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# cDNA Cloning and Expression of the Bovine Factor in the Germline Alpha (*FIGLA*) Gene in Oocytes and Ovarian Follicles

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**Abstract:** The factor in the germline alpha (FIGLA) is a basic helix-loop-helix transcription factor that is essential for folliculogenesis and regulates expression of zona pellucida genes in mouse and human. But bovine homologue has not as yet been confirmed experimentally. Here, researchers present the first cDNA cloning and transcript expression analysis of the bovine *FIGLA* gene. Using RT-PCR and quantitative real-time PCR, researchers revealed that expression within adult cattle tissues is limited to the ovary. The researchers found that Germinal Vesicle (GV) oocytes, Metaphase II (MII) oocytes, 4 and 8 cell embryos, morula and blastocysts were all shown to express mRNA for FIGLA and expression were different in bovine oocytes and IVF embryos at different stages ($p<0.05$) with the highest expression in Germinal Vesicle (GV) oocytes and lower expression in 8 cells and blastocysts embryos. There was no difference in mRNA levels for FIGLA gene among bovine IVF, PA (Parthenogenetic Activated) and NT (Nuclear Transfer) blastocysts ($p>0.05$). *In situ* hybridization the FIGLA RNA was only localized in ovarian follicle. The persistence of FIGLA in adult cowes suggests that it may regulate additional pathways that are essential for bovine ovarian and embryonic development.

**Key words:** *FIGLA* gene, oocytes, ovarian follicles, cattle, embryonic development, China

## INTRODUCTION

The mammal oocyte is a mysterious cell. Not only can it develop into a new embryo by fertilization with a spermatozoon but also can become an embryo in the absence of sperm and reprogram somatic nuclei to initiate embryonic development via parthenogenesis (Pangas and Rajkovic, 2006). However, the molecular mechanisms necessary for the development of the oocyte and embryo are largely unknown. Cells differentiate through the activation and repression of gene transcription. This transcription process might control oocyte and embryo-specific genes regulating transcriptional cascades (Pangas and Rajkovic, 2006; Choi and Rajkovic, 2006).

Transcriptional control of oocyte and early embryo must include expression of oocyte-specific genes necessary for oocyte growth and early embryonic development. Oocyte-specific genes are some of the most abundant transcripts in the oolemma, ooplasm and nucleus. Transcriptional control of the oocyte and early embryo have been performed to identify genes preferentially expressed and got two new key genes in mouse models including newborn ovary homeobox gene (*Nobox*) (Rajkovic *et al*., 2004). Besides, there are some other oocyte and embryo-specific genes including POU domain, class 5, transcription factor 1, Oct4 (Kehler *et al*., 2004), growth differentiation factor 9, Gdf9 (McGrath *et al*., 1995), maternal antigen that embryos require, mater (Tong *et al*., 2000), zygote arrest 1, Zar1 (Wu *et al*., 2003) and Neurotrophins (NTs) (Paredes *et al*., 2004).

FIGLA (Factor in the Germline, Alpha), a basic helix-loop-helix transcription factor was first identified to control the regulation of three genes (*Zp1-Zp3*) encoding proteins that form the zona pellucida surrounding ovulated oocytes (Liang *et al*., 1997). It was the first germ

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cell-specific transcription factor shown to affect primordial follicle formation. FIGLA is expressed as early as E13.5 in the female gonad in mouse models and appears exclusively confined to oocytes of germ cell clusters and throughout folliculogenesis. FIGLA knockout female mice are infertile (Soyal *et al*., 2000). These research suggested that FIGLA plays critical roles in female germline and follicle development (Bayne *et al*., 2004; Joshi *et al*., 2007) but bovine FIGLA homologue has not as yet been confirmed.

In the present study, researchers investigated the expression of FIGLA in the cattle ovary, follicle and in mature oocytes. Researchers also determined the structure of the gene and researched the expression of the corresponding transcripts in the cattle female germline. This study suggest that *FIGLA* gene has expressions and/or functions specifically associated with folliculogenesis, oogenesis and early embryogenesis.

## MATERIALS AND METHODS

**Reagents and media:** All of the chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO, USA), except TCM-199 powder Gibco BRL (Paisley, Scotland, UK), Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone Company, Logan, UT, USA). The IVM medium was TCM-199, supplemented with 26.2 mM $NaHCO_3$, 5 mM HEPES, 5% estrous Cow Serum (OCS, self preparation), 2% Bovine Follicular Fluid (BFF, collected without regard to the stage of the reproductive cycle) and 0.1 μg $mL^{-1}$ FSH (7000 IU $mg^{-1}$). The embryo Culture Medium (CM) was TCM-199, supplemented with 3% OCS. The basic micromanipulation medium was TCM-199, supplemented with 5 mM $NaHCO_3$, 5 mM HEPES and 5% OCS. The fertilization medium was Tyrode's medium supplemented with 50 g $mL^{-1}$ heparin, 2.5 mM caffeine. All of the media were supplemented with 60 μg $mL^{-1}$ Penicillin G and 100 IU $mL^{-1}$ Streptomycin sulfate then sterilized by passing through a 0.20 μM filter and stored at 4-8°C for up to 4 weeks (Lu *et al*., 2005).

