The Journal of Veterinary Medical Science

FULL PAPER
Theriogenology

Bone morphogenetic protein 1 is expressed in porcine ovarian follicles and promotes oocyte maturation and early embryonic development

Xiaocan LEI[1,2], Kuiqing CUI[1], Xiaoyan CAI[1], Yanping REN[1,2], Qingyou LIU[1]* and Deshun SHI[1]*

[1]State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi University, Nanning 530004, Guangxi, China
[2]School of Basic Medical Sciences, Zunyi Medical University, Zunyi 563000, Guizhou, China

ABSTRACT. In the present study, we tried to determine whether bone morphogenetic protein 1 (BMP1) plays a role in ovarian follicular development and early embryo development. We systematically investigated the expression and influence of BMP1 during porcine follicle and early embryonic development. Immunohistochemistry demonstrated that the BMP1 protein is expressed in granular cells and oocytes during follicular development, from primary to pre-ovulatory follicles, including atretic follicles and the corpus luteum. The mRNA expression of BMP1 significantly increased as the porcine follicles grew. Immunofluorescence analysis indicated that BMP1 was expressed in cumulus-oocyte complexes (COCs), oocytes and porcine embryos during early *in vitro* culture. qPCR and western blot analysis showed that the expression of BMP1 was significantly up-regulated in mature porcine oocytes and COCs compared to immature oocytes and COCs. BMP1 is expressed in early porcine embryos, and its expression reaches a peak at the 8-cell stage. To determine the effect of BMP1 on the maturation of oocytes and the development of early embryos, various concentrations of BMP1 recombinant protein or antibody were added to the *in vitro* culture media, respectively. BMP1 significantly affected the porcine oocyte maturation rate, the cleavage rate and the blastocyst development rate of embryos cultured *in vitro* in a positive way, as well as the blastocyst cell number. In conclusion, BMP1 is expressed throughout porcine ovarian follicle development and early embryogenesis, and it promotes oocyte maturation and the developmental ability of embryos during early *in vitro* culture.

KEY WORDS: BMP1, early embryo expression, follicular porcine

J. Vet. Med. Sci.
79(2): 258–266, 2017
doi: 10.1292/jvms.16-0277

Received: 30 May 2016
Accepted: 8 November 2016
Published online in J-STAGE: 25 November 2016

Bone morphogenetic proteins (BMPs) were first identified as a complex of proteins capable of inducing ectopic bone formation [2]. Further, it was found that they could regulate the differentiation of granulosa cells and delay the luteinization process [18, 25]. BMP1 was first isolated from extracts of bovine bone in association with other BMPs [32]. BMP1 differs from the other BMPs in that it is a metalloproteinase rather than a transforming growth factor beta (TGFβ)-like protein [4, 30]. This characterization is based on a unique 18-amino acid signature—HEXXHXXGFXHEXXRXDR—which includes zinc-binding motifs. According to a protein structure analysis, the BMP1 protein contains a signal peptide sequence that targets the protein for secretion, and the pro-domain binds to BMPs [17]; further, the EGF and CUB domains are characteristic of extracellular proteins, with high specificity [33, 36]. The current evidence indicates that EGF-CUB proteins, including BMP1, have extracellular functions [5, 28, 29].

To date, *BMP1* and its homologous genes have been identified in numerous species [1, 9, 22]. These genes belong to the astacin family and encode smaller proteins that contain a protease domain and have been described in fish, reptile and avian species as enzymes necessary for hatching [37]. Recently, the sheep ovary was used as a model system, and it was shown that BMP1 is expressed in sheep ovaries throughout the early fetal stages, up to adulthood (17). Further, the study showed that BMP1 was present in granulosa cells at all stages of follicular development, from primordial to large antral follicles [6]. In the chick, BMP-1/Tolloid is expressed in the early embryo in the delaminating and mesodermal cells of gastrulating embryos and later in premigratory neural crest cells, at the ectodermal neural/non-neural boundary, and in the dermatome and myotome of somites [22]. BMP1-like proteinases also reportedly play important roles in activating growth factors, such as BMP2/4 [13], growth and

*Correspondence to: Liu,Q. and Shi, D., State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi University, Nanning 530004, Guangxi, China. e-mail: qyliu2002@126.com; ardsshi@gxu.edu.cn

©2017 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

differentiation factor (GDF) GDF8 (also known as myostatin) [31], GDF11 (also known as BMP-11) [12] and transforming growth factor β1 [10] by cleaving extracellular antagonists and the potential complex.

Because BMP1 is expressed in sheep ovaries throughout the early fetal stages to adulthood and it activates various factors, such as BMP2/4 and GDF8 [12], we predicted that BMP1 may play an important role in porcine folliculogenesis and early embryogenesis. In this study, we utilized the pig as a model to systematically examine the expression pattern of BMP1 during follicular development and early *in vitro* embryonic culture. The effect of BMP1 on oocyte maturation and early embryonic development was also determined by adding BMP1 recombinant protein or antibody to the *in vitro* culture medium.

MATERIALS AND METHODS

Immunohistochemistry

Immunohistochemistry was performed as previously reported with minor adjustments [23]. Porcine ovaries were collected at the local slaughterhouse of NanNing City and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 24 hr at 4°C. The fixed porcine ovaries were dehydrated in graded ethanol, dealcoholized with xylene and embedded in paraffin. The paraffin-embedded tissues were sectioned into 6-μm thick slices, mounted onto poly-L-lysine-coated slides and dried at 45°C overnight in air. The sections were deparaffinized and rehydrated in graded ethanol, and the sections were then washed three times for 5 min in 0.1% Tween-20 in PBS on a horizontal shaker. The slides were permeabilized with 1% Triton X-100 in PBS for 30 min at room temperature, boiled in 100 mM sodium citrate (pH 6.0) three times for 6 min each at 5-min intervals for antigen retrieval and then washed in 3% hydrogen peroxide for 30 min to remove endogenous peroxidase; this was followed by blocking for 1 hr in 5% bovine serum albumin (BSA) at room temperature. The slides were first incubated overnight at 4°C with primary goat polyclonal BMP1 antibody (sc-27324, Santa Cruz Biotechnology Inc., Dallas, TX, U.S.A.) diluted to 1:200 in the blocking solution and then rewarmed for 45 min at 37°C at the next day. Following three washes with 0.1% Tween-20 in PBS, the samples were incubated with rabbit anti-goat biotin-SP-conjugated antibody (SA00004-4, Protein Tech Group Inc., Wuhan, China) diluted to 1:100 in the blocking solution for 45 min at room temperature and 45 min at 37°C. The immunoreactive signals were detected using streptavidin-horseradish peroxidase (HRP) and diaminobenzidine (DAB Map Kit, Ventana Medical Systems, Inc., Tucson, AZ, U.S.A.). The primary antibody was replaced with normal goat IgG (diluted to 1:200), which served as a negative control. After preparation, the sections were observed with an Olympus DP70 digital camera (Olympus, Tokyo, Japan) mounted on a Leica DMR microscope (Leica, Mannheim, Germany) with Nomarski optics.

