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Expression Patterns of *BMP15* Gene in Folliculogenesis of Buffalo (*Bubalus bubalis*)

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Authors' Contribution
QL designed the experiment. JL cloned the *BMP15* gene and drafted the manuscript. YC analyzed the expression pattern of *BMP15* gene in folliculogenesis. TF analyzed the data. CL analyzed the expression pattern of *BMP15* gene in embryogenesis. PZ did the immunohistochemistry work. LS revised the manuscript.

Key words
Buffalo, *BMP15*, Expression pattern, Folliculogenesis, Embryogenesis.

ABSTRACT

In the present study, *BMP15* gene of buffalo was cloned, analyzed, and its' expression pattern was further determined. It consists of 1185 nucleotides that encodes 394 peptides bond. The *BMP15* gene was observed to be expressed in cumulus tissue, granular tissue, hypophysis, genital ridge and tissues of ovary and testis. Moreover, QRT-PCR results showed that *BMP15* was expressed in the whole process of embryogenesis and folliculogenesis, early high level and then down regulated. It was significantly expressed higher level in COCs of middle diameter sized follicles than that of small and large sized follicles. *BMP15* gene expression enhanced until morula stage but it fell sharply at blastula stage. Immunohistochemistry exhibited *BMP15* protein was located in germ cells of testis, in primordial granulosa cells, primary, secondary, and antral follicles of ovary, and none in theca cells. The more conspicuous reaction for *BMP15* was observed in germ cells than cumulus cells and granulosa cells, particularly in primordial germ cells of genital ridge or in foetus ovary of buffalo. The expression pattern of *BMP15* suggested that it may play a key role in the formation of primordial follicles as well as in the development and maintaince of early embryos in buffalo.

INTRODUCTION

In Asia, livestock including buffalo has been considered a vital part of agriculture for over 5000 years. But, in spite of its pronounced significance in the fiscal zone, it makes available milk, meat, and draft for agriculture, buffalo remains quite ignored. The reproductive efficacy also remains deprived chiefly due to late puberty, reduced expression of estrus, summer anoestrus, long postpartum service period and low conception rate (Singh, 2012). So to shed light on the mechanism of folliculogenesis and embryogenesis are central for enlightening the reproductive efficacy of buffalo. The method of ovarian folliculogenesis consists of production and distinction of the constitutive cells in developing germ cells. Ovarian folliculogenesis is categorized by the development of oocytes from the primordial germ cells stage to the mature ovulating Grafian follicle stage.

The whole process is organized by both extra ovarian elements, *e.g.* pituitary gonadotropins, and locally produced paracrine factors, bidirectional interaction between the oocyte and the adjacent somatic cells, it is broadly acknowledged that oocyte directs the differentiation of granulosa cell and promote the development of follicles. Oocyte is able to secrete the solvable paracrine growth factors (such as *BMP15* and GDF9) by performing on its immediate granulosa cells, in turn to control the self-development of oocyte. In pre-antral follicles, the oocyte leads granulosa cells to control the development of oocyte. The interaction of oocyte-cumulus cell has been described to accomplish the prevention of cumulus cell luteinization and regulating steroidogenesis, inhibit the synthesis and suppressing LH receptor manifestation.

Bone morphogenetic protein 15 (*BMP15* also known as GDF9B) are interrelated members of the transforming

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development element b (TGFb) superfamily that are formed by the ovary and have intense effects on fertility (Elvin *et al*., 2000; McNatty, 2003). In mice, GDF9 (Dong, 1996) but not *BMP15* (Yan, 2001) is crucial for usual follicular growth. But, in sheep, both GDF9 and *BMP15* are vital for typical follicular development (Juengel, 2002). Several studies (Otsuka *et al*., 2000, 2001; Otsuka and Shimasaki, 2002) also recognized three most important biological purposes of *BMP15* in the ovary. Firstly, *BMP15* is a forceful stimulator of GC mitosis; Secondly, *BMP15* prevents the manifestation of FSH receptor mRNA in GCs, causing the consequent suppression of FSH-induced progesterone synthesis as well as FSH-induced manifestation of a battery of mRNAs in GCs; and Thirdly, *BMP15* excites the expression of kit ligand mRNA in GCs. The significance of *BMP15* in fertility in sheep was clarified that naturally taking place alterations in the *BMP15* gene in Inverdale ($FecX^{I}$) and Hanna ($FecX^{H}$) ewes triggered the rise in the rate of ovulation in heterozygotes due, in part, to an increased FSH sensitivity of GCs but initiated infertility in homozygotes due to a block in the primary stage of folliculogenesis (Galloway, 2000).

Mice with directed omissions in the *BMP15* gene are sub-fertile, indicating the significance of *BMP15* in the mouse fertility. The sound effects of transmutations in the *BMP15* gene on the development of placenta and the fertility of cattle, especially in ovarian folliculogenesis, are unfamiliar and well-intentioned of advance study. In this research work, the duplicating and manifestation study of the water buffalo *BMP15* were studied.

MATERIALS AND METHODS

Collection of animal tissues samples

The water buffaloes were slaughtered by exsanguination and the required tissues including cardiac, alveolar, renal, hepatic, neural, oocytes, spermatocytes, skin, bone, muscle tissue, genital ridge, hypophysis, hypothalamus, granulosa tissues and cumulus tissues were removed. After that the required tissues were instantaneously freezed in liquid nitrogen and stored at -80°C before handling for the isolation of RNA.

Preparation of RNA and cDNA synthesis

The total RNA was extracted from removed tissues by using the RNAiso Plus reagent (TAKARA, Daliang, China) succeeding the manufacturer's specifications. The concentration, purity and integrity of RNA were detected by NanoDrop 2000 (GENE, USA) and agarose gel electrophoresis, separately. Synthesis of cDNA was executed using the PrimeScript 1st Strand cDNA Synthesis (TAKARA, Daliang, China) from 2 µg of total RNA from each the removed tissues.

