To confirm the presence of BMP-5 in the oviductal lumen, Western blot analyses were carried out with samples of bovine oviductal fluid from ipsilateral oviducts

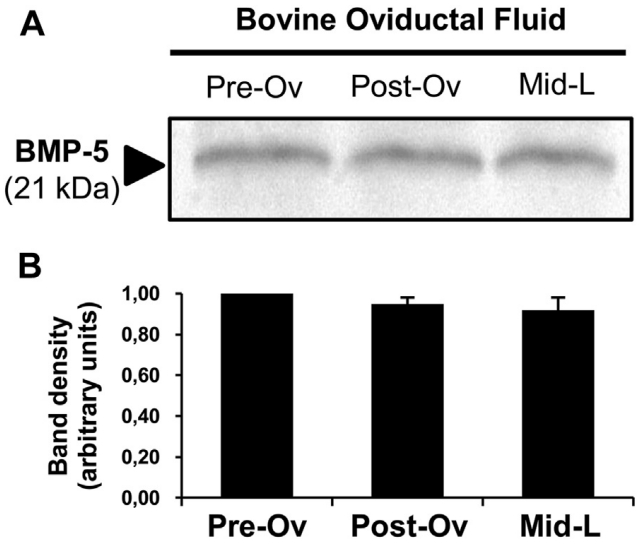

Fig. 4. Western blot analysis of BMP-5 in bovine oviductal fluid during the estrous cycle. (A) Representative immunoblot showing the presence of BMP-5 in bovine oviductal fluid pools obtained during the preovulatory (Pre-Ov), postovulatory (Post-Ov), and mid-luteal (Mid-L) phase of the estrous cycle. Each lane represents a pool of oviductal fluid of five animals for each stage of the estrous cycle analyzed (90 μg per lane). Molecular weight of the band is indicated on the left. (B) Densitometric analyses of BMP-5 protein levels normalized against preovulatory oviductal fluid samples. BMP, bone morphogenetic protein.

during the Pre-Ov, Post-Ov, and Mid-L phase obtained as described in Section 2. Under reducing conditions, BMP-5 was detected as a 21 kDa band in oviductal fluid pools of the three stages of the estrous cycle analyzed (Fig. 4 A). The molecular weight (MW) of the specific protein detected agreed with the expected MW for the mature monomeric form of BMP-5. Protein levels of BMP-5 were relatively constant during the estrous cycle considering that no significant differences were observed between the oviductal fluid pools of the three stages of the estrous cycle analyzed (Fig. 4 B).

4. Discussion

Bone morphogenetic proteins play a crucial role in embryonic development and mammalian reproduction. The expression of BMPs, BMP receptors, and SMAD signaling molecules in the ovary and their activities in the regulation of ovarian follicular development has been extensively explored [21,48]. Bone morphogenetic protein signaling is also implicated in female reproductive tract differentiation, blastocyst implantation in the uterus, and morphogenesis and organogenesis during embryo development [7,19,20]. However, the expression and the action of BMPs in the oviduct, where preimplantation events take place, have not been elucidated yet.

In the present study, it has been demonstrated that different members of the BMP family are expressed in the bovine oviduct during the estrous cycle. RT-PCR analysis showed expression of BMP-2, -3, -4, -7, -10, and -15 mRNA in bovine oviduct epithelial cells from ampulla and isthmus regions. Interestingly, only one of the members analyzed, BMP-5 was exclusively detected in isthmus epithelial cells, showing a differential expression in the anatomic regions of the bovine oviduct. It is known that the ampulla and isthmus are two anatomically and functionally different regions of the oviduct that may show differences in mRNA expression levels and in the synthesis and secretion of various proteins [49–51]. Therefore, region-specific expression of BMP-5 mRNA in the bovine oviduct would suggest a different regulatory mechanism that may result in a specific action in the isthmus region.