Separation of follicles

To analyze each follicle independently, the ovaries from the slaughterhouse were washed two to three times in PBS under aseptic conditions, the ovarian mesenteries were removed, and the ovaries were dissected into small pieces containing complete follicles. Then, a pair of ophthalmic forceps was used to carefully separate follicles of different diameters under a stereomicroscope. We selected healthy follicles for total RNA extraction and qRT-PCR analysis; healthy follicles were identified by their pink or slightly yellow color and the presence of blood vessels all around them and follicular fluid inside. The follicles were randomly divided into four groups of different diameters: 1–2 mm, 2.5–3.5 mm, 3.5–4.5 mm and 4.5–6 mm (Supplement 1).

Oocyte recovery, in vitro maturation, activation and embryo culture

Cumulus-oocyte complexes (COCs) were aspirated from the antral follicles (2–6 mm in diameter) and cultured in 150-μl droplets of maturation medium (TCM-199 with 10% porcine follicular fluid, 0.1 mg/m*l* cysteine, 1% non-essential amino acids and 0.2 mM pyruvate) with hormonal supplementation (10 IU/m*l* eCG and 10 IU/m*l* PMSG) at 38.5°C in air containing 5% CO_2 with humidity at saturation point.

After 44 hr of *in vitro* maturation, the cumulus cells were removed by gently pipetting with a fine-bore pipette in CCM (TCM-199 containing 2% FBS and 5 mM HEPES) supplemented with 0.1% hyaluronidase and washed three times in the same medium. Oocytes with a visible polar body were selected. Briefly, the oocytes were washed and pre-incubated for 20 sec in activation medium (0.25 M mannitol solution, 0.1 g/*l* BSA, 0.5 mM HEPES, 0.1 mM $CaCl_2 \cdot 2H_2O$ and 0.1 mM $MgCl_2 \cdot 6H_2O$ [pH 7.2]) at room temperature. The oocytes were then transferred to two 0.2-mm diameter platinum electrodes with a 0.5-mm gap and covered with the activation medium in a chamber connected to an electrical pulsing machine (BTX 2000 Electro Cell Manipulator; Biotechnologies and Experimental Research Inc., San Diego, CA, U.S.A.). The oocytes were exposed to 80-μs pulses at 1.0 KV/cm DC. After activation treatment, the oocytes were thoroughly washed and then cultured in 35-μl drops of the culture medium PZM-3 (supplemented with 0.2 M Na-Pyruvate, 4.4 mM hypotaurine, 1% nonessential amino acids, 1% essential amino acids and 3 mg/m*l* BSA) for 5–7 days at 38.5°C in a humidified atmosphere containing 5% CO_2 (the medium was not changed).

To determine the effect of BMP1 on the development of oocytes and embryos, various concentrations of BMP1 recombinant protein (0, 5, 15, 25 and 50 *n*g/m*l*) or anti-BMP1 antibody (0, 25, 50, 100 and 200 *n*g/m*l*) were added to the COC culture medium.

Immunofluorescence

BMP1 was detected in PFA-fixed oocytes, COCs and parthenogenetic embryos at different stages, as described previously with slight modifications [16]. After washing three times in PBS containing 1% TritonX-100 and 0.3% BSA (TBP) for 5 min, the samples were permeabilized with 1% Triton X-100 in PBS for 10 min at RT and then blocked in PBS supplemented with 1% BSA at RT for 1 hr. The samples were incubated with the primary goat polyclonal anti-BMP1 (sc-27324, 1:200; Santa

Cruz Biotechnology Inc.) antibody at 4°C overnight. After washing the oocytes three times in TBP, they were incubated with fluorescein-conjugated rabbit anti-goat IgG (H+L) (SA00003-2, 1:100; Protein Tech Group Inc.,) for 1 hr at RT (darkness). The samples were then washed three times with TBP and counterstained with 10 μg/m*l* propidium iodide (Sigma, st. Louis, MO, U.S.A.) for 10 min at RT (darkness). After three more washes, the samples were mounted on slides with anti-fade reagent. The samples were then observed under a laser-scanning confocal microscope (Zeiss, Heidelberg, Germany).

RNA extraction and reverse transcription

Total RNA was extracted from follicles of varying diameters using Trizol reagent according to the manufacturer's protocol. The RNA concentrations were measured on a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, U.S.A.). The cDNA was synthesized with the TransScript II One-Step gDNA Removal and cDNA Synthesis SuperMix kit (Transgen Biotech, Beijing, China) according to the manufacturer's protocol.

For the oocytes, COCs and early parthenogenetic embryos, five cells from each stage were immersed in Cells-to-cDNA II cell lysis buffer (Life Technologies, Carlsbad, CA, U.S.A.) to induce lysis of the cells, and the extract was stored at −80°C until the experiment. Prior to the synthesis of cDNA, an optional DNAse step was performed to avoid DNA contamination. The reverse transcription step was then performed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's protocols. For all the samples, one cDNA reaction was performed without reverse transcriptase to check for contamination with genomic DNA.

Quantitative PCR

Quantitative PCR analyses for the gene expression level of BMP1 were carried out using the SYBR Green assay system. The PCR reaction mixtures contained 1 μl cDNA, 0.5 μM of the appropriate forward and reverse primers, and 10 μl of SYBR Green PCR Master Mix (Applied Biosystems, Froster City, CA, U.S.A.), to which RNase-free water was added to make a final volume of 20 μl. The constitutively expressed 18S gene was used as an internal control. The thermal cycling conditions included initial sample incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 30 sec. Water was substituted for the template cDNA to serve as a negative control. All samples were amplified in triplicate. The cycle threshold values (CT) indicated the quantity of the target gene in each sample, and the expression level of the target gene was determined in real time using the Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, U.S.A.). The specificity of amplification was tested at the end of the PCR with a melting-curve analysis. Real-time RT-PCR was performed using the following primers: BMP1: 5′-AAGTATCTGAGGGCTTCCA-3′ and 5′-GTCATCGGGCTTCTCGTA-3′ (which generated a 155-bp fragment); the internal control 18S: 5′-GAAGGAAGTCCAATGTCCA-3′ and 5′- GATGGGCGGCGGAAAATTG- 3′ (which generated a 97-bp fragment).