Cloning of BMP15 *gene in water buffalo*

RT-reactions were implemented as earlier defined by our lab (Huang, 2010), one pair of primers was considered and chosen for the final PCR amplification based on the *Bos taurus BMP15* sequence (GenBank: NM_001031752.1) (Table I). The PCR was accomplished using the PrimeScript RT-PCR Kit (TAKARA, Daliang, China) at 94°C 5min, 94°C /30s, 55°C/30s and 72°C/1.5 min for a total of 35 cycles, with a final extension at 72°C for 7 min in a Biometra thermocycler. The PCR products were cloned into pMD-18T (TAKARA, Daliang, China) and then were sequenced by Invitrogen. The sequences were counting by DNAStar 7.1 that have been deposited to Gene Bank under accession number JQ326273.1.

Software for bioinformatics analysis

All sequence outputs (ABI trace files) were examined with the DNAStar Seqman module. Sequences were trimmed at high stringency. Software for bioinformatics analysis was list in (Table II). The BLAST search program was used to find homologies with nucleic acids and protein sequences. The ORF Finder was used to confirm open reading frames and translated to protein sequences. The physical and chemical properties of the putative *BMP15* protein were predicted using the software on the ExPASy server.

Table I.- Primers used to amplify the *BMP15* gene and expression analysis.

Products	Amplicon length (bp)	Primer name	Sequences (5'→3')	Annealing temp. (°C)	Note
BMP15	1185	*Bmp15*-F1	ATGGTCCTTCTGAGCATCC	55	CDS cloning
		Bmp15-R1	TCACCTGCATGTACAGGACT		
q*BMP15*	156	*Bmp15*-qRT-F	AAAGCCCAACCAATCACT	55	RT-qPCR
		Bmp15-qRT-R	GACACACGAAGCGGAGTC		
qβ-actin	199	β-actin-qRT-F	ACCGCAAATGCTTCTAGG	55	RT-qPCR
		β-actin-qRT-R	ATCCAACCGACTGCTGTC		

Table II.- Software for bioinformatics analysis.

Software	Website	Function
BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi	Assemble sequences
ORF Finder	http://www.ncbi.nlm.nih.gov/gorf/gorf.html	Find CDS
MEGA5.0	-	Constructed phylogenetic tree
DNAMAN	-	Multiple sequence alignment
EXPASY	http://web.expasy.org/protparam/	Predict protein property
SMART[1] (Letunic, 2017)	http://smart.embl—heidelberg.de	Predict protein domains
SignIP[2] (Petersen, 2011)	www.cbs.dtu.dk/services/SignalP/	Predict signal peptides
Softberry	http://linux1.softberry.com/berry.phtml?topic=protcompan&group =programs&subgroup=proloc	Predict the sub-cellular localization of protein
DNAStar	-	Predict secondary structure of protein
InterProScan	http://www.ebi.ac.uk/interpro/search/sequence-search	Predict protein domains
I-TASSER[3] (Roy, 2010)	http://zhanglab.ccmb.med.umich.edu/I-TASSER/	Predict protein structure

Tissue distribution of water buffalo BMP15 *mRNA*

The removed tissues included cardiac, alveolar, renal, hepatic, neural, skin, bone, muscle, genital ridge, ovary, testis, hypophysis, hypothalamus, granulosa cell and cumulus cell as temples to study the distribution of the water buffalo *BMP15* mRNA using the q*BMP15* primers (Table I). The PCR mixtures contained 1 uL 50 ng/μL cDNA, 0.3 μL each of 10 μM forward and reverse primers, 8.7 μL PCR-grade Water, and 10 μL of Premix Taq™ (TAKARA, Daliang, China). Reaction conditions were 95°C for 5 min; 40 cycle of 95°C for 10 s, 55°C for 30 s, 72°C for 30 s; with a final extension at 72°C for 7 min in a Biometra thermocycler.

Collection of COC and embryo and reverse transcription

Cumulus-oocyte-complexes (COC), parthenogenetic activation, embryo culture, collection and reverse transcription were according to the method reported by our lab (Li *et al*., 2005; Li, 2006; Shi, 2007). COC were collected from different diameter follicles, including 0-2 mm, 2-4 mm, 4-6 mm, 6-8 mm and more than 8 mm.

Quantitative real-time PCR

To study the distribution of the water buffalo *BMP15* mRNA during folliculogenesis and embryogeneis using the primers (Table I). The PCR mixtures contained 1 uL 50 ng/μL cDNA, 0.3 μL each of 10 μM forward and reverse primers, 8.7 μL PCR-grade Water, and 10 μL of SYBR Master Mix (TAKARA, Daliang, China). Reaction conditions were 95°C for 5 min; 40 cycle of 95°C for 10 s, 55°C for 30 s, 72°C for 30 s; 95°C for 5 s, 65°C for 1 min, 97°C continuous, 1 cycles; 40°C for 10 s. SYBR Green was used to detect specific PCR products. Amplification and detection of SYBR Green were performed with the ABI 7500 Instrument. At least three sets of embroys were analyzed for *BMP15* examined, and all PCRs were conducted in triplicates. The comparative C_T method was used for relative quantification of target gene expression levels. The quantification was normalized to the control *β-actin* gene. The calculation of $\Delta\Delta C_T$ involved using the <2 mm sample ΔC_T value as an arbitrary constant to subtract from all other ΔC_T sample values. The ΔC_T value was determined by subtracting the *β-actin* ΔC_T value for each sample from the *BMP15* gene ΔC_T value of the sample. Fold-changes in the relative mRNA expression of the target gene were determined using the formula $2^{-\Delta\Delta}$CT.

