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Full Length Article

Molecular Cloning and Expression Patterns Study of Growth Differentiation Factor 9 Gene (*GDF9)* in Water Buffalo (*Bubalus bubalis*)

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Abstract

The Gene Growth Differentiation Factor 9 (*GDF9*) is far most important gene for oogenesis. Few studies have been reported concerning buffalo oogenesis and embryogenesis. In current study, complete coding region of a gene of the buffalo *GDF9* gene was cloned and sequenced, which was found to have 1,362 nucleotides long encoding of 453 amino acid proteins. The expression pattern analysis results showed that *GDF9* existed spanning the entire stage of folliculogenesis and embryogenesis. The *GDF9* gene increased in the initial stage then decreased during follicular development. The gene was significantly higher in the Cumulus-Oocyte-Complexes (COC) of small and middle diameter follicles compared to large follicles. Its expression level was significantly higher in embryogenesis rather than folliculogenesis except the blastula stage. Immunohistochemistry showed that the *GDF9* protein was found in testis and in follicles of oocytes including primordial granulose cells, primary, secondary and antral follicles, and also in theca cells. The study of the *GDF9* gene provided an important base knowledge for further study regulation mechanism of *GDF9* gene in buffalo oogenesis. © 2019 Friends Science Publishers

Keywords: Buffalo; *GDF9*; Expression pattern; Oogenesis

Introduction

Buffaloes (*Bubalus bubalis*) are playing central role in livestock and agriculture in Asia for 5000 years. Buffalo provide high quality milk, meat and draft for agriculture in the economic sector. However, Buffaloes remain neglected due to poor reproductive efficiency, delayed puberty, poor estrus expression, long postpartum service period and low conception rate (Singh *et al*., 2012; Abulaiti *et al*., 2018). So, the mechanism clarification of folliculogenesis and embryogenesis is very important for improving buffalo reproductive efficiency (Zaidi and Anwar, 2018).

Ovarian folliculogenesis is characterized by the growth of follicles from primordial follicles up to the mature ovulating Graafian follicles (Silva *et al*., 2016). During ovarian folliculogenesis, follicle development is proscribed by both extra ovarian factors, *e.g*., pituitary gonadotropins and locally produced paracrine factors, *e.g*., Growth Differentiation Factor 9 **(***GDF9*) and Bone Morphogenetic Protein 15 (BMP15), which bidirectional communication between the oocyte and the adjoining somatic cells (Zhai *et al*., 2018). It is widely accepted that oocytes involved in granulose cell differentiation and promotes follicle growth (Gilchrist *et al*., 2004).

GDF9, a soluble porcine growth factors secreted by oocyte, is far most important gene for oogenesis. In animals, *GDF9* is proficient in oocyte and acts on oocytes' adjacent granulosa cells, resulting in regulating oocyte self-development (Belli and Shimasaki, 2018). *GDF9* are the associated members of Transforming Growth Factor b (TGFβ) super family which produced within ovary and have profound upshot on fertility (Wang *et al*., 2017; Liu *et al*., 2018; Lou *et al*., 2018). In mice, *GDF9* is crucial for normal follicular development (Dong *et al*., 1996) while BMP15 is not (Yan *et al*., 2001). However, in sheep, for regular follicular growth both *GDF9* and BMP15 are important (Wang *et al*., 2017; Tang *et al*., 2018).

Previous studies also identified some major biological characteristics of *GDF9* in ovary, as a potential stimulator of GC mitosis; restrain the expression of FSH receptor mRNA

in GCs, resulting in the subsequent inhibition of FSH-induced progesterone synthesis as well as FSH-induced expression of mRNAs battery in GCs; and initiate the expression of kit ligand mRNA in GCs (Karagül *et al*., 2017). In mice the *GDF9* gene with targeted deletions causes sub-fertility indicating the significance of *GDF9* in fertility. Mutations in *GDF9* gene effects on placental development and cattle fertility, but few studies have been reported *GDF9* concerning buffalo oogenesis and embryogenesis, especially its function in ovarian folliculogenesis, are unknown and worthy for further research. In current study, the cloning and expression analysis of *GDF9* were studied in water buffalo.