**Tissues:** Bovine ovaries were collected from a local abattoir and kept warm during transportation. In the laboratory, ovaries were washed in Dulbecco's phosphate-buffered saline at 35-37°C.

**Oocyte maturation *in vitro*:** Bovine Cumulus-oocytes Complexes (COCs) were recovered by aspiration of bovine follicles (diameter, 2-6 mm) using a 10 mL disposable syringe with an 18 gauge needle. Only oocytes possessing a compact complete cumulus oophorus and evenly granulated cytoplasm were selected for IVM (maturation *in vitro*). Then, COCs were washed twice in the IVM medium and cultured in a 30 mm glass dish containing 1.5 mL IVM medium for 20-22 h under a humidified atmosphere of 5% $CO_2$ in air at 38.5-38.8°C.

**Nuclear transfer:** Firstly, the researchers made a preparation of granulosa cells as donor karyoplasts. The granulosa cells were obtained by removing surrounding cumulus cells after IVM of oocytes. Granulosa cells were pretreated with 0.1 mg $mL^{-1}$ aphidicolin for 24 h and then cultured in DMEM+0.5% FBS for 6-9 days before use as donor nuclei.

Bovine donor granulosa cells were placed into a droplet of 100 μL manipulation medium containing 8% polyvinyl pyrrolidone dialyzed and 0.2 mM sucrose. The cytoplasmic membrane of donor cells was ruptured (as indicated by a rough surface) by repeatedly pipetting using a 10 mm inner diameter injection pipette before transferring to another manipulation drop containing enucleated oocytes in the injection medium. Thereafter, the donor cell was injected directly into the cytoplasm of a recipient oocyte. After microinjection, the reconstructed oocytes were incubated in CM under a humidified atmosphere of 5% $CO_2$ in air at 38.5-38.8°C for up to 26-28 h after the start of maturation. The activation of reconstructed oocytes was induced by exposure to 5 mM ionomycin in CM for 5 min and subsequent incubation in 2 mM 6-dimethylaminopurine for 3 h at 38.5-38.8°C and 5% $CO_2$ in air. Meanwhile, bovine oocytes matured *in vitro* for 26-28 h were activated by using the same method described above and were considered as the parthenogenetic control.

**Culture of IVF and parthenogenetic activated embryos:** After IVM, the oocytes were cultured in fertilization medium. IVF was performed by using frozen semen and fertilization medium. The straws of frozen semen were thawed in water at 37.5°C for 0.5 min. The obtained sperm was suspended in the fertilization medium for 30 min. Next, the supernatant were taken to other centrifuge tube and centrifuged for 5 min (1600 g). The supernatant were discarded. Sperm were obtained and transferred to the fertilization medium with oocytes under a humidified atmosphere of 5% $CO_2$ in air at 38.5-38.8°C. After 24-26 h, the surrounding cumulus cells and sperm were removed by manual pipetting in the CM. After IVM, surrounding cumulus cells were removed by manual pipetting in the CM, the reconstructed oocytes were incubated in CM under a humidified atmosphere of 5% $CO_2$ in air at 38.5-38.8°C for up to 26-28 h after the start of maturation. The activation of reconstructed oocytes was induced by

Table 1: SMART-PCR primers used for amplification and cloning of putative bovine *FIGLA* gene transcripts

| Genes | Sequence 5'-3' | Reference sequence |
|---|---|---|
| *F1* | 5' CTCAAGCGGCTGCCTTCGTC 3' | XM_607327 |
| *F2* | 5' CTGGTGCCATTTCTTCCC 3' | XM_607327 |
| *R1* | 5' TGATCGGGGTCTCGTTTCTC 3' | XM_607327 |
| *R2* | 5' GACCCTTCCTTCTCATTCTTCA 3' | XM_607327 |
| 3' outer primer | 5' GAGGTGCAACTGAATACATACAA 3' | XM_607327 |
| 3'inner primer | 5' CAAAGACTCCGAGAAACGAGACCC 3' | XM_607327 |
| 5'outer primer | 5' GGGGTCTCGTTTCTCGGAGTCTTT 3' | XM_607327 |
| 5'inner primer | 5' CCGCTTGAGCCGGCAGATGGTG 3' | XM_607327 |
| *GAPDH* forward | 5' CCAACGTGTCTGTTGTGGATCTGA 3' | NM_001034034 |
| *GAPDH* reverse | 5' GAGCTTGACAAAGTGGTCGTTGAG 3' | NM_001034034 |

Table 2: Details of primers used for RT-PCR

| Genes[a] | Sequence 5'–3' | Product size (bp) | Reference sequence |
|---|---|---|---|
| *FIGLA* | Forward 5'-CTGGTGCCATTTCTTCCC-3"<br>Reverse 5'-GACCCTTCCTTCTCATTCTTCA-3' | 245 | XM_607327 |
| *SDHA* | Forward 5'-GCAGAACCTGATGCTTTGTG-3'<br>Reverse 5'-CGTAGGAGAGCGTGTGCTT-3' | 185 | NM_174178 |

[a]Gene specific primers' Tm (°C) are 60°C in both FIGLA and SDHA

exposure to 5 mM ionomycin in CM for 5 min and subsequent incubation in 2 mM 6-Dimethylaminopurine (DMAP).