Morphologic, functional, and gene expression changes have been described in the oviduct as a consequence of the hormonal fluctuations during the estrous cycle [42,52]. To evaluate whether the differential expression of BMP-5 mRNA could be associated with a particular stage of the bovine estrous cycle, expression of this factor was analyzed in bovine oviductal epithelial cells from ampulla and isthmus regions during the Pre-Ov, Post-Ov, and Mid-L phase. Expression of BMP-2, -4, and -7 mRNA was also analyzed to determine if gene expression of BMP-5 during the estrous cycle showed a similar regulation pattern to factors belonging to the same subfamily (BMP-7) or to a closely-related subfamily (BMP-2/4) [6]. Our results showed that BMP-5 mRNA was exclusively expressed in the isthmus throughout the estrous cycle, whereas BMP-2, -4, and -7 mRNA were expressed both in ampulla and isthmus. Furthermore, highest expression of these four factors was detected during the Pre-Ov stage, suggesting a possible estrogen-regulated expression. Considering that the Pre-Ov phase is characterized by growth and differentiation of the oviduct mucosal cells to prepare for the presence of gametes and embryos, the active expression of BMP-2, -4, -5, and -7 genes in the oviductal epithelium during this stage suggests their possible participation in the events around the time of ovulation. During the Post-Ov and Mid-L phase, gene expression of BMP-5 decreased showing a similar regulation to that of BMP-7. In contrast, gene expression of BMP-2 and -4, both of which belong to another BMP subfamily, showed a different regulation. After ovulation, BMP-2 and -4 mRNA expression decreased in the ampulla but remained high in the isthmus epithelial cells, presenting a different anatomic behavior during the sexual cycle. Studies in rat uterus have shown that contrary to BMP-7, -2, and -4 mRNA expression undergo dynamic changes in the estrous cycle, and significantly low BMP-2 and high BMP-4 expression levels have been detected during the Post-Ov phase [12]. In the case of BMP-5 mRNA, to our knowledge, there is no evidence reporting an estrous cycle regulation of its expression in other reproductive tissues.

Coexpression of different BMP family members in the bovine oviduct epithelial cells could suggest a coordinated regulation with overlapping functions. It has been proven that coexpression of BMP-2, -4, -5, and -7 mRNAs can lead to the formation of heterodimers that can activate different BMP receptor combinations allowing a much more potent biological activity than that achieved through the activation of a single type of receptor [53]. *In vitro* tests have shown that these dimers, like BMP-2/7, BMP-4/7, BMP-2/5, and BMP-15/GDF-9 combinations, perform greater activity than their respective homodimers [54,55]. Consequently, it may be considered that epithelial

coexpression of BMP ligands in the bovine oviduct allows regulation of different functions by combinatorial heterodimer formation and receptor activation. However, further studies are necessary to confirm this hypothesis.

Considering the differential expression of BMP-5 mRNA in the bovine oviduct, the protein localization of this factor was evaluated by immunohistochemistry in ampulla and isthmus sections. The BMP-5 protein was detected during the estrous cycle in the isthmus epithelium, and a less positive signal was observed in the lamina propria and in muscle cells. This result is in agreement with that observed by other authors who found that the protein distribution of other BMPs was observed in all oviduct cell types but mainly in the epithelium, as has been described for BMP-4 in mouse oviduct [13]. It is important to mention that in the case of BMP-5, the intense positive staining in the luminal epithelial cell layer was restricted to the isthmus region, which agrees with the differential expression of BMP-5 mRNA previously observed in the isthmic epithelial cells obtained by scraping. Although the scraping of the oviductal epithelium can also produce removal of nonepithelial cells present in the lamina propria (fibroblast, endothelial cells, lymphocytes, etc.), the immunohistochemistry confirms that bovine oviduct epithelial cells are the principal source of BMP-5 expression. In addition, immunohistochemical analysis showed that BMP-5 was uniformly detected in the isthmic epithelium during the estrous cycle suggesting that this factor is synthesized in both ciliated and secretory epithelial cells. On the other hand, epithelial cell origin of BMP-2 mRNA was also confirmed by immunohistochemistry detection of BMP-2 in epithelial cells of both ampulla and isthmus during the estrous cycle. Differences in the epithelial distribution of BMP-2 and -5 could suggest different regulatory expression mechanisms at least for these two BMPs in the bovine oviduct.