Materials and Methods

Preparation of RNA and cDNA Synthesis

The total RNA was extracted from collected ovary samples by using RNA iso-Plus reagent (TAKARA, Dalian, China) following the manufacturer's specifications. Synthesis of cDNA was performed by using the Prime Script 1st Strand cDNA Synthesis from 2 μg of total RNA from each of the collected sample tissues (TAKARA, Dalian, China).

Cloning of *GDF9* Gene in Water Buffalo

RT-reactions were performed as described by our lab (Zhu *et al*., 2018). One pair of primers were designed and chosen for the final PCR amplification based on the *Bos taurus GDF9* sequence (GenBank: NM_174681.2; Table 1). The PCR was performed by the usage of Prime Script RT-PCR Kit (TAKARA, Dalian, China) at 94°C/5 min, 94°C/30 s, 56°C/30 s and 72°C/90 s for a total of 35 cycles in a Biometra thermo cycler. The PCR products were cloned into pMD-18T (TAKARA, Dalian, China) and then were sequenced by Invitrogen. The sequences have been deposited to Gen Bank under accession number JQ326274.1.

Cumulus-Oocyte-Complexes (COC) and Embryo Collection and Reverse Transcription

Previous reported method of our lab was applied on COC, Parthenogenetic activation, embryo culture, collection and reverse transcription (Sun *et al*., 2015). COC were collected from different diameter follicles, including 0–2 mm, 2–4 mm, 4–6 mm, 6–8 mm and >8 mm.

Bioinformatics Analyses

All sequence outputs (ABI trace files) were analyzed with the DNAStar Seqman module. Sequences were trimmed at high stringency. Software for bioinformatics analysis was list in Table 2. The BLAST search program was used to identify 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 *GDF9* protein were predicted using the software on the ExPASy server. The signal peptide was predicted using the SignalP 4.1 Server. The 3D ectodomain structure of *GDF9* protein was constructed using I-TASSER server. The phylogenetic tree was constructed with Mega 6.06 using the Neighbour-Joining method based on sequence alignments of *GDF9* with other homologous sequences.

Quantitative Real-time PCR

Primers were used for the distribution of the water buffalo *GDF9* mRNA during folliculogenesis and embryogenesis (Table 1). The PCR mixtures contained 1 μL (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, Dalian, China). Reaction conditions were maintained on 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 and 40°C for 10 s with the ABI 7500 Instrument. At least three sets of embryos were analyzed and examined for *GDF9*. The comparative CT method was used for relative quantification of target gene expression levels. The quantification was normalized to control the β-actin gene. The calculation of $^{\triangle\triangle}$CT involved in the <2 mm sample $^{\triangle}$CT value as an arbitrary constant to subtract from all other $^{\triangle}$CT sample values. Fold-changes in the relative mRNA expression of the target gene were determined using the formula $^{2-\triangle\triangle}$CT.

Immunohistochemical Localization of *GDF9*

The *GDF9* at different stages in COC and parthenogenetic embryos and PFA-fixed oocytes was detected as previously described with slight modifications (Lei *et al*., 2016). The samples washing three times in PBS solution that contains 0.3% BSA and 1% Triton X-100 used for 5 min, 10 min treatment also with 1% Triton X-100 in PBS for permeabilized by RT and blocked for 1hr in supplemented with 1% BSA at RT. Primary goat polyclonal anti-*GDF9* antibody used for samples incubation overnight at 4°C (sc-12244, 1:200; Santa Cruz Biotechnology Inc.). Samples were incubated for 1 h at RT (darkness) with fluorescein-conjugated rabbit anti-goat IgG (H+L) after three washing of oocytes in TBP (SA00003-2, 1:100; Protein Tech Group Inc.,). For 10 min RT darkness samples again three times treated with TBP and counterstained with 10 μg/mL propidium iodide (Sigma, St. Louis, M.O., U.S.A.). Samples fixing with antiskid regents on slides after three more washes. Confocal microscope laser scanning was used for samples observation (Zeiss, Heidelberg, Germany).