***In vitro* culture of embryos:** After fertilization, parthenogenesis or nuclear transfer activation, oocytes were placed into co-culture with granulosa cell monolayers in a 30 μL droplet of CM overlaid with mineral oil under a humidified atmosphere of 5% $CO_2$ in air at 38.5-38.8°C. The granulosa cell monolayers were established at 48-72 h before introduction of embryos. After introduction of embryos, half of the medium was replaced with fresh medium every 24 h. After 2 days of co-culture, cleavage of embryos was verified and the number of developed blastocysts was recorded within 9 days of co-culture.

**Complementary cDNA cloning and sequencing of FIGLA:** Total RNA was extracted with Trizol (Invitrogen Co., Carlsbad, CA) from cow ovaries according to the manufacturer's instructions. The cDNA sequences were compared with the sequences in the GenBank databases by using the BLAST program. Based on the cDNA sequence of Expressed Sequence Tags (ESTs) identified, gene-specific PCR primers were designed and the targeted genes were amplified by RT-PCR by using SMART PCR cDNA synthesis kit (Takara Clontech, Tokyo, JPN) and an advantage 2 PCR kit (Takara Clontech, Tokyo, JPN). The complete sequence of the gene was constructed by using the EST database by 5'-RACE and 3'-RACE (rapid amplification of cDNA ends). The GenBank Accession number of the cDNA is EU581635. Specific primers for each gene are shown in Table 1 and Fig. 1.

**Extraction of RNA and synthesis and amplification of cDNA:** Total RNA was extracted by by using a RNA easy micro kit (Qiagen, Crawley, UK) as previously described from oocytes and preimplantation embryos. First-strand cDNA was synthesized from 2 ng of total RNA from the oocytes or embryos. Reverse transcription was conducted with the PrimeScript™ 1st Strand cDNA synthesis kit (Takara, Tokyo, JPN). PCR system included the TaKaRa Ex Taq (TaKaRa Ex Taq 0.25 μL, 10×Ex Taq Buffer ($Mg^{2+}$ free) 5 μL, $MgCl_2$ (25 mM) 1.4 μL, dNTP mixture (each 2.5 mM) 4 μL, cDNA 2 μL, forward and reverse primers each 1 μL, $ddH_2O$ up to 50 μL) (Takara, Tokyo, JPN). PCR was conducted with Gene Amp. PCR system 9700 (ABI, MO, USA). Specific primers for each gene are shown in Table 2.

**Quantitative real-time PCR:** After total RNA was extracted and first-strand cDNA was synthesized, quantitative real-time PCR was conducted with SYBR Premix Ex Taq(2×SYBR Premix Ex Taq 12.5 μL, forward and reverse primers each 0.5 μL, cDNA 2 μL, $ddH_2O$ up to 20 μL) (Takara, Tokyo, JPN). The program used for all genes consisted of a denaturing cycle of 30 sec at 95°C; 45-50 cycles of PCR (95°C for 5 sec, 60°C for 15 sec and 72°C for 10 sec); a melting cycle consisting of 95°C for 0 sec, 65°C for 15 sec, 95°C for 0 sec and a step cycle starting at 65°C until 95°C with a 0.2°C $sec^{-1}$ transition rate and finally, a cooling cycle of 40°C for 30 sec.

***In situ* hybridization:** All the reagents used for ISH were purchased from Sigma. All the glass and plastic wares used were autoclaved and baked and all solutions and water were treated with Diethylpyrocarbonate (DEPC) to inactivate RNase prior to use. For ISH, the sections were deparaffinized in xylene, hydrated. After washing in PBS, the slides were incubated in 2×SSC (1×SSC 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7) for 15 min at room temperature. Prehybridization was carried out at room temperature for 2 h in a prehybridization solution containing 50% formamide, 4×SSC, 5×Denhardt's