Western blotting

Porcine oocytes, COCs and parthenogenetic embryos at different stages were collected in different tubes and then frozen in liquid nitrogen and thawed three times with RIPA lysis buffer containing 0.1% PMSF. After lysis on ice for 30 min, the samples were denatured in boiling water for 10 min with SDS-PAGE loading buffer.

The samples were separated by SDS-PAGE and transferred to NC membranes (BIO-RAD Membrane, 0.22 μm) in a semi-dry transfer cell (BIO-RAD, Hercules, CA, U.S.A.). The membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% nonfat milk at 4°C overnight. After washing three times in TBST for 10 min each, the membrane was probed with specific primary goat polyclonal anti-BMP1 (AF-1927, R&D System, Minneapolis, MN, U.S.A.) antibody at 37°C for 2 hr. After washing with TBST, the membranes were incubated for 1 hr at room temperature with a 1:2,000 horseradish peroxidase-conjugated rabbit anti-goat IgG. Finally, the membranes were processed using the enhanced chemiluminescence detection system and exposed in a ChemiDoc-TM MP Imaging System (BIO-RAD).

Statistical analyses

Statistical analyses were performed with SPSS16 (IBM, Armonk, NY, U.S.A.). The relative mRNA expression level of BMP1 was calculated based on the comparative cycle threshold method as previously reported [21]. All percentile data were normalized with an arcsine transformation prior to statistical analysis. Differences between treatments with regard to the maturation rate, cleavage rate, blastocyst rate and total number of cells were analyzed using a general linear model (SAS Institute, Cary, NC, U.S.A.). Differences between groups were analyzed using one-way ANOVA and least significant difference (LSD) post-hoc test and are reported as the mean ± SEM value from three independent observations.

RESULTS

Location and expression level of BMP1 in porcine follicles

Immunohistochemistry was used to localize BMP1 within the adult porcine ovary. An anti-BMP1 antibody was used to detect BMP1 signals produced predominantly by cells in the granulosa cell layer of all follicles, from primary to pre-ovulatory follicles (Fig. 1), including atretic and luteal follicles. A strong staining signal was also observed on the membrane of the oocytes in secondary follicles (Fig. 1C and 1E). Theca cells showed faint staining or no signal (Fig. 1). Immunopositive signals were absent in negative control sections incubated with the commercial antisera (Fig. 1I).

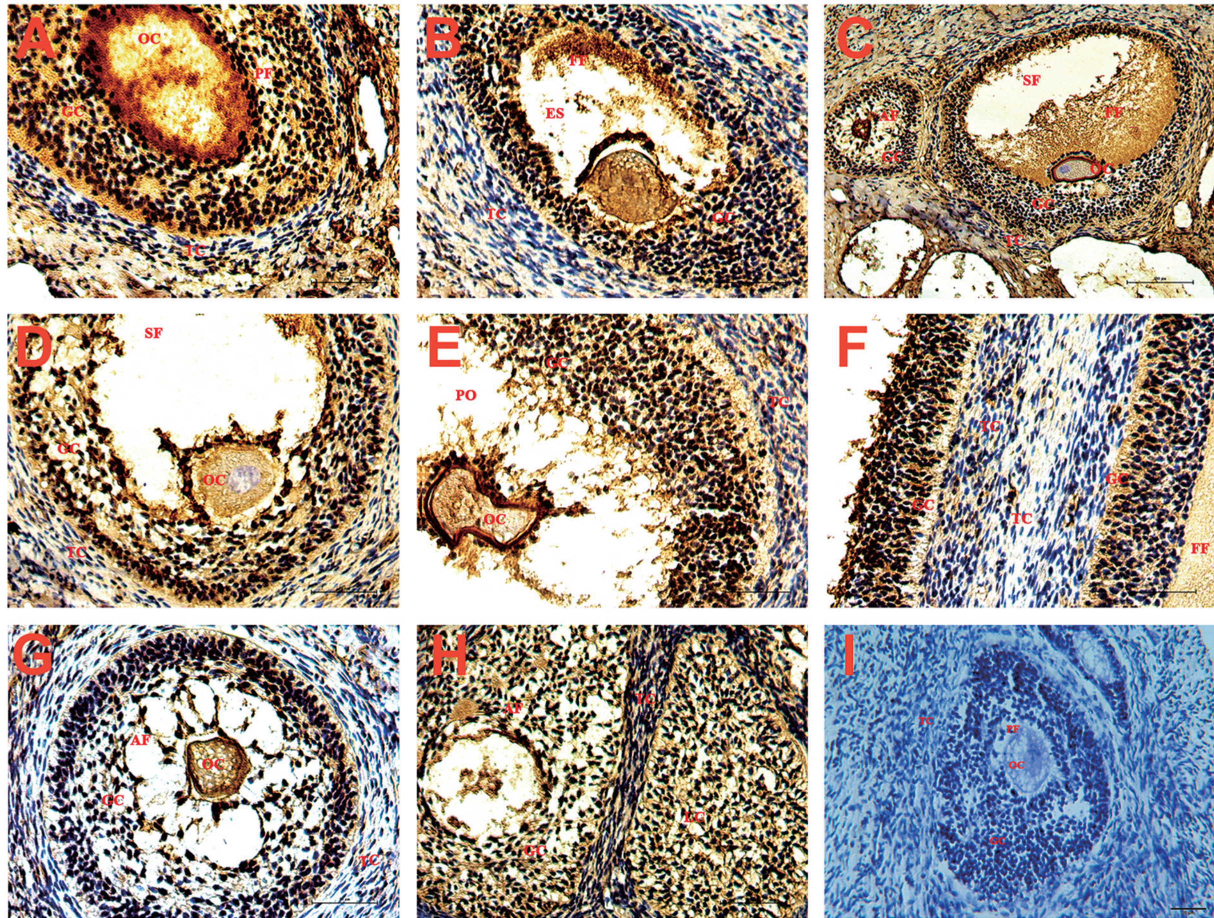

Fig. 1. Immunohistochemistry for detecting the BMP1 expression pattern in the porcine ovary. The BMP1 protein was stained brown in the presence of the BMP1 antibody (A–H) or the pre-immuno serum with diluents (negative control, I). PF: primary follicle; ES: early secondary follicle; SF: secondary follicle; PO: pre-ovulatory follicle; AF: atretic follicle; GC: granular cell; OC: oocyte; TC: theca cell; LC: luteal cell.

