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Characterization of the coding region of basic fibroblast growth factor (FGF2) c-DNA (open reading frame) in buffalo cumulus cells

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Fibroblast growth factors (FGFs) are a family of growth factors involved in angiogenesis, wound healing and embryonic development. FGFs are heparin-binding proteins and have interactions with cell-surface associated heparin sulphate proteoglycans, which are essential for FGF signal transduction pathway. Basic fibroblast growth factor (bFGF), also known as FGF2, is found in ovary, modulates steroidogenesis in granulosa cells and increases tissue plasminogen activator (tPA) activity in endothelial cells. FGF2 acts as an intraovarian inducer of granulosa cell tPA gene expression and oocyte maturation (LaPolt *et al*. 1990). Several workers have used FGF2 in the culture medium of embryo production, but the effect of FGF2 on blastocyst yield is so variable. It has been found that FGF @20ng/ml increases cumulus cell expansion and nuclear maturation in *in vitro* produced buffalo oocytes (Nandi *et al*. 2003). FGF2 (1–100ng/ml) also increases the interferon tau (IFNT) m-RNA and protein abundance in bovine trophectodermal cells, but did not affect the blastocyst cell numbers (Michael *et al*. 2006). Neira *et al*. (2010) found that TGF-α1, FGF2, granulocyte-macrophage colony-stimulating factor (GM-CSF), and LIF used alone in culture medium significantly improved the yield of hatched blastocysts in cattle. Furthermore, there was no effect of FGF2 on cleavage or blastocyst development rates when 5 or 100 ng/ml FGF2 was supplemented after fertilization (Fields *et al*. 2011).

The expression of FGF2 in oocytes and embryos of cattle was reported (Lazzari *et al*. 2002); but information on expression of FGF2 transcript in buffalo is very limited. Few sequences of the FGF2 were reported in the buffalo (Gene Acc. Nos. JQ326277 and JN399071, 468 bp mRNA). In our previous study, supplementation of the FGF2 improved the cumulus cell expansion and cleavage rate of buffalo embryos; but did not improve the blastocyst yield (unpublished data). The reason of low blastocyst yield in buffalo needs to be investigated in details. Therefore, the present study was designed to characterize the full coding region of the FGF2 (open reading frame) in buffalo cumulus cells.

In vitro matured buffalo oocytes with expanded cumulus cells were transferred into 1.5ml micro-centrifuge tube containing 500µl hyaluronidase (0.5mg/ml) in T2 medium (where T denotes TCM–199 supplemented with 2.0mM sodium pyruvate, 50µg/ml gentamicin and 2% FBS) and incubated at 38.5°C for 1 min. It followed vortexing at high speed for 2–3 min. The content of tube was transferred into a 35 mm dish containing T2 medium, and completely denuded oocytes were discarded. The cumulus cells were transferred into 25 cm^2 culture flask and cultured in DMEM medium supplemented with 10% FBS, 0.0001g/ml L-glutamine, and 50µg/ml gentamicin. After 70–80% confluency, the cumulus cells were detached by 0.25% trypsin EDTA and subsequently sub-cultured for studying the characterization of the FGF2 coding region.

Buffalo FGF2 ORF was amplified by the FGF2 full length forward primer (5'- ATGGCCGCCGGGAGCATCAC-3') and reverse primer (5'- TGAGATCAGATGCTGCCATT-3'). The primers were designed based on the cattle FGF2 sequence (Gene Acc. NM_174056.3). The total RNA was isolated from buffalo cumulus cells by TRI reagent and the c-DNA was prepared by single strand c-DNA synthesis kit. The PCR amplification reaction components were as follows; 1× PCR buffer, 1.5mM $MgCl_2$, 200µM dNTPs, 10µM each FGF2 full length forward and reverse primer, 1.5U Go Taq Flexi DNA polymerase, and 3.0 µl buffalo cumulus cell c-DNA. The amplification conditions were as follows: initial denaturation 95°C for 3 min, denaturation at 95°C for 40sec, annealing at 58°C for 40sec, extension at 72°C for 1.30 min for 35 cycle followed by final extension at 72°C for 10 min. The primers amplified the 492bp product including ORF

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region from buffalo cumulus cells.