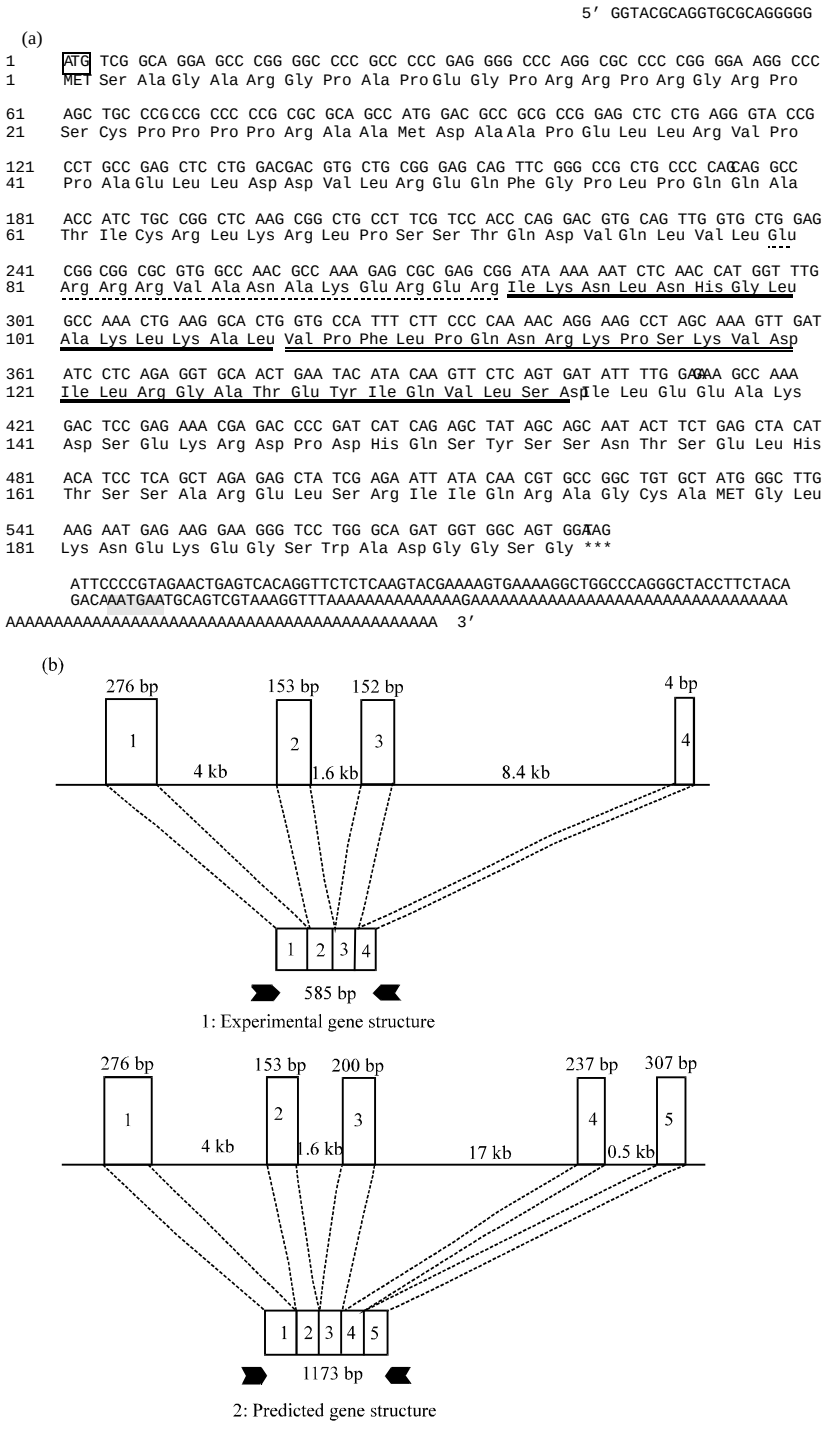


Fig. 1: Primary structure of FIGLA mRNA and protein. a) The nucleic acid sequence of the near full-length FIGLA cDNA (585 bp) was used to deduce the amino acid sequence of the resultant protein. The initiator AUG and stop codon, UAG are boxed and a variant polyadenylation signal is overlined. The longest open reading frame beginning at the initiator methionine was translated into a 194 amino polypeptide, the residues of which are represented with 5 and 3'UTR. The basic region is dotted line; the two helices are single underlined and the loop region is indicated with double underlined. b) Genomic structure of bovine FIGLA sequences. The previously published and current experimental exon/intron structures are shown with expected splice products

solution, 0.25% yeast tRNA, 0.5% sheared salmon sperm DNA and 10% dextran sulfate. The oligo probe used in this study was synthesized and labeled commercially (TBDSCIENCE, China). After prehybridization, the sections were hybridized overnight at 37°C with the labeled probe diluted in the same solution at a concentration of 2.5 pm $mL^{-1}$. Next day, the sections were stringently washed in varying concentrations of SSC (2×SSC for 10 min and 0.1×SSC for 10 min at 42°C) and blocked for 2 h at room temperature in blocking solution containing 2% normal sheep serum and 0.1% Triton X-100 in 0.1 M Tris-HCl buffer, pH 7.5. The sections were then incubated 2 h at room temperature in alkaline phosphatase conjugated anti-digoxigenin antibody diluted (1:500) in the above blocking solution. The signal was detected with Detection kit (for color detection with DAB) (BOSTER, Wuhan, China). The sections were viewed under microscope (Olympus, Tokyo, Japan). Sections incubated with sense FIGLA probe served as negative controls.

**Statistical analysis:** Relative levels of FIGLA mRNA were analyzed by ANOVA (GraphPad software, V2.02). Significant results were analyzed by Tukey's multiple comparison test. Results were considered significant at $p<0.05$. Each experiment was repeated at least three times.