Immunohistochemical localization of BMP15

Swamp Buffalo ovaries and testis were sampled from the local slaughterhouse, and instantly fixed for 24 h in PBS containing 4% PFA (paraformaldehyde) in 4°C, and then administered in steadily alcohol and xylene, infiltrated with paraffin in a Biological Tissue Automatic Dehydration Machine, last embedded in paraffin. Serial sections 5-7 uM thick, were cut from laica RM 2235 rotary microtome, and then mounted on poly-L-lysine coated slides, dried in the 50°C drying oven overnight. Five to ten sections were processed in 1:49 APES: acetone for 60s to prevent the sections fall off, then deparafinized in xylene, and rehydrated. Then three 5-min washes with 0.1% Tween-20 in PBS (PBS-T) in a horizontal shaker, endogenous peroxidase was removed by incubating the deparaffinized sections in 3% hydrogen peroxide in methanol for 30 min. After three 5 min washes with PBS-T on a horizontal shaker, sections were treated with microwave heat-induced epitope retrieval three 6-min and 90% firepower with 5 min interval and cooled for 2 h to room temperature. Wash three times 5 min each in PBS-T, sections were incubated in PBS-T contain 1% Triton X-100 for 30 min, then wash three times 5 min each in PBS-T, wiped off the surrounding liquid of the sections, drawn a circle around the tissue sections with a Super Pub

Pen (ZLI-9305, Beijing Solarbio Science and Technology Co., Ltd.), then dripped 5% BSA onto the sections incubate for 45min in room temperature to minimize non-specific binding. After that, sections were incubate in 4°C overnight with the rabbit polyclonal *BMP15* (sc-27324, SANTA) antibody diluted 1:80 in PBS-T. The next day, the sections were incubated in 37°C for 45 min to ensure the antibody for the best combination with the target antigens. After three times washes, the sections were incubated with goat anti-rabbit Biotin-SP-conjugated antibody (SA00004-4, Protein Tech Group, Inc.) for 45 min at room temperature and 45 min at 37°C. Sections were washed three times in PBS-T before being incubated with Peroxidase-conjugated Streptavidin (SA00001-0, Protein Tech Group, Inc.) for 45 min at 37°C. After three 5 min washes with PBS-T, sections were incubated with DAB color development kit for 2 min at room temperature and then counterstained with hematoxylin, dehydration in a grade alcohol and mounted with Neutral Balsam. Negative controls were performed in which the primary antibody was replaced with rabbit IgG. All other incubations and washes were performed on a horizontal shaker except antibodies incubation. The sections were observed with a Nikon ECLIPSE E800 photomicroscope (Nikon, Tokyo, Japan).

Data of analysis

The expression of mRNA was investigated using SPSS software and SigmaPlot. One-way repeated-measures analysis of variance, followed by multiple pairwise comparisons using Student–Newman–Keuls Multiple Comparisons Test, was used for analysis of differences in mRNA expression assayed by QRT-PCR. A P-value of less than 0.05 was considered to be significant.

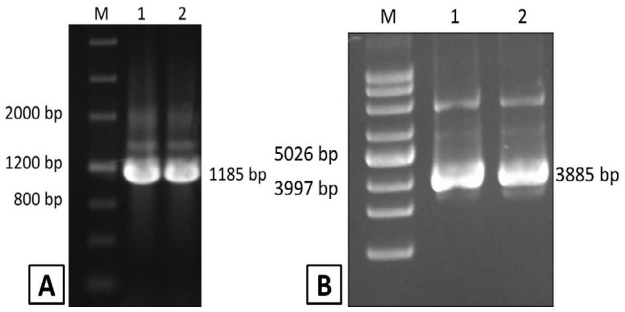

Fig. 1. RT-PCR results for the buffalo *BMP15* gene. **A**: Lane M, marker III; Lane 1-2, RT-PCR product; **B**: Lane M1, supercoiled DNA ladder; Lane 1-2, pMD-18T-*BMP15*.