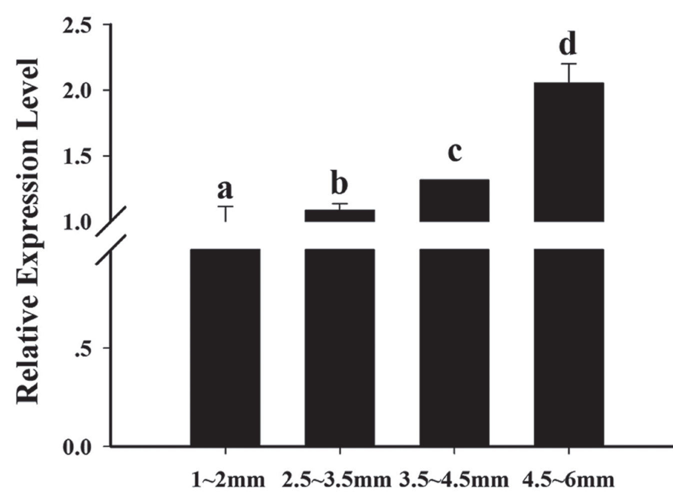

Fig. 2. The relative expression level of BMP1 in porcine follicles of different diameters by qPCR. The vertical bars represent the standard error of three replicates. The different lowercase letters indicate significant differences (P<0.05).

The expression of BMP1 was further assayed at multiple stages of follicle development by qPCR. RNA was extracted from follicles of different diameters, i.e., 1–2 mm, 2.5–3.5 mm, 3.5–4.5 mm and 4.5–6 mm. As shown in Fig. 2, the mRNA expression of BMP1 significantly varied between follicles of different sizes ($P<0.05$): it was low in the 1- to 2-mm follicles and gradually increased to reach a peak in the 4.5- to 6-mm follicles (in the 4.5- to 6-mm follicles, it was 2.05-fold the value in the 1- to 2-mm follicles, $P<0.05$).

Expression pattern and level of BMP1 in oocytes, COCs and early parthenogenetic embryos

Immunofluorescence staining was utilized to explore the expression pattern of BMP1 in oocytes, COCs and early parthenogenetic embryos. BMP1-positive fluorescence was detected in the granulosa cells of immature COCs and mature COCs (Fig. 3). Positive fluorescence signals were also observed on the membranes of mature and immature oocytes. During the development of *in vitro* cultured parthenogenetic embryos, positive fluorescence signals were detected from the 2-cell stage to the blastocyst embryo stage (Fig. 3).

The expression level of BMP1 in oocytes, COCs and early parthenogenetic embryos was also assayed using qRT-PCR and western blotting. According to qPCR analysis, the BMP1 mRNA expression of mature oocytes and COCs was significantly upregulated compared with the expression in immature oocytes and COCs (Fig. 4A and 4B: in mature COCs, the expression was 4.55-fold that in immature COCs, $P<0.05$). BMP1 was also expressed in early parthenogenetic porcine embryos; its expression first increased and reached a peak at the 8-cell stage and then declined at the blastocyst stage (in the 8-cell stage, it was 1.69-fold the expression in the 2-cell stage, $P<0.05$). Western blot analysis of the corresponding stage samples showed that expression of the BMP1 protein was up-regulated in mature oocytes and COCs. It was expressed throughout the early stages *in vitro* cultured embryos, and its expression obviously peaked at the 8-cell stage (Fig. 5). The protein expression pattern of BMP1 was consistent with that of BMP1 mRNA in porcine COCs and early parthenogenetic embryos.

Influence of BMP1 on porcine oocyte maturation and embryo development

To determine the effect of BMP1 on porcine oocyte maturation and embryo development, various concentrations of BMP1 recombinant protein (0, 5, 15, 25 and 50 *ng*/m*l*) were added to the *in vitro* maturation culture medium. BMP1 could significantly increase the porcine oocyte maturation rate (Table 1) (56.98 ± 4.31 with 0 *ng*/m*l* protein vs. 67.77 ± 1.17 with 25 *ng*/m*l* protein, $P<0.05$), the cleavage rate (78.96 ± 5.44 with 0 *ng*/m*l* protein vs. 87.22 ± 1.67 with 25 *ng*/m*l* protein, $P<0.05$) and blastocyst development rate (28.11 ± 3.13 with 0 *ng*/m*l* protein vs. 34.08 ± 4.96 with 25 *ng*/m*l* protein, $P<0.05$) of *in vitro* cultured embryos, as well as the blastocyst cell number (45 ± 2 with 0 *ng*/m*l* protein vs. 55 ± 3 with 25 *ng*/m*l* protein, $P<0.05$). On the contrary, various concentrations of BMP1 antibody (0, 25, 50, 100 and 200 *ng*/m*l*) were added to the *in vitro* maturation culture medium. The results indicated that the BMP1 antibody could significantly decrease the porcine oocyte maturation rate (Table 2) (65.71 ± 3.61 with 0 *ng*/m*l* protein vs. 36.08 ± 3.1 with 200 *ng*/m*l* protein, $P<0.05$), the cleavage rate (75.54 ± 6.2 with 0 *ng*/m*l* protein vs. 34.89 ± 2.1 with 200 *ng*/m*l* protein, $P<0.05$) and the blastocyst development rate (43.45 ± 10.7 with 0 *ng*/m*l* protein vs. 15.97 ± 7.2 with 200 *ng*/m*l* protein, $P<0.05$) of *in vitro* cultured embryos, as well as the blastocyst cell number (42 ± 4 with 0 *ng*/m*l* protein vs. 32 ± 3 with 200 *ng*/m*l* protein, $P<0.05$). These findings are presented in Table 1 and Table 2 and Supplement 2, and the sample used to count the number of blastocysts, which were stained with Hoechst, can be seen in Supplement 3. The negative effect of the BMP1 antibody on the maturation rate of oocytes, and the cleavage rate and blastocyst rate of porcine embryos appeared to be dose-dependent.