## RESULTS AND DISCUSSION

**cDNA cloning of the bovine *FIGLA* gene:** Researchers cloned a full-length cDNA for bovine FIGLA from bovine ovary and identified the 585 bp the protein sequence region (CDS) cDNA as bovine FIGLA (Fig. 1a). The 3' untranslated region contains one AATGAA polyadenylation signal start, 23 bases upstream from the poly (A) addition site. The amino acid sequence deduced from a full-length bovine FIGLA cDNA is 194 amino acids. This suggested that the sequence discrepancy researchers observed was not the result of a PCR mistake or generation of a chimeric clone. Screening for the new FIGLA sequences in the predicted genomic sequence (Accession No. XM_607327) indicated that sequences matching the new 3' end were present in the genomic clone and that their boundaries matched consensus splice sites. This suggests that the *FIGLA* gene is composed of four rather than 5 exons (Fig. 1b). Exons 1 and 2 remain as predicted in GenBank (Accession No. XM_607327) as do sequences present in exon 3 but shortening a splice donor site 48 bp downstream of the predicted exon 3 by this length. An entirely new exon 4 was found between the predicted exons 3 and 4 (Fig. 1b). The amino acid sequence of this protein was compared to the sequences of FIGLA homologues from mouse (NP_036143), human (XP_232137), chimpanzee (XM_001138053), zebra fish (NM_198919) and toad (NM_001016342) (Fig. 2a). Conservation in the mammalian homologues remains high over the majority of the protein length.

The predicted basic helix-loop-helix region amino acids sequence of bovine FIGLA exhibited similarity of 91% to human FIGLA (XM_496553) and 90% to mouse FIGLA (NM_012013) (Fig. 2b). This indicated that the bHLH domain is highly conserved between all species. Researchers submitted this mRNA sequence to the Genbank (EU581635).

**Germ cell-specific expression of *FIGLA*:** RT-PCR analyses by using primers specific for FIGLA and GAPDH (positive control) transcripts, were performed on total RNA from 10 different tissues (Fig. 3). GAPDH as expected was ubiquitous among bovine tissues but FIGLA_transcripts were detected only in the ovary (Fig. 3, lane 4).

The FIGLA transcripts were further localized within the ovary by *in situ* hybridization. Ovaries from three 2 years old cows were fixed, sectioned and hybridized with anti-sense FIGLA probes. FIGLA transcripts were most notably localized in oocytes and granulosa cells of preantral and antral follicles (Fig. 4A-H). While FIGLA transcripts in preantral follicles was only faint in the surrounding follicle cells (Fig. 4A, C) and FIGLA transcripts in oocyte cytoplasm was only faint in ovarian tissue sections it was also marked in isolated cumulus cells (Fig. 4E, G) No staining was detected in negative controls (Fig. 4 B, D, E and H).

**Expression analysis of bovine FIGLA in cDNA derived from oocytes and preimplantation embryos:** Researchers intended to know whether mRNA for FIGLA could be detected in RNA isolated from bovine Germinal Vesicle (GV) oocytes, Metaphase II (MII) oocytes, 4 and 8 cell, morula and blastocysts, repectively. Amplification products of the expected size corresponding to FIGLA (245 bp) and Succinate Dehydrogenase flavoprotein subunit A (SDHA) (185 bp) were detected in cDNA sequence which was reversely transcripted by RNA extracted from bovine oocytes and embryos (Table 2). Results from the quantitative analysis of the *FIGLA* gene expression are shown in Fig. 5. There was no difference in mRNA levels for *FIGLA* Gene between bovine Vesicle (GV) oocytes and morula ($p>0.05$). While mRNA levels were different at other stages ($p<0.05$ with the exception of $p>0.05$ between metaphase II oocytes and 4 and 8 cell and blastocysts) with the highest expression in vesicle (GV) oocytes and lower expression in 8 cells and blastocysts embryos (i.e., GV oocytes,