Data Analyses

The mRNA expression was analyzed by using SPSS software and Sigma Plot. One-way analysis of variance,

Table 1: Primers used to amplify the *GDF9* gene and expression analysis

Products	Amplicon length (bp)	Primer name	Sequences (5’→3’)	Annealing temperature (°C)	Note
GDF9	1473	*GDF9*-F1	CTTGCTAATTCTTCCAAGCC	56	CDS cloning
		GDF9-R1	GACACATGAAACTTCCTCCC		
q*GDF9*	195	*GDF9*-qRT-F	AGGACTGCGTTGGAATCT	55	RT-qPCR
		GDF9-qRT-R	CCCCTCCTTGGTAGCATA		
qβ-actin	199	β-actin-qRT-F	ACCGCAAATGCTTCTAGG	55	RT-qPCR
		β-actin-qRT-R	ATCCAACCGACTGCTGTC		

Table 2: Software used for bioinformatics analysis

Software	Website	Purpose
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	/	Construct Phylogenetic tree
DNAMAN	/	Multiple sequence alignment
EXPASY	http://web.expasy.org/protparam/	Predict Protein property
SMART (Letunic *et al*., 2012)	http://smart.embl—heidelberg.de	Predict protein domains
SignIP (Petersen *et al*., 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 (Roy *et al*., 2010)	http://zhanglab.ccmb.med.umich.edu/I-TASSER/	Predict protein structure

followed by multiple pair wise comparisons using Student–Newman–Keuls Multiple Comparisons Test, was used for analysis of differences in mRNA expression accessed by quantitative real-time PCR. The P-value less than 0.05 were considered to be significant.

Results

Cloning and Sequencing Analysis of *GDF9* in Water Buffalo

Full-length Coding Sequence (CDS) of *GDF9* gene from the buffalo ovary cDNAs were obtained by RT-PCR with direct sequencing, one pair of the primer was designed for conserved regions of the *B. taurus*, namely *GDF9*-F1/*GDF9*-R1, for the amplification of water buffalo *GDF9* gene. The obtained PCR products were visualized by agarose/TAE gel electrophoresis (Fig. 1) that demonstrated the expected size of products was 1473 bp at reliability. Although, sequencing results and Open Reading Frames (ORF) Finder software analysis represented that the ORF length size of water buffalo *GDF9* gene was 1, 362 bp and encoded 453 amino acids (Fig. 2).

The water buffalo *GDF9* protein is of equal size as that of the *B. taurus*, *Capra hircus* and *Ovis aries* (453 aa), more than that of *Equus caballus* (451 aa), *Sus scrofa* (444 aa) *Rattus norvegicus* (440 aa) and *Felis catus* (452 aa), less than that of *Gorilla gorilla gorilla* (455 aa) and *Homo sapiens* (454 aa), all they have the conserved regions in *GDF9* proteins. The Inter Pro software (EMBL-EBI Services) revealed that protein configuration of the water buffalo *GDF9* contains the TGFB 352-453 (Fig. 3 and 5).