RESULTS

Cloning and sequence analysis of the water buffalo BMP15

The full-length CDS of *BMP15* gene was attained from the buffalo ovary cDNAs by RT-PCR and sequencing. One pair of primers was designed in the conserved regions of the *Bos taurus*, namely *BMP15*-F1/*BMP15*-R1, for amplifying the water buffalo *BMP15* gene. Specific PCR products were obtain as seen by agarose/TAE gel electrophoresis (Fig. 1). The size of the products was 1185 bp, consistent with those expected. The sequence results and ORF Finder software analysis showed that the open reading frame (ORF) length size of water buffalo *BMP15* gene was 1, 185 bp, and encoded 394 amino acids (Fig. 2).

```
   1  ATGGTTCTTCTGAGCATCCTTAGAATCCTTCTTCTTTGGGGACTGGCGCTTTTTATGGAACATAGGGTCCAAATGACACAGGTAGGGCAG
   1   M  V  L  L  S  I  L  R  I  L  L  L  W  G  L  A  L  F  M  E  H  R  V  Q  M  T  Q  V  G  Q
  91  CCCTCTATTGCCCACCTGCCTGAGGCCCCTACCTTGCCCCTGATTCAGGAGCTGCTAGAAGAAGCCCCCCGGCAAGCTGCAGAGGAAGCCG
  31   P  S  I  A  H  L  P  E  A  P  T  L  P  L  I  Q  E  L  L  E  E  A  P  G  K  L  Q  R  K  P
 181  CGGGTCTTAGGGCATCCCTTACGGTATATGCTGGAGTTGTACCACCGTTCAGCTGACGCAAGTGGACACCCTAGGGAAAACCGCACCATT
  61   R  V  L  G  H  P  L  R  Y  M  L  E  L  Y  H  R  S  A  D  A  S  G  H  P  R  E  N  R  T  I
 271  GGGGCCACCATGGTGAGGCTGGTGAGGCCACTGGCTAGTGTAGCAAGGCCTCTCAGAGGCTCCTGGCACGTACAGACCCTGGACTTTCCT
  91   G  A  T  M  V  R  L  V  R  P  L  A  S  V  A  R  P  L  R  G  S  W  H  V  Q  T  L  D  F  P
 361  CTGAGACCAAACCGGGTAGCATACCAACTAGTCAGAGCCACTGTGGTTTACCGCCATCAACTTCACCTAACTCATTCCCACCTCTCCTGC
 121   L  R  P  N  R  V  A  Y  Q  L  V  R  A  T  V  V  Y  R  H  Q  L  H  L  T  H  S  H  L  S  C
 451  CATGTGGAGCCCTGGGTCCAGAAGAGCCCAACCAATCACTTTCCTTCTTCAGGAAGAGGCTCCACAAAGCCTTCCCTGTCGCCCAAAGCT
 151   H  V  E  P  W  V  Q  K  S  P  T  N  H  F  P  S  S  G  R  G  S  T  K  P  S  L  S  P  K  A
 541  TGGACAGAGATGGATATCATGGAACATGTTGGGCAAAAGCTCTGGAATCACAAGGGGCGCAGGGTTCTACGACTCCGCTTCGTGTGTCAG
 181   W  T  E  M  D  I  M  E  H  V  G  Q  K  L  W  N  H  K  G  R  R  V  L  R  L  R  F  V  C  Q
 631  CAGCCAAGAGGTAGTGAGGTTCGTGAGTTCTGGTGGCATGGCACTTCATCATTGGACACTGTCTTCTTGTTACTGTATTTCAATGACACT
 211   Q  P  R  G  S  E  V  R  E  F  W  W  H  G  T  S  S  L  D  T  V  F  L  L  L  Y  F  N  D  T
 721  CAGAGTGTTCAGAAGACCAAACCTCTCCCTAAAGGCCTGAAAGAATTTACAGAAAAAGACCCTTCTCTTCTCTTGAGGAGGGCTCGTCAA
 241   Q  S  V  Q  K  T  K  P  L  P  K  G  L  K  E  F  T  E  K  D  P  S  L  L  L  R  R  A  R  Q
 811  GCACGCAGTATTGCATCTGAAGTTCCTGGCCCCTCCAGGGAGCATGACGGGCCTGAAAGTAACCAGTGTTCCCTCCACCCTTTTCAAGTC
 271   A  R  S  I  A  S  E  V  P  G  P  S  R  E  H  D  G  P  E  S  N  Q  C  S  L  H  P  F  Q  V
 901  AGCTTCCAGCAGCTGGGCTGGGATCACTGGATCATCGCTCCCCATCTCTATACCCCAAACTACTGTAAGGGAGTATGTCCTCGGGTACTA
 301   S  F  Q  Q  L  G  W  D  H  W  I  I  A  P  H  L  Y  T  P  N  Y  C  K  G  V  C  P  R  V  L
 991  CACTATGGTCTCAATTCTCCCAATCATGCCATCATCCAGAACCTTGTCAATGAGCTGGTGGATCAGAGTGTCCCTCAGCCTTCCTGTGTC
 331   H  Y  G  L  N  S  P  N  H  A  I  I  Q  N  L  V  N  E  L  V  D  Q  S  V  P  Q  P  S  C  V
1081  CCTTATAAGTATGTTCCCATTAGCATCCTTCTGATTGAGGCAAATGGGAGTATCTTGTACAAGGAGTATCAGGGTATGATTGCCCAGTCC
 361   P  Y  K  Y  V  P  I  S  I  L  L  I  E  A  N  G  S  I  L  Y  K  E  Y  Q  G  M  I  A  Q  S