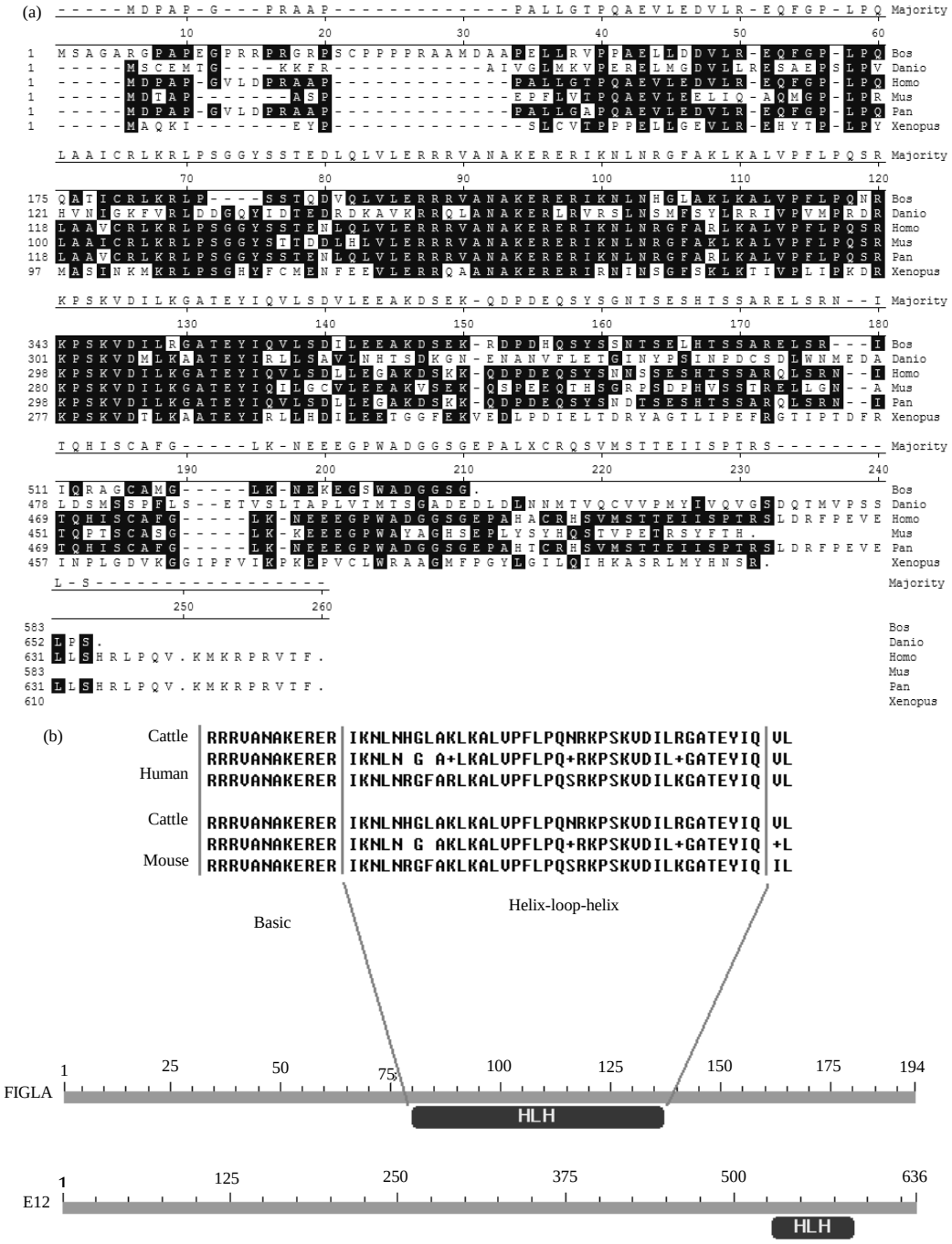


Fig. 2: a) Comparison of FIGLA protein sequences from human, mouse, chimpanzee, danio rerio and toad. Amino acid residues conserved across species are highlighted in blue grey; b) Schematic diagram of FIGLA and related basic helix-loop-helix transcription factors. Comparison of FIGLA protein sequences from human, mouse and cattle. The down red rectangle represents with the basic helix-loop-helix region in the FIGLA protein. The amino acid sequence alignment of the bHLH domain of FIGLA and transcription factors representative of E12 bHLH classes is shown. The GenBank Accession No. of the FIGLA cDNA is EU581635

1.00±0.17; MII oocytes, 0.42±0.10; 4 cell, 0.53±0.06; 8 cell, 0.15±0.04; morula, 0.99±0.23; blastocysts, 0.08±0.07; Fig. 5). There was no difference in mRNA levels for *FIGLA* gene among bovine parthenogenetic *in vitro* fertilized

and nuclear transfer blastocysts (i.e., IVF, 0.08±0.012; PA, 0.07±0.02; NT, 0.09±0.017; p>0.05; Fig. 6). FIGLA is a basic helix-loop-helix transcription factor discovered in a screen

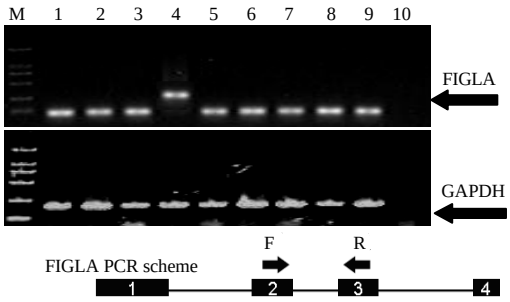


Fig. 3: Expression of the bovine *FIGLA* gene in a range of normalized cDNA samples derived from various bovine tissues: muscle (lane 1); spleen (lane 2); testicle (lane 3); ovary (lane 4); uterus (lane 5); lung (lane 6); liver (lane 7); heart (lane 8); kidney (lane 9); no RNA (lane 10). M, DNA 2000 bp Marker. Only was the band sequenced and verified as being FIGLA amplicons as indicated (245 bp) in the 4th lane. Results are compared with PCR mplification for the housekeeping glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene for the same tissues

to identify transcription factors that bind Zona pellucida (Zp) promoters (Choi and Rajkovic, 2006; Liang *et al*., 1997). FIGLA is the first germ cell-specific transcription factor shown to affect primordial follicle formation. FIGLA can bind a promoter motif called E-box (CANNTG), located upstream of transcription starts sites of the Zona pellucida (Zp) proteins and interacts with the ubiquitous transcription factor E12. Zp1-Zp3 are major components of the extracellular zona matrix that surrounds the developing oocytes and zona pellucida proteins are required for fertilization.