The overall similarity between the water buffalo

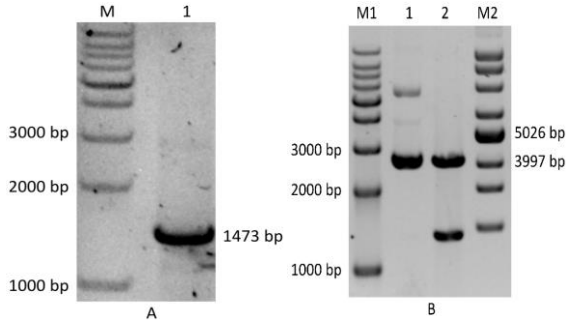

Fig. 1: RT-PCR results for the buffalo *GDF9* gene. A）M: 1 kb DNA Marker, 1: RT-PCR product. B）M1:1 kb DNA marker, 1: pMD-18T-*GDF9*, 2: The pMD-18T-*GDF9* vector digested by restriction enzyme BglII and EcoRI, M2: Supercoiled DNA Ladder

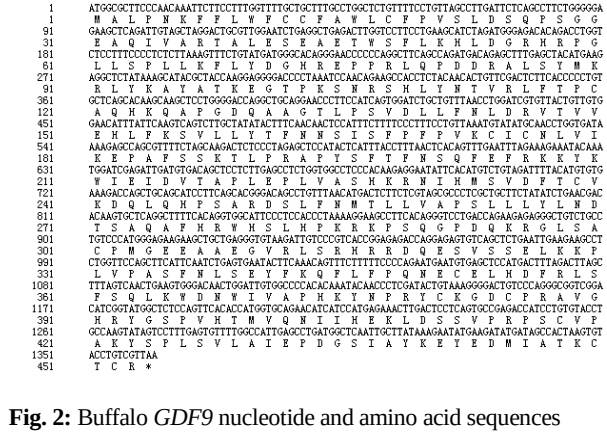

Fig. 2: Buffalo *GDF9* nucleotide and amino acid sequences

GDF9 and others’ was very high that displayed 82–99%

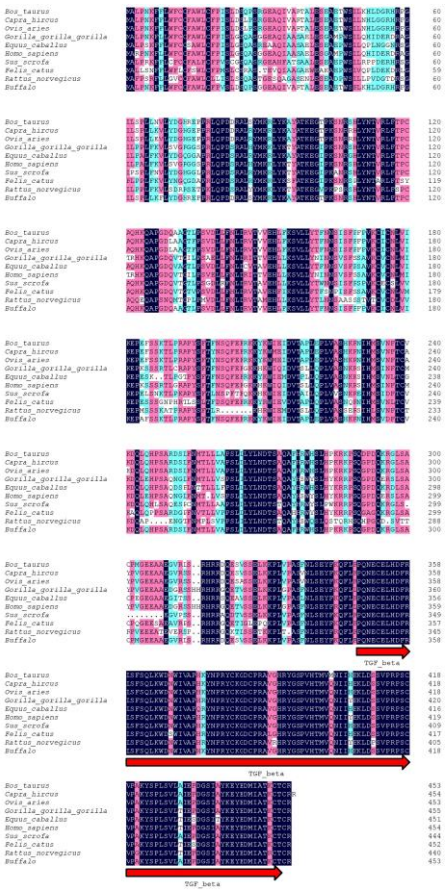

Fig. 3: Multiple alignment and analysis of deduced amino acid sequence of *GDF9*

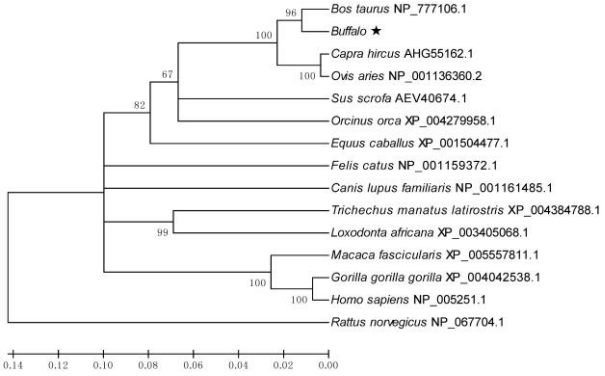

Fig. 4: Neighbor-joining phylogenetic tree based on the GDF9 protein among some species

similarity, showed 99, 97, 97, 88, 85, 85, 85, 83 and 82% identity with that of *B. taurus, O. aries, C. hircus, E. caballus, S. scrofa, G. gorilla gorilla, H. sapiens, F. catus* and *Canis lupus* respectively. Amino acids based phylogenetic tree (Fig. 4) represents sequence similarity. The water buffalo belonging to *Bovidae* family have close link with *B. taurus* than any of the other species.

