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BMP-1 participates in the selection and dominance of buffalo follicles by regulating the proliferation and apoptosis of granulosa cells

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ABSTRACT

BMP1/TLD-related metalloproteinases play a key role in morphogenesis *via* the proteolytic maturation of a number of extracellular matrix proteins and the activation of a subset of growth factors of the transforming growth factor beta superfamily. Recent data indicated that *BMP1* is expressed in sheep ovarian follicles and showed a protease activity. The aim of the present study was to characterize the function of the buffalo *BMP1* gene in folliculogenesis. A 3195-bp buffalo *BMP1* mRNA fragment was firstly cloned and sequenced, which contained a whole 2967-bp codon sequence. The multialigned results suggested that *BMP1* is highly conserved among different species both at the nucleic acid and the amino acid level. *BMP1* is located in the oogonium of the fetal buffalo ovary and in the granulosa cells (GCs) and the oocytes of adult ovary from the primordial to the large antral follicles. Further study showed that *BMP1* promoted cell cycle and proliferation and inhibited apoptosis in IVC GCs. Adding BMP1 recombinant protein to the culture medium of the GCs increased the expression of the key cell cycle regulators such as *cyclin D1* and *cyclin D2* and downregulated the expression of cell apoptosis pathway genes such as *Cytochrome C*, *Fas*, *FasL*, and *Chop*, both at the mRNA and at the protein levels. It also upregulated the expression of *PAPP-A*, *IGF* system, and *VEGF*, and so forth, which play important roles in the selection and dominance of growth follicles. The opposite results were observed by adding BMP1 antibody to the investigation groups. This study suggests that *BMP1* regulates the proliferation and apoptosis of IVC GCs by changing the expression pattern of related genes and may potentially promote the selection and dominance of the buffalo follicles.

1. Introduction

Bone morphogenetic proteins (BMPs) comprise an extensive group of phylogenetically conserved growth factors, of which, over 20 members have been identified [1]. Bone morphogenetic protein 1 (*BMP1*) was originally identified in bone extracts capable of inducing bone formation at ectopic sites [2]. Unlike the other BMPs such as *BMP2* and *BMP4*, which belong to the transforming growth factor beta (*TGF-β*) super family, *BMP1* is a zinc-dependent metalloproteinase that belongs to the astacin family [3]. *BMP1* has been reported in different species, including human [4], mouse [5], xenopus [6], drosophila [7], sea urchin [8], and chick [9], as having a similar structure. It contains an NH_2-terminal activation region and an astacin-like protease domain and is followed by different numbers of EGF-like motifs and a CUB protein–protein interaction domains [5], and a mammalian homolog of tolloid was

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identified (mTLD), which turned out to be a splice variant encoded by the same gene as *BMP1* [10].

BMP1 is expressed and has distinct temporal functions in different isoforms during the morphogenesis [11,12]. Three *XBMP1* transcripts (2.9, 5.2, and 6.6 kb) were found in the blastula and gastrula stages of xenopus by Northern blot [6], which increased gradually from the morula to the swimming tadpole stages [7]. The overexpression of *BMP1* in early xenopus embryos inhibited the development of dorsoanterior structures [13]. In sea urchin, the highest level of *suBMP-1* mRNA was expressed at the hatched blastula stage and was located on the surface of all cell types in late gastrula stage of the embryos, which suggested that *suBMP-1* is a secreted protein that subsequently associates with a cell surface component [8]. Furthermore, *BMP1*-null mice generated a syndrome of a persistent herniation of the gut in the umbilical region and were embryonic lethal [14,15].

BMP1 is the prototype of a family of putative proteases that is implicated in pattern formation during development in diverse organisms [16]. *BMP1* cleaves human and mouse *IGFBP3* at a single conserved site, resulting in a markedly reduced ability of cleaved *IGFBP3* to bind insulin-like growth factors-I (*IGF-I*) or to block *IGF-I*–induced cell signaling. In contrast, such cleavage is shown to result in the enhanced *IGF-I*–independent ability of cleaved *IGFBP3* to block fibroblast growth factor (*FGF*)-induced proliferation and to induce Smad phosphorylation [17]. *BMP1* plays a major role in the cleavage of latent *TGF-β* binding proteins, which releases the complex formed by *TGF-β1* and LAP (propeptide) from the extracellular matrix (ECM) [18]. It was also shown that *BMP1* contributes to maintaining high levels of active *TGF-β1* in tissues by promoting the degradation of two *TGF-β* antagonists (soluble betaglycan and CD109 [19]). *BMP1* activates several other members of the *TGF-β* or *IGF* superfamilies, such as *GDF-8/11* [20,21], *BMP-2/4* (chordin; [22,23]), and *IGF-1/2* (*IGFBP3*, [24]). Similarly, *BMP1* cleaves several proteins, including endorepellin, which is endowed with strong angiogenic properties [25]. In addition, *BMP1* can turn prolactin and growth hormone into potent antiangiogenic molecules [26,27] and abolish the prometastatic potential of angiopoietin-like protein 2 [28]. Recently, it was reported that *BMP1* was present in granulosa cells (GCs) at all stages of sheep follicular development both at the mRNA and the protein level [29], which suggested a new physiological role for *BMP1* metalloproteinases in mammalian folliculogenesis, but the underlying mechanisms need to be explored.