Individually, Zp1, Zp2 and Zp3 mouse knockouts can form primordial through antral follicles (Rankin *et al*., 1998, 1999, 2001). Now it is not known whether the triple knockout for the *Zp* genes disrupts early folliculogenesis. FIGLA deletion does not affect the transcription of other genes preferentially expressed in the oocyte including growth differentiation factor 9 (Gdf9), bone morphogenetic protein (Bmp15), kit receptor (kit) and fibroblast growth factor 8 (Choi and Rajkovic, 2006). These findings suggest that FIGLA likely regulates the expression of other downstream target genes that are critical in early folliculogenesis. Moreover, continual expression of FIGLA throughout folliculogenesis suggests that it is required to sustain transcription of its

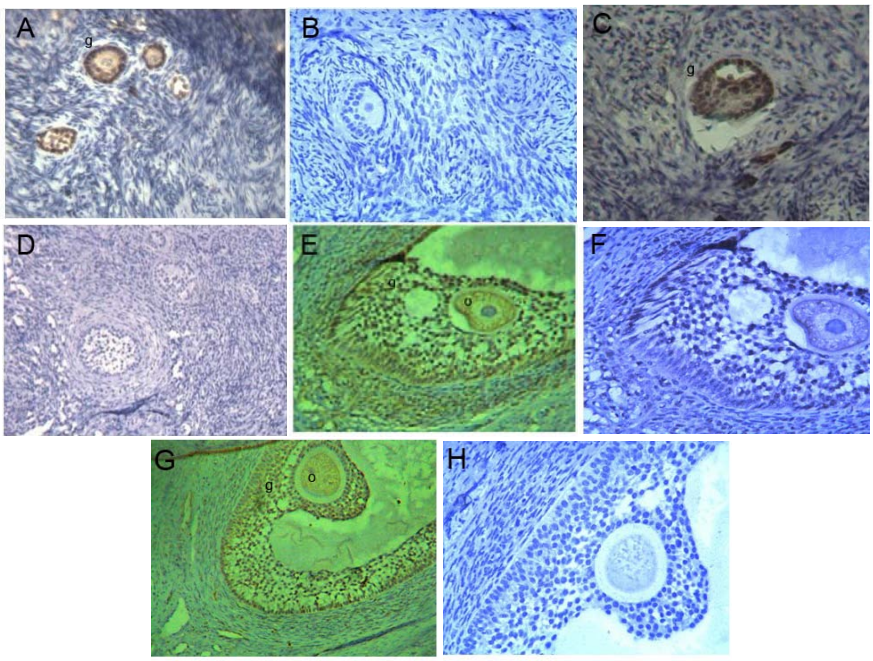


Fig. 4: Detection of FIGLA. By *in situ* hybridization of cow ovaries. The positive (A, C, E, G) and control (B, D, F, H) photomicrographs were obtained for sections hybridized with FIGLA specific anti-sense and sense probes. FIGLA Transcripts were detected in oocytes within growing follicles throughout the ovary but were particularly concentrated in the in both oocytes (o) and granulosa (g) cells of preantral (A, C) and antral (E, F) follicles. While it was only marked in isolated cytoplasm in oocytes (o)

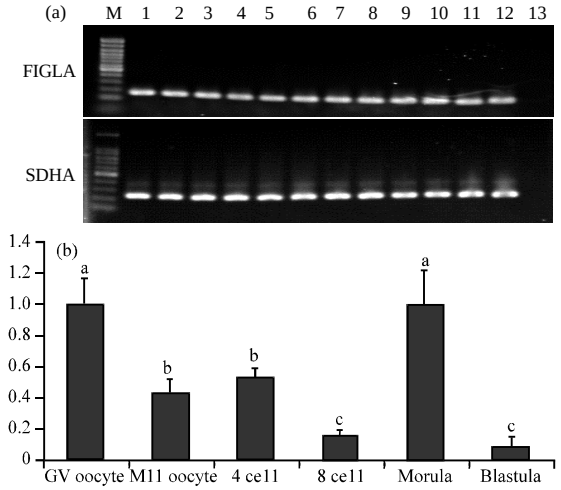


Fig. 5: a) The expression of *FIGLA*, *SDHA* gene transcripts in cDNA samples derived from oocytes and embryos. Stripped single Germinal Vesicle (GV) oocytes (lanes 1 and 2), stripped single Metaphase II (MII) oocytes (lanes 3 and 4), single preimplantation embryos 4 cell (lanes 5 and 6), 8 cell (lanes 7 and 8), morula (lanes 9 and 10) and blastocyst (lanes 11 and 12). Lanes 13 represent negative controls omitting added sample. b) Quantitative real-time RT-PCR of FIGLA mRNA expression in bovine oocytes and *in vitro* fertilization embryonic at different preimplantation stages. Each developmental stage was done in triplicate and 50 GV or MII oocytes , 40, 4 cells, 30, 8 cells , 20 morulaes and 10 blastocysts were used for each PCR run. The relative mRNA levels represent the amount in femtograms of mRNA expression corrected to the ratio of *SDHA* gene mRNA. Expression of FIGLA was used for normalization of Ct values and the $2^{-\Delta\Delta Ct}$ methods were used to calculate the relative expression. The different captital letter indicated highly statistical difference (The different lower case letter indicated highly statistical difference, $p<0.05$)