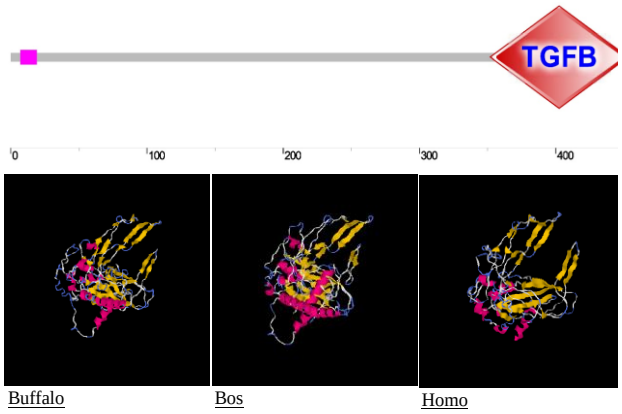

Fig. 5: The tertiary structure of buffalo, bovine and homo*GDF9*protein

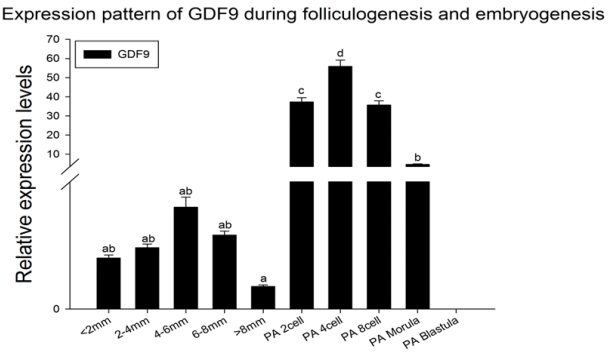

Fig. 6: The expression pattern of *GDF9* during buffalo folliculogenesis and embryogenesis

For amino acid sequence of GDF9 the deducted formula, theoretical Mw and pI were $C_{2341}H_{3600}N_{640}O_{650}S_{21}$, 51.78 kDa and 9.05, respectively. The Pro Scale software analysis showed that water buffalo GDF9 protein is weak alkaline in nature. Signal peptides predicted showed GDF9 had signal peptide at 25–26 sites by SignIP soft and Smart soft. The Softberry online tool predicted GDF9 is an extracellular protein.

Tissue Distribution of the Water Buffalo GDF9

In water buffalo the distribution of GDF9 mRNA during folliculogenesis and embryogenesis, COC of different diameter follicles and embryos of different development phase were tested by using RT-qPCR (Fig. 6). The results illustrated that GDF9 existed spanning the entire stage of folliculogenesis and embryogenesis. During follicular development, initially GDF9 gene expression was increased and then decreased, that's significantly higher in the COC of middle diameter follicles versus large follicles. It also exhibited the same expression trend in parthenogenetic embryos at different developmental stages, increased until 8 cell stage, and decreased at blastula stage.

Immunohistochemistry illustrated that GDF9 protein

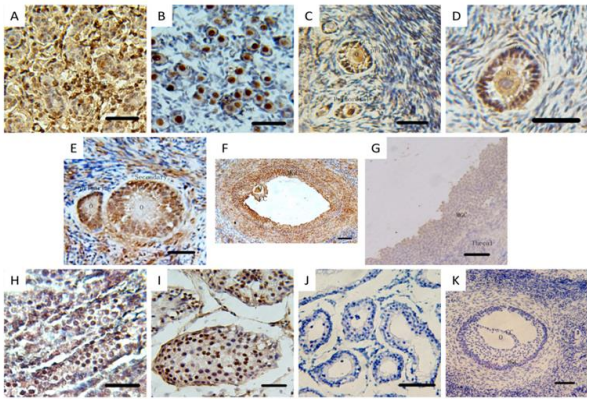

Fig. 7: *GDF9* immunoreactivity in the different structures found within buffalo ovaries and testis