1171  TGCACATGCAGGTGA
 391   C  T  C  R  *
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Fig. 2. Buffalo *BMP15* nucleotide and amino acid sequences.

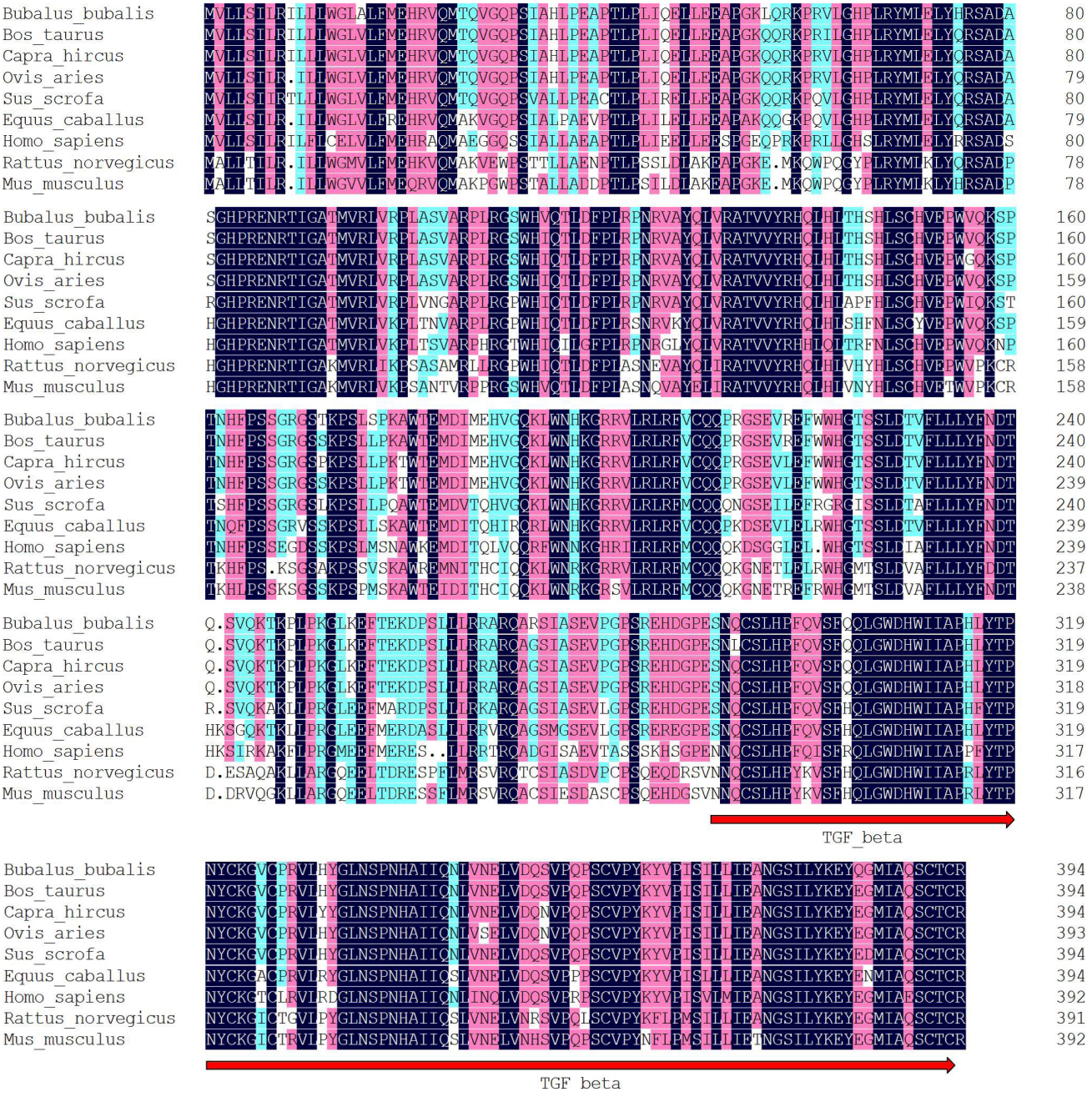

Fig. 3. Multiple alignment and analysis of deduced amino acid sequence of *BMP15*.

The water buffalo *BMP15* protein is the same size as that of *Bos taurus*, *Capra hircus*, *Equus caballus* and *Sus scrofa* (394 aa), more than that of *Ovis aries* (393 aa), *Homo sapiens* (392 aa), *Rattus norvegicus* (391 aa) and *Mus musculus* (392 aa), which contained the conserved domains of *BMP15* proteins. The InterPro software on the EMBL-EBI Services revealed that protein structures of the water buffalo *BMP15* contained the TGFB (278-394) (Figs. 3, 5). The overall similarity between the water buffalo *BMP15* and others' was very high that displayed 79%-98% similarity, showed 98%, 98%, 90%, 79% and 81% identity with that of *Bos taurus, Ovis aries*, *Sus scrofa, Mus musculus* and *Homo sapiens*. Figure 4 showed the phylogenetic tree based on amino acid sequence similarity. The water buffalo has the nearest relationship with *Bos taurus* than with those of other species, belonging to *Bovidae*.

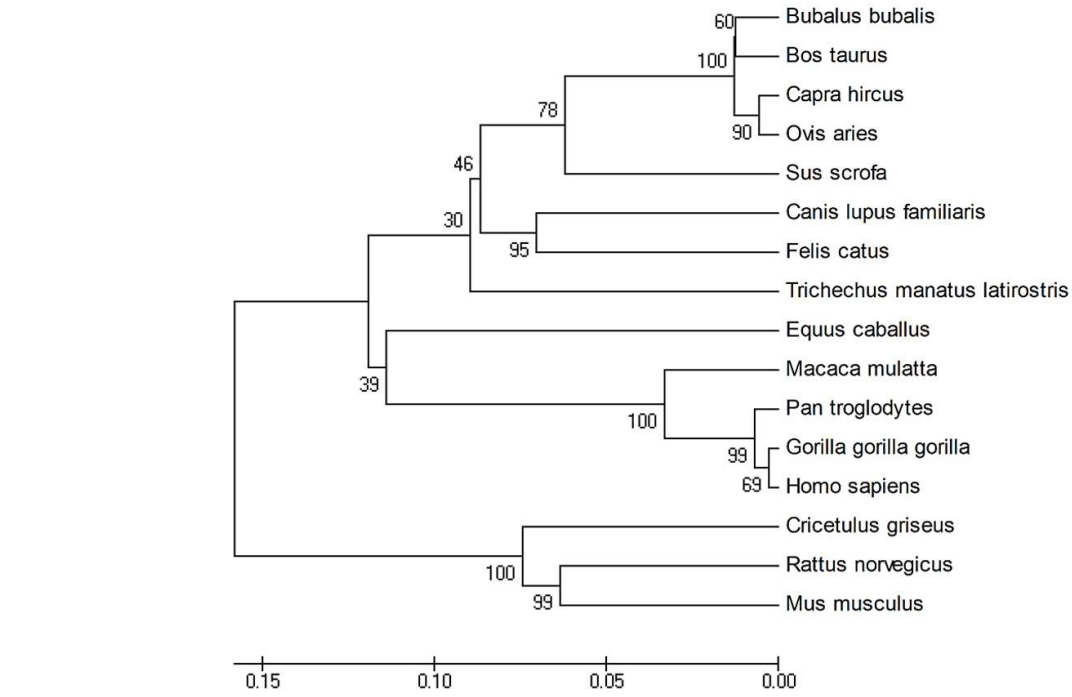

Fig. 4. Neighbor-Joining phylogenetic tree based on the *BMP15* protein among some species.

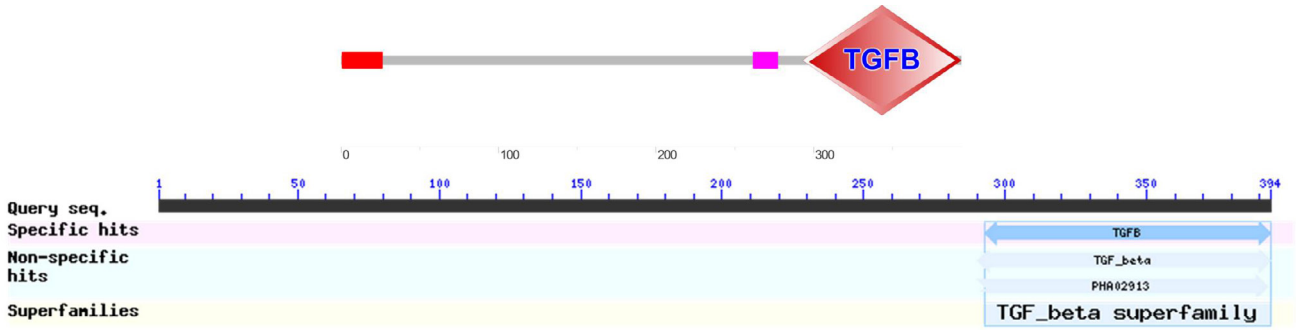

Fig. 5. The protein domain of *BMP15*.

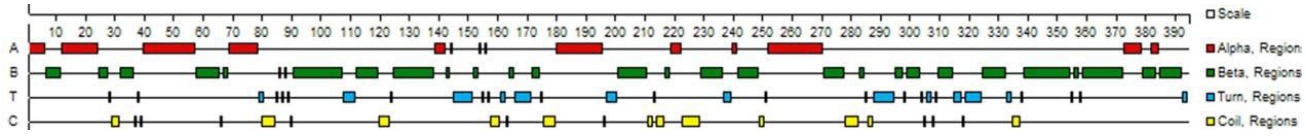

Fig. 6. The secondary structure of buffalo *BMP15* protein.

The formula, theoretical Mw and pI for the deduced amino acid sequence of *BMP15* were $C_{2027}H_{3188}N_{586}O_{549}S_{16}$, 45.06 kDa and 9.65, respectively. The ProScale software analysis exhibited the water buffalo *BMP15* protein was a weakly alkaline protein. Signal peptides predicted showed *BMP15* had signal peptide at 25-26 sites by SignIP soft and Smart soft. The Softberry online tool predicted *BMP15* was an extracellular protein.

The results of secondary structure prediction indicated that the deduced water buffalo *BMP15* contained 14 alpha helices, 29 beta helices, 31 turns, and 21 random coils (Fig. 6). The tertiary structure of buffalo *BMP15* protein prediction by I-TASSER server showed that the four species proteins were similar (Fig. 7).

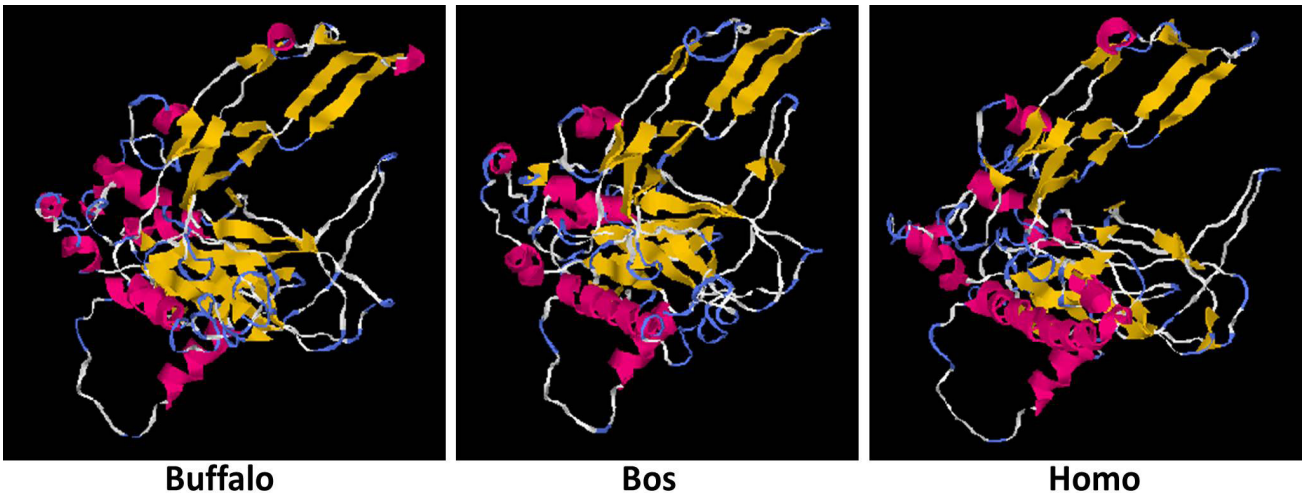

Fig. 7. The tertiary structure of buffalo, bovine and homo *BMP15* protein.

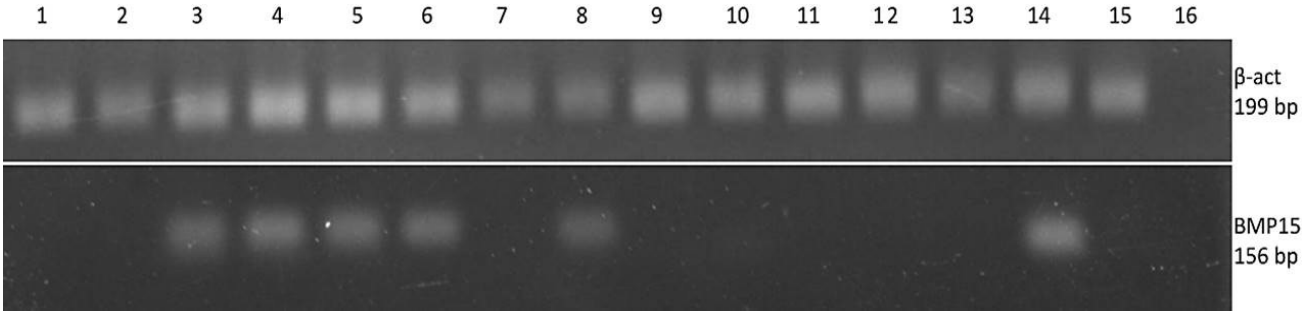

Fig. 8. Agarose gel electrophoresis of PCR product of buffalo *ALK*6 in different tissues. 1, skin; 2, bone; 3, granular cells; 4, cumulus cells; 5, ovary; 6, testis; 7, muscle; 8, genital ridge; 9, heart; 10, liver; 11, lung; 12, kidney; 13, brain; 14, hypophysis; 15, hypothalamus; 16, water.

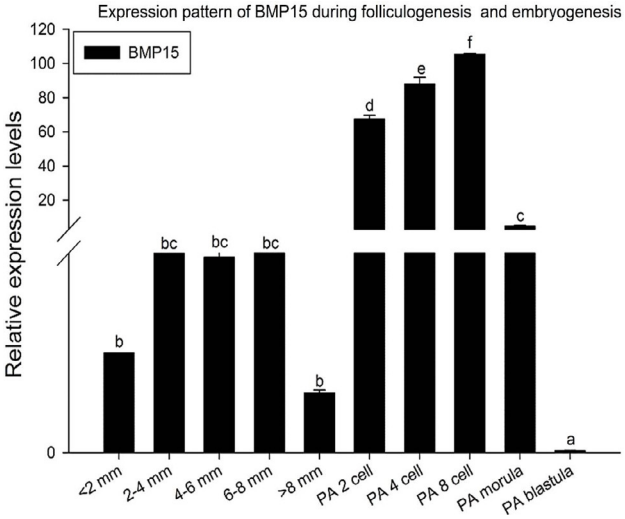

Fig. 9. The expression pattern of *BMP15* during buffalo folliculogenesis and embryogenesis.

Tissue distribution of the water buffalo BMP15