DISCUSSION

BMP1 is a vertebrate metalloproteinase of the astacin family. An increasing number of reports have indicated its ability to promote BMP signaling by releasing the BMP antagonist chordin [3, 27]. The BMP signaling pathway has also been shown to play an important role in oogenesis and embryo development. Numerous matrix metalloproteinases (MMPs) are reportedly involved in diverse aspects of ovarian function in mammals [8]. Thus, BMP1 could play a role in ovarian development by working in synergy with pro-collagen N-proteinases (namely, ADAMTS2, ADAMTS3 and ADAMTS14) to promote the deposition and maturation of collagen fibrils [11, 15, 24].

However, only one study has reported that BMP1 was present in granulosa cells at all stages of ovine follicular development, from primordial to large antral follicles, and that the final follicle selection mechanism did not affect the levels of BMP1 [6]. In the present study, the location and expression pattern of BMP1 in different stages of porcine follicle development were first examined. These results are consistent with observations in sheep [6], except that the positive signal was stronger in oocytes than in granular cells. Members of the BMP family [35], such as BMP2, BMP4 [38], BMP6 [26], BMP7 [20] and BMP15, are strongly expressed in granular cells and oocytes during follicular development in different animals. However, the relative BMP1 mRNA expression gradually increased in healthy porcine follicles as the diameter increased (2.05-fold difference between the 4.5–6 mm and 1–2 mm follicles), while the level of BMP1 decreased in large ovine follicles [6]. This finding reflects those in MMPs, such as MMP3 and MMP9, which are more strongly expressed as chicken follicles mature [39]. Taken together, these data indicate that BMP1 may play an important role in follicle development in pigs, especially during the process of follicle dominance and selection, as well as during the atresia of follicles.

This study is the first to show the presence and expression pattern of BMP1 in denuded oocytes, COCs, mature denuded oocytes, mature COCs and early embryos using immunofluorescence. A clear positive staining signal was observed in granular

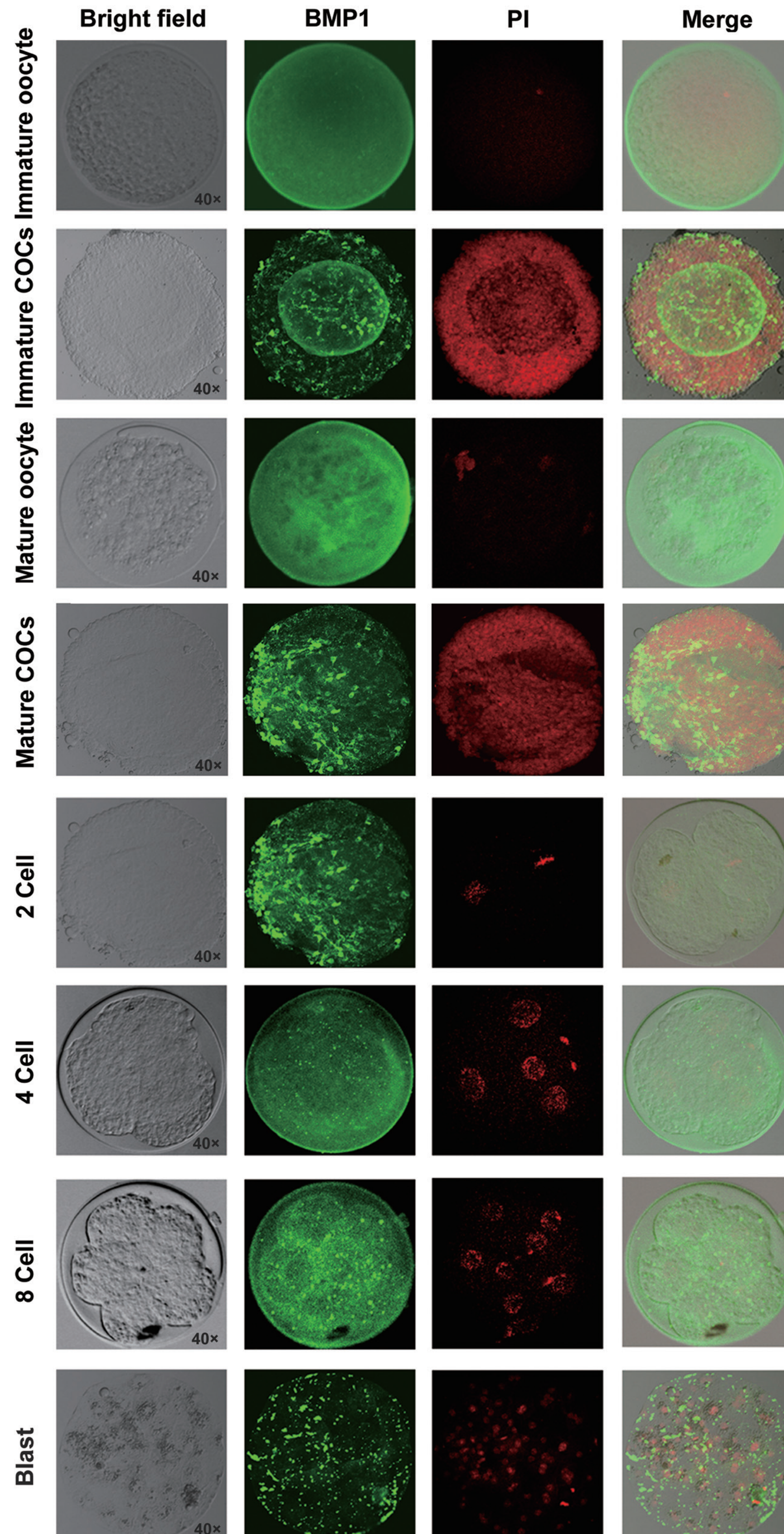

Fig. 3. Immunofluorescence staining results for porcine oocytes, cumulus–oocyte complexes (COCs) and embryos. Immunofluorescence staining using an anti-BMP1 antibody at different stages of oocyte and embryo development as observed by a confocal microscope. The yellow color corresponds to the merging of fluorescein (green) and propidium iodide (red) staining; Blast, blastocyst.