Considering that epithelial cells play an active role in the secretion of components to form the oviductal fluid [56,57], the localization of BMP-5 in the luminal epithelium suggests that this factor could be synthesized and released into the oviductal lumen. Western blot analysis confirmed the presence of this protein in the bovine oviductal fluid, showing a 21 kDa protein during the three phases of the estrous cycle assayed. According to the deduced amino acid sequence of bovine BMP-5 (GenBank accession no. XP_ 002697216), this protein is synthesized as a 454 aa preproprotein that is cleaved to yield a mature polypeptide with a predicted MW of 16 kDa [58]. The mature molecule contains seven cysteines plus three potential N-linked glycosylation sites and because of glycosylation, the protein can migrate as a band of >16 kDa under reducing conditions in SDS-PAGE, as has been observed in the present study. This is consistent with the MW of the BMP-5 mature domain reported by other authors in humans and mice [59,60]. Although significant changes in BMP-5 mRNA levels were observed in the isthmus during the estrous cycle, there were no clear differences in the levels of protein detected by immunohistochemistry and Western blot probably because the changes in protein levels between the different stages of the estrous cycle were at the limits of detection of these techniques. Also, it is important to mention that the correlation between mRNA and protein abundance depends on various biological factors that sometimes can determine a different regulation of expression at mRNA and protein level [61].

The high expression of BMP-5 in the epithelial cells and its presence in the oviductal fluid during the estrous cycle support the hypothesis that this factor is synthesized in the oviduct epithelium and secreted into the luminal fluid, and may be present in the oviductal microenvironment during fertilization and early embryo development. However, its exact function needs to be investigated. It is known that BMP-5 is required for normal growth and patterning of skeletal structure [62], but information about its role in female reproductive mechanisms is scarce. BMP-5 is expressed in rat granulosa cells, and exerts specific biological effects on proliferation and steroidogenesis of these cells in an autocrine manner [63]. Despite its expression in the ovary, mice suffering natural or experimental loss of function mutation in BMP-5 are viable, and have no apparent reproductive abnormalities [62]. However, early embryonic lethality has been shown in double mutant mice lacking BMP-5 and BMP-7 [24]. In the uterus, BMP-5 mRNA shows expression in the stroma close to the myometrium and in the myometrial connective tissue [64]. Interestingly, Pfendler, et al. [65] provide genetic evidence that BMP-5 and the TGFβ-related protein, Nodal, play a potential role during implantation, and appear to facilitate adequate embryo spacing within the uterus, although the precise mechanism of their action is unknown.

At present, there is no information about the BMP-5 function in the oviduct of mammalian species. Considering that BMP receptors have been detected in bovine oocytes [14] and that the isthmus is involved in sperm storage and embryo transport toward the uterus [29,66], the differential expression of BMP-5 in this anatomic region and its presence in the oviductal fluid suggest a possible paracrine interaction with the gametes and the developing embryo during their transit through the oviduct. The high expression levels of BMP-5 mRNA observed in the isthmus epithelium during the periovulatory period also leaves open the question of whether this factor is involved in the molecular pathways that control the storage, selection, and capacitation of sperm in the bovine oviduct. In this context, the action of growth factors locally produced in the isthmus is not totally clear, but there are studies reporting that growth factors can regulate acrosome reaction, motility, and viability of bovine sperm [67–69]. In addition, as BMPs perform important functions in the regulation of proliferation, differentiation, and apoptosis in other reproductive tissues [45,70], BMP-5 could also play an autocrine role in the regulation of oviductal tissue remodeling during the estrous cycle.

4.1. Conclusions

The present study demonstrates for the first time that different members of the BMP family are expressed in the bovine oviduct during the estrous cycle. One of these factors, identified as BMP-5, shows differential expression in isthmus epithelial cells, and is present in the oviductal fluid during the estrous cycle. These findings suggest that this factor is produced and secreted by bovine oviduct epithelial

cells, and could act as a new autocrine and/or paracrine regulator of the reproductive events that occur in the oviductal environment. Further studies to elucidate the physiological role of the BMP system in sperm storage and/or selection, fertilization, and early embryonic development in the oviduct are currently in progress.

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Competing interests

The authors declare that they have no competing interests.

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