To observe the differential distributions of the *BMP15* in water buffalo tissues, 15 buffalo tissues or cells were tested by RT-PCR (Fig. 8). The expression pattern exploration results showed that buffalo *BMP15* expressed in the six of 15 tested samples, including ovary, testis, granular cells, cumulus cells, hypophysis and genital ridge. To study the distribution of the water buffalo *BMP15* mRNA during folliculogenesis and embryogeneis, COCs of different dimeter follicles and embryos of different development phase were tested by RT-PCR (Fig. 9). The results indicated that *BMP15* existed spanning the entire stage of folliculogenesis and embryogenesis. During follicular development, *BMP15* firstly up-regulated and then down-regulated, was significantly higher in the COCs of middle dimeter follicles than small and large follicles. Also, it demonstrated the same expression trend in parthenogenetic embryos at different stages of development, continued to rise until morula stage,

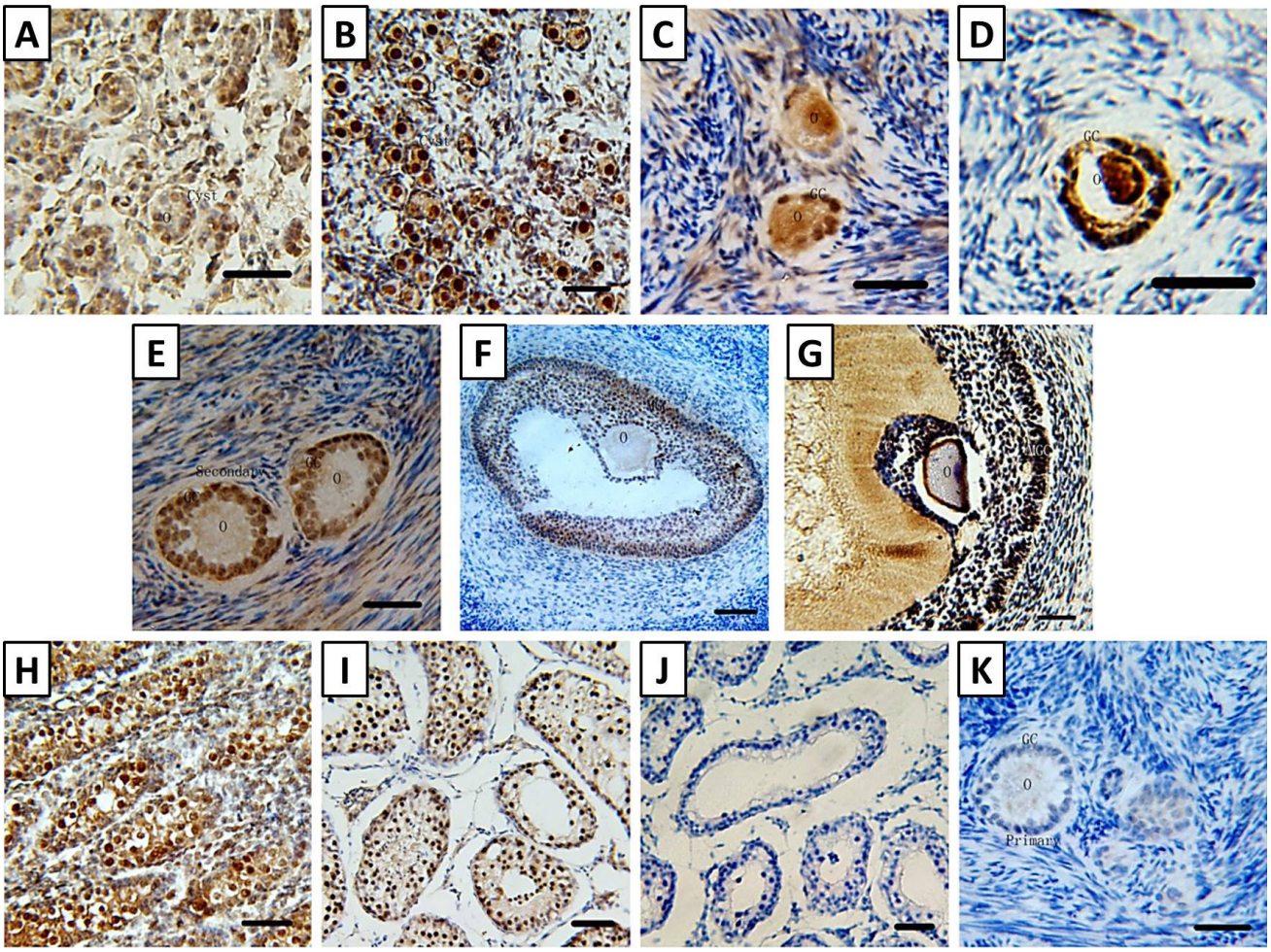

Fig. 10. *BMP15* immunoreactivity in the different structures found within buffalo ovaries and testis. **A**, Primordial follicle in genital ridge of foetus between 60-90 days post concep tion (dpc); **B**, Primordial follicle in ovary of foetus; **C**, Primordial follicle in ovary of adult buffalo; **D**, primary follicle; **E**, secondary follicle; **F**, small antral follicle; **G**, COC of a large antral follicle; **H**, testis of foetus; **I**, testis of adult buffalo; **J**, negative control of testis; **K**, negative control of ovary. Cyst, primordial cyst; O, oocyte; GC, granulosa cells; CC, cumulus cells; MGC, mural granulosa cells. Scale bars represent 20 µm.

and fell sharply at blastula stage. The gene expression was very significantly greater in embryogenesis than folliculogenesis, with the exception of blastula stage. Immunohistochemistry showed *BMP15* protein was bring into being in all types of follicles (Fig. 10), primordial granulosa cells (Fig. 10C), primary (Fig. 10D), secondary (Fig. 10E), and antral follicles (Fig. 10G) but weak reaction for cumulus cells of antral follicles (Fig. 10G), and absent in theca cells (Fig. 10F, G). *BMP15* protein was earliest noticed in primordial cyst of genital ridge of female buffalo foetus (body slanting length was 6-8 cm, amplification of the SRY Gene for sex identification by PCR, data not shown) (Fig. 10A), it exhibited a further conspicuous reaction for *BMP15* in follicles than that in granulosa cells and cumulus cells, especially in primordial follicles of ovary of female buffalo foetus (body slanting length was 30-40 cm, data not shown) (Fig. 10B). Also, it exhibited a more conspicuous reaction for *BMP15* in primary and secondary follicle than that in small and large antral follicle (Fig. 10B-G). In addition, *BMP15* protein was noticed in buffalo foetus testis (body slanting length was 30-40 cm, data not shown) and adult buffalo (Fig. 10H, I).

DISCUSSION

Water buffalo are an earliest constituent of domestic livestock possessions, and it gives milk and meat, even though which is very little in contrast to cattle, grown up in many countries, such as China, India, Turkey (Yilmaz

et al., 2012; Kaplan, 2018), Egypt (Wilson, 2012) and so on. The present study focused on molecular cloned buffalo *BMP15* gene and estimated the dispersal of *BMP15* mRNA and protein in buffalo tissues to conclude whether *BMP15* may play a role in the development of follical and embryogenesis in the buffalo.