A) Primordial follicle in genital ridge of foetus between 60-90 days post conception (dpc); **B**) Primordial follicle in ovary of foetus; **C**) Primordial and primary follicles in ovary of adult buffalo; **D**) primary follicle; **E**) secondary and primary follicles; **F**) large antral follicle; **G**) mural granulosa and theca cells from 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

present in all types of follicles (Fig. 7), including granulosa cells of primordial (Fig. 7C), primary (Fig. 7D), secondary (Fig. 7E) and antral follicles (Fig. 7F), also reaction for theca cells (Fig. 7F and G). Compare with the negative control of testis and ovary(Fig. 7J and K), the *GDF9* protein was detected the earliest in primordial cyst of genital ridge of female buffalo fetus (body slanting length was 6–8 cm, amplification of the SRY Gene for sex identification by PCR, data not shown in Fig. 7A), it showed a more prominent reaction for *GDF9* in oocytes than that in granulosa cells and cumulus cells, especially in oocytes of primordial follicles of ovary of female buffalo fetus (body slanting length was 30–40 cm, data not show; Fig. 7B). Also, it showed a more prominent reaction for *GDF9* in primordial, primary and secondary follicle than that in small and large antral follicle (Fig. 7B, C, D, E, F and G). Additionally, *GDF9* protein was also detected in the testis of buffalo foetus (body slanting length was 30–40 cm, data not shown Fig. 7H and I) and adult buffalo.

Discussion

Water buffalo are antiques of domestic livestock and their production of milk and meat is low compared with cattle, but are raised up in different countries like China, Pakistan, India, Turkey and Egypt (Naveena and Kiran, 2014). However, Buffaloes remain neglected due to poor reproductive efficiency, so the mechanism clarification of folliculogenesis and embryogenesis is very important for improving buffalo reproductive efficiency. Previous studies had identified *GDF9* is far most important gene for oogenesis, but few studies have been reported *GDF9* concerning buffalo oogenesis and embryogenesis, especially its function in ovarian folliculogenesis, are unknown and worthy for further research. In current study, the molecular cloned buffalo *GDF9* gene and the distribution of *GDF9* mRNA and protein in buffalo tissues were evaluated to determine whether *GDF9* may have role in follicular development and embryogenesis in buffalo.

The first BMPs were isolated and cloned by Wozney *et al*. (1988), after that, more and more members of TGFβ were discovered and cloned, and GDF9 was cloned firstly by McPherron and Lee (1993). However, there hasn't had report of cloning buffalo *GDF9* gene, in present study, the full-length coding sequence of *GDF9* gene was accessed from the buffalo ovary cDNAs by RT-PCR with direct sequencing. It was observed that 1,185 nucleotides encoding a protein of 394 amino acid residues with 45.06 kDa molecular weight and pI of 9.65 that showed it was weak alkaline with signal peptide at 25–26 sites is an extracellular protein, earlier study showed all TGF-β superfamily members including *GDF9* and BMP15 are synthetized as large preproproteins composed of a signal peptide, a prodomain that directs their dimerization and a mature domain (Belli and Shimasaki, 2018). The homologous comparison showed buffalo *GDF9* coding sequence had similarity index with different species as described in results that are consistent with the results of the zootaxy. In addition, the sequence similarity among species to a certain degree, conceded the phylogenetic relationships and divulged the stability of protein coding gene was important in different species structures as function of organisms. The phylogenetic analyses revealed that the nearest relationship between the water buffalo and *B. taurus* and the function of the buffalo *GDF9* protein could be predict through bioinformatics analysis by using *B. taurus GDF9* protein information. The SMART online tool predicted the buffalo *GDF9* protein contained the TGFB domain (352–453). A distinctive configuration aspect of TGF-β superfamily is the occurrence of seven conserved cysteines that is involved in developing a unique cystine knot that act as three-dimensional structure (Schlunegger and Grutter, 1992). An interesting phenomenon is that *GDF-9* and BMP-15 have only six conserved cysteines rather than seven; both not have the fourth cysteine that is needed for inter subunit-disulfide bridge (Dube *et al*., 1998).