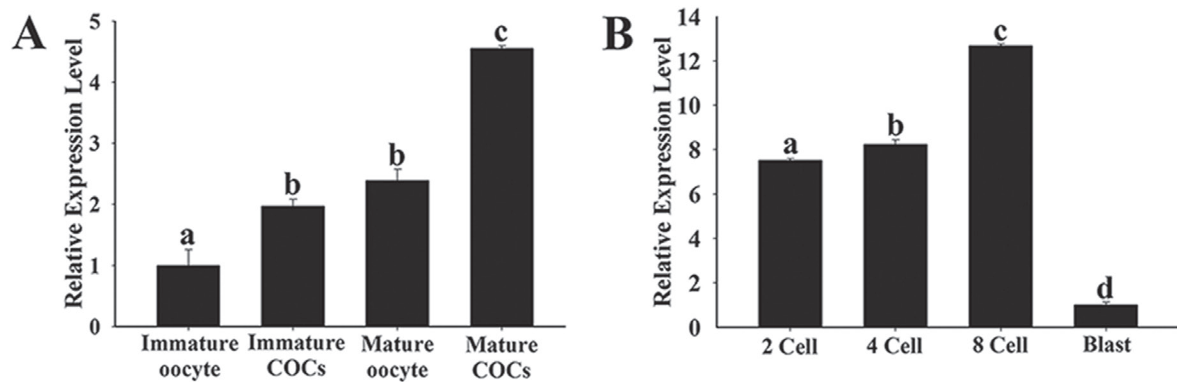

Fig. 4. The relative expression level of BMP1 in porcine oocytes and cumulus oocyte complexes (COCs), as well as parthenogenetic embryos by qPCR. The vertical bars represent the standard error of three replicates. The different lowercase letters indicate significant differences (P<0.05).

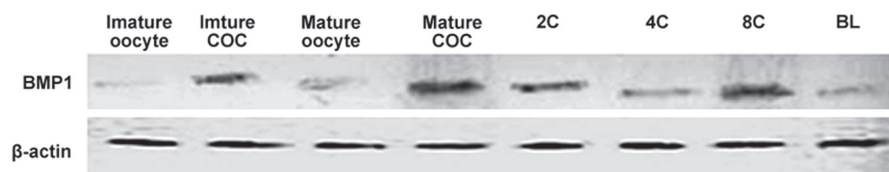

Fig. 5. Western blot analysis showed the expression of BMP1 in porcine oocytes and cumulus–oocyte complexes (COCs), as well as in parthenogenetic embryos.

Table 1. Effect of different concentrations of BMP1 recombinant protein on oocyte *in vitro* maturation and embryo development (Mean ± SEM). All developmental rates are in percentages

BMP1 concentration (*ng*/m*l*)	Total No. of oocytes used	Maturation % (n)	Development ability		
			Cleavage rate % (n)	Blastocysts rate % (n)	Cell number (n)
0	254	56.98 ± 4.31$^{a)}$ (144)	78.96 ± 5.44$^{a)}$ (113)	28.11 ± 3.13$^{a)}$ (32)	45 ± 2$^{a)}$ (25)
5	241	55.50 ± 4.14$^{a)}$ (133)	80.59 ± 5.15$^{a)}$ (107)	26.03 ± 2.96$^{a)}$ (28)	48 ± 3$^{a,b)}$ (26)
15	206	60.96 ± 2.30$^{a,b)}$ (126)	81.81 ± 3.03$^{a,c)}$ (103)	31.28 ± 4.91$^{a)}$ (32)	49 ± 3$^{b)}$ (24)
25	235	67.77 ± 1.17$^{b)}$ (159)	87.22 ± 1.67$^{b,c)}$ (139)	34.08 ± 4.96$^{b)}$ (47)	55 ± 3$^{c)}$ (23)
50	231	56.90 ± 2.09$^{a)}$ (131)	73.02 ± 3.18$^{d)}$ (95)	23.31 ± 4.16$^{c)}$ (22)	44 ± 3$^{a,d)}$ (20)

There is a significant difference (P<0.05) between the top and bottom results. Data were analyzed by using the one-way ANOVA. Three replications were used for detection of the effect.

Table 2. Effect of different concentrations of BMP1 antibody on oocyte *in vitro* maturation and embryo development (Mean ± SEM). All developmental rates are in percentages

BMP1 concentration (*ng*/m*l*)	Total No. of oocytes used	Maturation % (n)	Development ability		
			Cleavage rate % (n)	Blastocysts rate % (n)	Cell number (n)
0	242	65.71 ± 3.61$^{a)}$ (159)	75.54 ± 6.2$^{a)}$ (120)	43.45 ± 10.7$^{a)}$ (52)	42 ± 4$^{a)}$ (32)
25	236	63.89 ± 3.15$^{a)}$ (151)	59.39 ± 0.7$^{a,b)}$ (90)	37.69 ± 9.1$^{a)}$ (34)	41 ± 4$^{a)}$ (23)
50	229	55.95 ± 2.3$^{b)}$ (128)	52.28 ± 1.5$^{b)}$ (67)	28.44 ± 0.5$^{b)}$ (19)	34 ± 6$^{b)}$ (15)
100	228	41.84 ± 1.2$^{c)}$ (95)	48.02 ± 5.4$^{b)}$ (46)	26.01 ± 2.9$^{b,c)}$ (12)	33 ± 2$^{b)}$ (12)
200	226	36.08 ± 3.1$^{d)}$ (82)	34.89 ± 2.1$^{c)}$ (29)	15.97 ± 7.2$^{d)}$ (5)	32 ± 3$^{b)}$ (5)

There is a significant difference (P<0.05) between the top and bottom results. Data were analyzed by using the one-way ANOVA. Three replications were used for detection of the effect.

cells, oocytes and early embryos. We further assessed BMP1 mRNA and protein expression by qPCR and western blotting, the results of which were consistent. BMP1 expression was up-regulated at the mRNA and protein levels in mature porcine COCs. In early porcine embryos, the BMP1 expression level first increased, reached a peak at the 8-cell stage and then declined at the

blastocyst stage. However, matrix metalloproteinase-8 (MMP8) reportedly enhances vascular smooth muscle cell migration and proliferation [34], and matrix metalloproteinase-1 (MMP1) promotes lung alveolar epithelial cell proliferation/migration, protects these cells from apoptosis and represses the oxygen consumption rate and ROS production [14]. The above findings indicated that BMP1 may act as a metalloproteinase to improve embryonic development, especially at the 8 cell-stage, which is an essential stage of embryonic differentiation. Furthermore, in one study, BMP1 was purified with BMP2 extracted from bone [33], and it activated BMP2/4, which participates in *in vitro* oocyte maturation and embryo development. We hypothesize that the endogenous expression of BMP1 could play an important role in the maturation of porcine oocytes and embryonic development.