The name bone morphogenetic protein (BMP) was first given in 1965 by Urist (1965) to the active components in demineralized bone and bone extracts that are capable of inducing bone formation at ectopic sites. In 1988, the first BMPs were isolated, and their cDNAs were cloned by Wozney (1988). In this study, the full-length CDS of *BMP15* gene was obtained from the buffalo ovary cDNAs by RT-PCR with direct sequencing, and was found to be 1,185 nucleotides encoding a protein of 394 residues. The homologous comparison showed buffalo BMP15 coding sequence had 98%, 98%, 90%, 79% and 81% identity with that of *Bos taurus, Ovis aries*, *Sus scrofa, Mus musculus* and *Homo sapiens*. This is consistent with the results of the zootaxy that further confirmed the reliability of the buffalo *BMP15* cDNA sequence. Moreover, the sequence homology levels among species to a certain extent, reflected the phylogenetic relationships, and revealed the stability of the gene encoding protein has significance for the function of organisms in different species structures. The phylogenetic analyses exposed that the nearest relationship existed between the water buffalo and *Bos taurus*, which revealed that using information of the *Bos taurus BMP15* protein to expect the function of the buffalo *BMP15* protein by bioinformatic analysis. The SMART online tool and InterPro software on the EMBL-EBI Services for prediction of the buffalo *BMP15* protein showed that this protein contained the TGFB domain (278-394). A distinguishing structural feature of the TGF-β superfamily is the presence of seven conserved cysteines, which are involved in folding the molecule into a unique three-dimensional structure called a cystine knot by Schlunegger and Grutter (1992). Interestingly, GDF-9 and *BMP15* have only six of the seven conserved cysteines; both lack the fourth cysteine that is required for the intersubunit-disulfide bridge (Dube, 1998).

We revealed the expression of mRNA *BMP15* gene in buffalo tissues, COCs and embryos as well as in ovary and testis. By using the RT-PCR we were able to demonstrate that ovary, testis, granular cells, cumulus cells, hypophysis and genital ridge expressed mRNA for *BMP15*. These results are similar to those reported by Silva (2005) for goats, rare minnow *Gobiocypris rarus* (Zhang, 2014), mouse (Otsuka and Shimasaki, 2002) where *BMP15* mRNA were found in ovary, testis, gramular cells, cumulus cells and hypophysis. In this research work, *BMP15* existed spanning the entire stage of folliculogenesis and embryogenesis. During follicular development, *BMP15* firstly enhanced and then reduced, was considerably greater in the COCs of middle dimeter sized follicles than small and large sized follicles. Also, it indicated the same expression trend in parthenogenetic embryos at different stages of development, sustained to rise until morula stage, and fell sharply at blastula stage. The gene expression was very considerably greater in embryogenesis than folliculogenesis, with the exception of blastula stage. Immunohistochemistry exhibited *BMP15* protein