We demonstrated that mRNA expression of *GDF9* in buffalo tissues, COC and embryos, and protein expression of *GDF9* in buffalo ovary and testis. By using RT-PCR this is inferred that ovary, testis, granular cells, cumulus cells, hypophysis and genital ridge expressed mRNA for *GDF9*. Similar results were also observed for goats (Silva *et al*., 2016), rare minnow *Gobiocypris rarus* (Zhang *et al*., 2014), mouse (Otsuka and Shimasaki, 2002), *GDF9* mRNA were found in ovary, testis, granular cells, cumulus cells and hypophysis. In present study, *GDF9* existed spanning the entire stage of folliculogenesis and embryogenesis, was significantly higher in the COC of middle diameter follicles versus large follicles, and also exhibited the same expression trend in parthenogenetic embryos at different developmental stages, increased until 8 cell stage and

decreased at blastula stage. Similar to Jain showed that *GDF9* expression at both mRNA and protein levels at different time points of IVM revealed that magnitude of mRNA abundance at 8 h of IVM was most important towards imparting development competence to buffalo oocytes (Jain *et al*., 2012). Interesting, the expression of *GDF9* in buffalo testis, suggested the function of *GDF9* in buffalo testis development. Previous study reveals that *GDF9* is a key regulator of bovine SCs through the modulation of the cell cycle, apoptosis and tight junction functions (Tang *et al*., 2017).

Immunohistochemistry of *GDF9* protein was present in all kind of follicles (primary, secondary, granulosa cells of primordial and antral) but weak reaction for cumulus cells of antral follicles and also absent in theca cells and similar findings were observed for goats (Silva *et al*., 2016), *Brushtail possum* (Eckery *et al*., 2002), *S. scrofa* (Li *et al*., 2008), whereas mRNA for *GDF9* found early in oocytes of primordial follicles, the expression of *GDF9* mRNA observed at low level in immature oocytes but enhanced with high 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 *GDF9* mRNA was found early as in oocytes of primordial follicles in goats earlier than that found in human, rat and mouse (Dube *et al*., 1998). Surprisingly and interesting, we detected the protein of *GDF9* in early genital ridge of the fetus which is fascinating and significantly maintain primordial follicles in buffalo, and this is the first time showed expression pattern of *GDF9* in buffalo fetus. Hosoe had showed the expression of BMP15 and *GDF9* in cumulus cells of adult ovaries (4–6 years-old cows) was significantly higher than that in calf ovaries (9–11 months-old calves) and *GDF9* expression in the oocytes of calf ovaries was significantly higher than in those of the adult ovaries (Hosoe *et al*., 2011).

Conclusion

The *GDF9* is far most important gene for oogenesis, few studies have been reported concerning buffalo oogenesis and embryogenesis. It is clear that, in the present study, buffalo *GDF9* gene cDNAs were successful cloned and its expression pattern was also studied. Buffalo *GDF9* has 1,362 nucleotides long encoding of 453 amino acid proteins, existed spanning the entire stage of folliculogenesis and embryogenesis, increased in the initial stage then decreased during folliculogenesis and embryogenesis, significantly higher in embryogenesis rather than folliculogenesis except the blastula stage. In a word, the present study provides an important significance for further mining molecular markers associated buffalo reproduction, exploring its function by TALENs or CRISPR/Cas systems, even in the breeding of new twinning buffalo varieties (Niu *et al*., 2018).

Acknowledgments

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