Thus, to evaluate the effect of BMP1 on porcine oocyte maturation, different concentrations of BMP1 recombinant protein or BMP1 antibody were added to the *in vitro* maturation culture medium. The addition of BMP1 recombinant protein (25 *n*g/m*l*) to the maturation medium increased the extrusion of polar bodies and improved the maturation rate of porcine oocytes, blastocyst rates and embryo quality *in vitro* by ameliorating the total number of cells. The BMP1 antibody exerted the opposite effect. Moreover, oocytes may have undergone apoptosis or stopped growing in response to 300 *n*g/m*l* and 400 *n*g/m*l* of BMP1 antibody; the mature oocytes at these two concentrations of BMP1 antibody can be observed in Supplement 3. We inferred that the oocyte membranes may contain a crucial substrate for BMP1 (Supplement 4). Because BMP1 was found at the center of a putative feedback loop that orchestrates TGF-β super-family ligand signaling in the ovary, it seems that BMP1, a candidate gene that belongs to the astacin family, regulates TGF-β signaling during oogenesis and embryo development in vertebrates [7, 10, 19].

In conclusion, the expression pattern of BMP1 metalloproteinases provides a solid base to further probe the function of this enzyme during follicular development and embryogenesis. Overall, these observations suggest a new physiological role for BMP1 metalloproteinases in the porcine ovary.

ACKNOWLEDGMENTS. This work was supported by a research grant from the China High-tech 863 Project (2013AA102504), National Natural Science Fund (NSFC 31260552), Innovation Project of Guangxi Graduate Education (YCBZ2014001) and Discipline Construction Project of Zunyi Medical University (0000015). Xiaocan Lei, Kuiqing Cui and Xiaoyan Cai contributed equally to this work. The authors have no conflicts of interest to declare that could be perceived as prejudicing the impartiality of the research reported.

REFERENCES

1. Balaban, N., Rudakova, N. and Sharipova, M. 2013. Structural features and functional importance of metzincin metalloproteinases. *Russ. J. Bioorganic Chem.* **39**: 493–497. [CrossRef]
2. Beederman, M., Lamplot, J. D., Nan, G., Wang, J., Liu, X., Yin, L., Li, R., Shui, W., Zhang, H., Kim, S. H., Zhang, W., Zhang, J., Kong, Y., Denduluri, S., Rogers, M. R., Pratt, A., Haydon, R. C., Luu, H. H., Angeles, J., Shi, L. L. and He, T. C. 2013. BMP signaling in mesenchymal stem cell differentiation and bone formation. *J. Biomed. Sci. Eng.* **6** 8A: 32–52. [Medline] [CrossRef]
3. Blader, P., Rastegar, S., Fischer, N. and Strähle, U. 1997. Cleavage of the BMP-4 antagonist chordin by zebrafish tolloid. *Science* **278**: 1937–1940. [Medline] [CrossRef]
4. Bond, J. S. and Beynon, R. J. 1995. The astacin family of metalloendopeptidases. *Protein Sci.* **4**: 1247–1261. [Medline] [CrossRef]
5. Bork, P. and Beckmann, G. 1993. The CUB domain. A widespread module in developmentally regulated proteins. *J. Mol. Biol.* **231**: 539–545. [Medline] [CrossRef]
6. Canty-Laird, E., Carré, G. A., Mandon-Pépin, B., Kadler, K. E. and Fabre, S. 2010. First evidence of bone morphogenetic protein 1 expression and activity in sheep ovarian follicles. *Biol. Reprod.* **83**: 138–146. [Medline] [CrossRef]
7. Chang, C. and Hemmati-Brivanlou, A. 1998. Cell fate determination in embryonic ectoderm. *J. Neurobiol.* **36**: 128–151. [Medline] [CrossRef]
8. Curry, T. E. Jr. and Osteen, K. G. 2003. The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. *Endocr. Rev.* **24**: 428–465. [Medline] [CrossRef]
9. Dale, L., Evans, W. and Goodman, S. A. 2002. Xolloid-related: a novel BMP1/Tolloid-related metalloprotease is expressed during early Xenopus development. *Mech. Dev.* **119**: 177–190. [Medline] [CrossRef]
10. Ge, G. and Greenspan, D. S. 2006. BMP1 controls TGFbeta1 activation via cleavage of latent TGFbeta-binding protein. *J. Cell Biol.* **175**: 111–120. [Medline] [CrossRef]
11. Ge, G. and Greenspan, D. S. 2006. Developmental roles of the BMP1/TLD metalloproteinases. *Birth Defects Res. C* **78**: 47–68.
12. Ge, G., Hopkins, D. R., Ho, W. B. and Greenspan, D. S. 2005. GDF11 forms a bone morphogenetic protein 1-activated latent complex that can modulate nerve growth factor-induced differentiation of PC12 cells. *Mol. Cell. Biol.* **25**: 5846–5858. [Medline] [CrossRef]
13. Goodman, S. A., Albano, R., Wardle, F. C., Matthews, G., Tannahill, D. and Dale, L. 1998. BMP1-related metalloproteinases promote the development of ventral mesoderm in early Xenopus embryos. *Dev. Biol.* **195**: 144–157. [Medline] [CrossRef]
14. Herrera, I., Cisneros, J., Maldonado, M., Ramírez, R., Ortiz-Quintero, B., Anso, E., Chandel, N. S., Selman, M. and Pardo, A. 2013. Matrix metalloproteinase (MMP)-1 induces lung alveolar epithelial cell migration and proliferation, protects from apoptosis, and represses mitochondrial oxygen consumption. *J. Biol. Chem.* **288**: 25964–25975. [Medline] [CrossRef]
15. Hopkins, D. R., Keles, S. and Greenspan, D. S. 2007. The bone morphogenetic protein 1/Tolloid-like metalloproteinases. *Matrix Biol.* **26**: 508–523. [Medline] [CrossRef]
16. Hussein, T. S., Sutton-McDowall, M. L., Gilchrist, R. B. and Thompson, J. G. 2011. Temporal effects of exogenous oocyte-secreted factors on bovine oocyte developmental competence during IVM. *Reprod. Fertil. Dev.* **23**: 576–584. [Medline] [CrossRef]
17. Jasuja, R., Ge, G., Voss, N. G., Lyman-Gingerich, J., Branam, A. M., Pelegri, F. J. and Greenspan, D. S. 2007. Bone morphogenetic protein 1 prodomain specifically binds and regulates signaling by bone morphogenetic proteins 2 and 4. *J. Biol. Chem.* **282**: 9053–9062. [Medline] [CrossRef]
18. Knight, P. G. and Glister, C. 2003. Local roles of TGF-β superfamily members in the control of ovarian follicle development. *Anim. Reprod. Sci.* **78**: 165–183. [Medline] [CrossRef]