was bring into being in all kinds of follicles, primordial granulosa cells, primary, secondary, and antral follicles but weak reaction for cumulus cells of antral follicles, and absent in theca cells. In oocytes exhibited a more conspicuous reaction for *BMP15* than in granulosa cells and cumulus cells, especially in primordial follicles. The mRNA expression was considerably higher in the COCs of middle dimeter sized follicles than small and large sized follicles, was reliable with the result of protein expression by immunohistochemistry, which displayed a more prominent reaction for *BMP15* in primary and secondary follicle than in small and large antral follicle. These results are similar to those reported for goats (Silva, 2005), brushtail possum (Eckery, 2002), Sus (Li, 2008) where *BMP15* mRNA were found as early as in oocytes of primordial follicles, *BMP15* mRNA expressed at low levels in immature oocytes and increased to the highest level at 18 h of IVM, which coincides with the time of cumulus cell expansion. These results explained low expression in large follicle (>8 mm), but high expression in 2 cell embryo stage in this study, at that time the buffalo genome hadn't been activated. The *BMP15* mRNA were found as early as in oocytes of primordial follicles in this study and elsewhere goats *et al*, is earlier than that found for the mouse, rat, and human (Aaltonen, 1999; Dube, 1998; Elvin *et al*., 2000; Erickson and Shimasaki, 2003; Jaatinen, 1999; Laitinen, 1998), where they were first observed in oocytes of primary follicles. Surprisingly, the discover of detecting protein of *BMP15* in the very early, genital ridge of foetus is intriguing and it suggests that *BMP15* maybe play an important fuction in formation, growth and maintenance of primordial follicles in buffalo, as Bodin (2007) showed that homozygous $FecX^{L}$ adult females displayed an infantile genital tract and the ovaries did not carry any obvious follicular structures in Lacaune sheep.

BMP15 may play diverse roles in regulating early follicular development in different species. Yan (2001) showed that mice lacking BMP15 are subfertile. In contrast, ewes that have naturally occurring inactivating mutations in the BMP15 gene showed follicular development arrested at primary follicle stage and are infertile by

Galloway (2000). *BMP15* is known to stimulate granulosa cell mitosis and early follicular development in rodents as described by Otsuka (2000). Furthermore, the importance of *BMP15* for early folliculogenesis is confirmed by the findings of Dong (1996) and Galloway (2000) which showed that *BMP15*-deficient sheep are infertile because follicle development does not proceed beyond the primary stage. Clearly, the buffalo *BMP15* gene cDNAs were successful cloned that provide an important significance for further mining molecular markers associated buffalo reproduciton, exploring its function by TALENs or CRISPR/Cas systems, even breeding new twinning buffalo varieties.

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Statement of conflict of interest

The authors declare no conflict of interest.

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