Buffalo is one of the most important domestic animals distributed in tropical and subtropical regions and provides better milk, meat, and draft for agriculture [30]. The Chinese swamp buffalo has a lower reproductive ability, and this limits the production of this species. Therefore, there is an urgent need to improve their production traits by genetic manipulation technology and to determinate the function of more genes involved in the folliculogenesis of buffalo. To our knowledge, expression pattern and function of *BMP-1* in the folliculogenesis of swamp buffalo were seldom reported. The present study was performed to investigate the expression pattern of *BMP-1* and its function during the folliculogenesis of the swamp buffalo.

2. Materials and methods

2.1. Cloning and analysis of buffalo BMP1

Three adult swamp buffalo ovaries were collected from the local slaughterhouse, and the total RNA was extracted using the Trizol reagent (Ambion, Life Technologies, NY, USA) according to the manufacturer's instruction. Three independent preparations were used. The first-stranded cDNA was synthesized from 2 μg of total RNA for RT-PCR by using the Prime Script 1st strand cDNA synthesis kit (Takara, Japan). A pair of specific primers (F:5′-CAGTCCTCCGCTTCCC-3′ and R:5′-GTCTCCCATCCCTGCC-3′) were designed based on the sequence of bovine BMP1 (XP_002689817.1). Then, a touchdown PCR was performed with annealing temperatures from 61 °C to 55 °C by going down 2 °C with each touchdown. All assessments were conducted in three biological replicates. The PCR products were purified using a TIAN Gen Mini Purification Kit (TIANGEN Biotech; Beijing CO., Ltd, Beijing, China), inserted into the pMD18-T vector (Takara, Japan) and transformed into DH 5α *Escherichia coli* (stored in the laboratory). The positive clones were sequenced by the automated sequencing method (BGI-Guangzhou, China).

The alignment of the nucleotide sequences was established with the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The open reading frame and protein prediction were performed using the NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The protein domain architecture was predicted on http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1. The alignment of the highly conserved sequence, the Zn^{2+}-binding sequence, in the signal peptide and metalloendopeptidase was performed onsite (http://www.uniprot.org/). The multialignments were carried out on Clustalx 1.83 (Conway Institute UCD, Dublin) and GeneDoc software (Pittsburgh Supercomputing Center), and a phylogenetic tree was constructed using the Neighbor-Joining method with the MEGA 5 program and visualized by TreeView software.

2.2. Immunohistochemistry

Fetal and adult swamp buffalo ovaries were obtained from the local slaughter house. They were fixed in 4% paraformaldehyde (PFA; P-6148, sigma), dehydrated, and paraffin embedded. Serial sections of 5-μm thickness were cut using a Leica RM 2235 rotary microtome. The sections were processed in 1:49 APES: acetone, deparafinized, and rehydrated. Then, the sections were incubated with 3% hydrogen peroxide in methanol, boiled in 10-mM sodium citrate buffer, and permeabilized in 1% Triton X-100. After blocking with 5% BSA, the sections were incubated with the goat polyclonal *BMP1* (sc-27324, SANTA) antibody (diluted 1:100 in 1% Tween-20 in PBS [PBS-T]) at 4 °C overnight. After three 5-minute washes with PBS-T, the sections were incubated with rabbit antigoat Biotin-SP-conjugated antibody (1:100, SA00004–4, Protein Tech Group, Inc., Wuhan) and Peroxidase-conjugated Streptavidin (1:100, SA00001–0, Protein Tech Group, Inc.) separately and followed another 5-minute washes in PBS-T. The sections were incubated with a DAB color development kit for 2 minutes and then counterstained with hematoxylin at room temperature