The FGF2 full length PCR products were gel purified using gel extraction kit as per manufacturer recommendations. The purified product was cloned into pJET 1.2/blunt cloning vector as per the manufacturer protocol. About 200µl aliquot of competent *E. coli* cells (XL-1–blue strain) was taken. Mix the ligated product with competent cells and kept on ice for 30 min. The mixture was heat shocked at 42°C for 90sec. About 400µl of SOC medium (containing 20% of glucose) was added in the mixture. The content was agitated in an incubator at 37°C for 1h. The whole content (*E. coli* cells) was spreaded on prewarmed LB plate containing ampicillin (100µg/ml) and incubated at 37°C for overnight. On the next day, few individual colonies were picked up and streaked on new ampicillin containing LB plate. The colony PCR was performed using the same components and amplification conditions (annealing 58°C) as they were used for FGF2 full length primers. The FGF2 positive colonies were transferred into LB broth for preparation of plasmid DNA through a kit. The FGF2 full length clones were sent for sequencing to confirm their sequence identity. The buffalo FGF2 ORF was confirmed and analyzed by Bio-Edit software. The buffalo FGF2 ORF sequence was slightly different from the cattle. The nucleotide changes in the sequence were as follows:

The nucleotide change were observed from G to C at 31 bp (alanine to proline), G to C at 69bp, and T to C at 282 bp position in the buffalo FGF2 sequence compared to cattle sequence. The nucleotide similarity was found as 99, 99, 95 and 90% with cattle, sheep, human, and mouse, respectively, whereas the amino acid similarity was found as 99, 99, 98, and 94% with cattle, sheep, human, and mouse, respectively.

The presence of FGF2 transcript was demonstrated in mature oocytes and *in vitro* produced bovine embryos (Watson *et al*. 1992, Yoshida *et al*. 1998). The m-RNA transcript for FGF2 was also detected in mature oocytes and in early stages of preimplantation bovine embryos (up to the 8-cell stage), but not in 16-cell stage through the blastocyst stage (Larson *et al*. 1992). However, FGF2 mRNA and its receptor (FGFR2) were present throughout an early bovine embryonic development, including the blastocyst stage (Daniels *et al*. 2000, Lazzari *et al*. 2002). Muñoz *et al.* (2009) showed the expression of FGF2R throughout the early bovine embryonic development *in vitro*. It was also found that FGF1R transcript predominantly expressed in cumulus cells, whereas FGF2R was most abundantly expressed in bovine oocytes (Zhang *et al*. 2010). It is well established that the FGF2 expressed in different cells/tissues of the cattle, but its expression has not been well reported in buffaloes.

Therefore, the purpose of characterization of FGF2 ORF

5'**ATG**GCCGCCGGGAGCATCACCACGCTGCCA**C**CCCCTGCCGGAGGACGGCGGCAGCGGCGCTTTCCCGCC**C**G
GCCACTTCAAGGACCCCAAGCGGCTGTACTGCAAGAACGGGGGCTTCTTCCTGCGCATCCACCCCGACGGCCG
AGTGGACGGGGTCCGCGAGAAGAGCGACCCACACATCAAACTACAACTTCAAGCAGAAGAGAGAGGGGTTGTG
TCTATCAAAGGAGTGTGTGCAAACCGTTACCTTGCTATGAAAGAAGATGGAAGATTACTAGCTTC**C**AAATGTGTT
ACAGACGAGTGTTTCTTTTTTGAACGATTGGAGTCTAATAACTACAATACTTACCGGTCAAGGAAATACTCCAGTT
GGTATGTGGCACTGAAACGAACTGGGCAGTATAAACTTGGACCCAAAACAGGACCTGGGCAGAAAGCTATACTT
TTTCTTCCAATGTCTGCTAAGAGC**TGA**3'

The amino acid similarity between buffalo, cattle, sheep, human and mouse is given below.