19. Lee, S., Solow-Cordero, D. E., Kessler, E., Takahara, K. and Greenspan, D. S. 1997. Transforming growth factor-β regulation of bone morphogenetic protein-1/procollagen C-proteinase and related proteins in fibrogenic cells and keratinocytes. *J. Biol. Chem.* **272**: 19059–19066. [Medline] [CrossRef]
20. Lee, W. S., Yoon, S. J., Yoon, T. K., Cha, K. Y., Lee, S. H., Shimasaki, S., Lee, S. and Lee, K. A. 2004. Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. *Mol. Reprod. Dev.* **69**: 159–163. [Medline] [CrossRef]
21. Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-ΔΔC(T)) Method. *Methods* **25**: 402–408. [Medline] [CrossRef]
22. Martí, E. 2000. Expression of chick BMP-1/Tolloid during patterning of the neural tube and somites. *Mech. Dev.* **91**: 415–419. [Medline] [CrossRef]
23. Nanjidsuren, T., Yun, S. J., Park, C. W., Kim, M. S., Kang, M. H. and Min, K. S. 2014. Expression and localization of 20α-hydroxysteroid dehydrogenase (20α-HSD) in porcine reproductive tissues during pregnancy. *Anim. Reprod. Sci.* **148**: 63–71. [Medline] [CrossRef]
24. Ohnishi, J., Ohnishi, E., Shibuya, H. and Takahashi, T. 2005. Functions for proteinases in the ovulatory process. *BBA-Proteins Proteom.* **1751**: 95–109.
25. Otsuka, F. 2013. Multifunctional bone morphogenetic protein system in endocrinology. *Acta Med. Okayama* **67**: 75–86. [Medline]
26. Otsuka, F., Moore, R. K. and Shimasaki, S. 2001. Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. *J. Biol. Chem.* **276**: 32889–32895. [Medline] [CrossRef]
27. Petropoulou, V., Garrigue-Antar, L. and Kadler, K. E. 2005. Identification of the minimal domain structure of bone morphogenetic protein-1 (BMP-1) for chordinase activity: chordinase activity is not enhanced by procollagen C-proteinase enhancer-1 (PCPE-1). *J. Biol. Chem.* **280**: 22616–22623. [Medline] [CrossRef]
28. Tsao, K. C., Tu, C. F., Lee, S. J. and Yang, R. B. 2013. Zebrafish scube1 (signal peptide-CUB (complement protein C1r/C1s, Uegf, and Bmp1)-EGF (epidermal growth factor) domain-containing protein 1) is involved in primitive hematopoiesis. *J. Biol. Chem.* **288**: 5017–5026. [Medline] [CrossRef]
29. Tu, C.-F., Tsao, K.-C., Lee, S.-J. and Yang, R.-B. 2014. SCUBE3 (Signal Peptide-CUB-EGF Domain-containing Protein 3) Modulates Fibroblast Growth Factor Signaling during Fast Muscle Development. *J. Biol. Chem.* jbc. M114. 551929.
30. Ugalde, A. P., Ordóñez, G. R., Quirós, P. M., Puente, X. S. and López-Otín, C. 2010. Metalloproteases and the degradome. *Methods Mol. Biol.* **622**: 3–29. [Medline] [CrossRef]
31. Wolfman, N. M., McPherron, A. C., Pappano, W. N., Davies, M. V., Song, K., Tomkinson, K. N., Wright, J. F., Zhao, L., Sebald, S. M., Greenspan, D. S. and Lee, S. J. 2003. Activation of latent myostatin by the BMP-1/tolloid family of metalloproteinases. *Proc. Natl. Acad. Sci. U.S.A.* **100**: 15842–15846. [Medline] [CrossRef]
32. Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M. and Wang, E. A. 1988. Novel regulators of bone formation: molecular clones and activities. *Science* **242**: 1528–1534. [Medline] [CrossRef]
33. Wu, B. T., Su, Y. H., Tsai, M. T., Wasserman, S. M., Topper, J. N. and Yang, R. B. 2004. A novel secreted, cell-surface glycoprotein containing multiple epidermal growth factor-like repeats and one CUB domain is highly expressed in primary osteoblasts and bones. *J. Biol. Chem.* **279**: 37485–37490. [Medline] [CrossRef]
34. Xiao, Q., Zhang, F., Grassia, G., Hu, Y., Zhang, Z., Xing, Q., Yin, X., Maddaluno, M., Drung, B., Schmidt, B., Maffia, P., Ialenti, A., Mayr, M., Xu, Q. and Ye, S. 2014. Matrix metalloproteinase-8 promotes vascular smooth muscle cell proliferation and neointima formation. *Arterioscler. Thromb. Vasc. Biol.* **34**: 90–98. [Medline] [CrossRef]
35. Yamamoto, Y. and Oelgeschläger, M. 2004. Regulation of bone morphogenetic proteins in early embryonic development. *Naturwissenschaften* **91**: 519–534. [Medline] [CrossRef]
36. Yang, R. B., Ng, C. K. D., Wasserman, S. M., Colman, S. D., Shenoy, S., Mehraban, F., Kömüves, L. G., Tomlinson, J. E. and Topper, J. N. 2002. Identification of a novel family of cell-surface proteins expressed in human vascular endothelium. *J. Biol. Chem.* **277**: 46364–46373. [Medline] [CrossRef]
37. Yasumasu, S., Mao, K. M., Sultana, F., Sakaguchi, H. and Yoshizaki, N. 2005. Cloning of a quail homologue of hatching enzyme: its conserved function and additional function in egg envelope digestion. *Dev. Genes Evol.* **215**: 489–498. [Medline] [CrossRef]
38. Zhu, G., Cui, Y., Wang, Q., Kang, Y., Lv, Y., Wang, J., Song, Y.and Cao., B. Guangqin, Z., Yihong, C., Jiangang, W., Yuxuan, S. and Binyun, C. 2013. Bone morphogenetic proteins (BMP) 2, 4, 6 and 7 affect ovarian follicular development through regulation of follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) expression in goat granulosa cells. *J Cell. Biol. Genet.* **3**: 14–21. [CrossRef]
39. Zhu, G., Kang, L., Wei, Q., Cui, X., Wang, S., Chen, Y. and Jiang, Y. 2014. Expression and Regulation of MMP1, MMP3, and MMP9 in the Chicken Ovary in Response to Gonadotropins, Sex Hormones, and TGFB1. *Biol. Reprod. biolreprod.* **113**.114249.