	5	15	25	35	45	55
Buffalo	—MAAGSITT	LPPLPEDGGS	GAFPPGHFKD	PKRLYCKNGG	FFLRIHPDGR	VDGVREKSDP
Cattle	—MAAGSITT	LPALPEDGGS	GAFPPGHFKD	PKRLYCKNGG	FFLRIHPDGR	VDGVREKSDP
Sheep	—MAAGSITT	LPALPEDGGG	GAFPPGHFKD	PKRLYCKNGG	FFLRIHPDGR	VDGVREKSDP
Human	GTMAAGSITT	LPALPEDGGS	GAFPPGHFKD	PKRLYCKNGG	FFLRIHPDGR	VDGVREKSDP
Mouse	—MAASGITS	LPALPEDGG–	AAFPPGHFKD	PKRLYCKNGG	FFLRIHPDGR	VDGVREKSDP
Clustal Co	***..**:	**.******	.**********	**********	**********	**********

	65	75	85	95	105	115
Buffalo	HIKLQLQAEE	RGVVSIKGVC	ANRYLAMKED	GRLLASKCVT	DECFFFERLE	SNNYNTYRSR
Cattle	HIKLQLQAEE	RGVVSIKGVC	ANRYLAMKED	GRLLASKCVT	DECFFFERLE	SNNYNTYRSR
Sheep	HIKLQLQAEE	RGVVSIKGVC	ANRYLAMKED	GRLLASKCVT	DECFFFERLE	SNNYNTYRSR
Human	HIKLQLQAEE	RGVVSIKGVC	ANRYLAMKED	GRLLASKCVT	DECFFFERLE	SNNYNTYRSR
Mouse	HVKLQLQAEE	RGVVSIKGVC	ANRYLAMKED	GRLLASKCVT	EECFFFERLE	SNNYNTYRSR
Clustal Co	*: ********	**********	**********	**********	: *********	**********

	125	135	145	155
Buffalo	KYSSWYVALK	RTGQYKLGPK	TGPGQKAILF	LPMSAKS
Cattle	KYSSWYVALK	RTGQYKLGPK	TGPGQKAILF	LPMSAKS
Sheep	KYSSWYVALK	RTGQYKLGPK	TGPGQKAILF	LPMSAKS
Human	KYTSWYVALK	RTGQYKLGSK	TGPGQKAILF	LPMSAKS
Mouse	KYSSWYVALK	RTGQYKLGSK	TGPGQKAILF	LPMSAKS
Clustal Co	**: *******	********.*	**********	*******

was to find out the nucleotide changes in the sequence of buffalo FGF2 as compared to cattle. Since the FGF2 (from bovine pituitary) were supplemented in the culture media of buffalo embryo production, but did not show any positive effect on morula and blastocyst production rate (unpublished data). It was thought that cattle FGF2 might have different sequence than the buffalo. Thus, buffalo FGF2 coding sequence was analyzed and it was found slightly different from the cattle sequence. This may be the reason that FGF2 from bovine pituitary could not bind effectively to the buffalo FGF2R receptor and may not initiate signaling. Thus, further study is required to characterize the buffalo FGF2R that is the binding site of FGF2 ligand molecule, which will give more insight about the binding of buffalo FGF2 with FGF2R and their role in buffalo embryo production.

SUMMARY

The 3 nucleotide changes were found in the buffalo FGF2 sequence as compared to cattle. These changes are— G to C at 31 bp, G to C at 69bp, and T to C at 282 bp positions. The nucleotide similarity of buffalo FGF2 was found as 99, 99, 95 and 90% with cattle, sheep, human, and mouse, respectively, whereas the amino acid similarity was found as 99, 99, 98, and 94% with cattle, sheep, human, and mouse, respectively. FGF2 ORF contains 468 bp nucleotides in the buffalo cumulus cells. The results indicated that the only one amino acid (from alanine to proline) was different in the buffalo sequence than the cattle. This single amino acid may cause variation in the binding site of FGF2 to its receptor (FGF2R) and hence could not be initiated FGF signaling, if this mutation is occurring at the FGF2 receptor biding site.

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