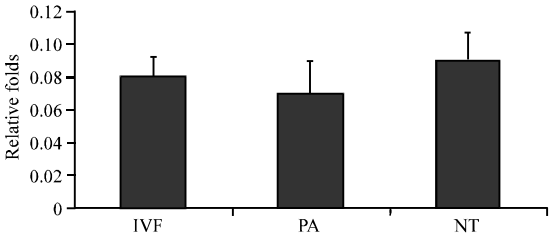


Fig. 6: Quantitative real-time RT-PCR of FIGLA bovine embryonic mRNA expression at different type blastocysts. Each developmental stage was done in triplicate and 10 *in vitro* fertilization, parthenogenetic activation or nuclear transfer blastocysts were used for each PCR run. The relative mRNA levels represent the amount in femtograms of mRNA expression corrected to the ratio of *SDHA* gene mRNA. Expression of FIGLA was used for normalization of Ct values and the $2^{-\Delta\Delta Ct}$ Ct methods were used to calculate the relative expression among blastocysts

target genes that may be critical both in folliculogenesis and early embryogenesis. One such candidate gene is the human and mouse homologue of FIGLA (Soyal *et al*., 2000). Expression of FIGLA mRNA in adult human and mouse oocytes has been demonstrated previously but no other data regarding expression or potential function in the cattle are available. Based on the full published bovine FIGLA coding sequence data predicted by automatic computational analysis and the homologous sequences derived by SMART and RT-PCR amplicons from samples. Researchers propose that the expression patterns reported truly represent the bovine *FIGLA* gene (Fig. 1a). The cattle gene is comprised of four exons, maps to chromosome 11 and encodes a peptide of 194 amino acids. The question remained as to whether this new sequence represents the only FIGLA transcript or whether alternative splicing occurs and the original predicted sequence is also represented.

The size of the product based on the new FIGLA sequence is 585 bp whereas that of the sequence predicted in the database is 1173 bp (Fig. 1b). In this study, the 3' untranslated region of the new sequence contains one AATGAA polyadenylation signal start, 23 bases upstream from the poly (A) addition site. This suggested that the sequence discrepancy. Researchers observed was not the result of a PCR mistake or generation of a chimeric clone obtained (Fig. 1b). This indicates that the new FIGLA coding sequence presented here is likely the sole transcript.

The amino acid sequence of this protein was compared to the sequences of FIGLA homologues from mouse, human, cattle and others species (Fig. 2a). Researchers found that the bHLH domain is highly conserved between all species (cattle: human, 96%, cattle: mouse, 96%, cattle: jocko, 96%, cattle: zebra, 84% and cattle: toad, 86%). Researchers think that is likely to be due to common essential functional requirements such as the binding of E-box motifs in the promoters of the *zona pellucida* genes and for dimerization with E12 or others bHLH proteins (Liang *et al*., 1997; Millar *et al*., 1991) (Fig. 2b). Real-time quantitative PCR is a sensitive and efficient technique to examine gene transcription

patterns in oocytes and embryos. The quantification of gene expression in tissue samples requires the use of reference genes to normalise transcript numbers between different samples. Reference gene stability may vary between different tissues and between the same tissue in different states. The function of SDHA is an electron transporter in the TCA cycle and respiratory chain. SDHA was used as reference gene in canine articular connective tissues, bovine polymorphonuclear leukocytes and human primary neuroblastoma (Fischer *et al*., 2005; De Ketelaere *et al*., 2006; Ayers *et al*., 2007; Yi *et al*., 2008). In order to optimize and validate the reaction to maximize the sensitivity and accuracy in quantitative real-time PCR, researchers chose SDHA as reference gene in the research because SDHA was found to be the most stable gene across the bovine embryonic stages. SDHA is an accurate normalization factor as reference gene (Goossens *et al*., 2005).

Researchers have measured FIGLA mRNA levels in oocytes and embryos *in vitro* fertilization (Fig. 5). The present study demonstrates that mRNA for FIGLA could be detected in RNA isolated from bovine Germinal Vesicle (GV) oocytes, Metaphase II (MII) oocytes, 4 and 8 cell embryos, morula and blastocysts, respectively suggesting that this factor could be important for oocytes and embryos (Pangas and Rajkovic, 2006). There was lower expression for FIGLA in 8 cell and blastocysts stage. During 8-16 cell period of bovine embryos, the maternal control of development maybe succeeded by zygotic control and the formation of a chromatin-mediated transcriptionally repressive state is beginning (Heikinheimo and Gibbons, 1998; Young *et al*., 1998). There was higher expression in Vesicle (GV) oocyte and morula stage, suggesting the effect of FIGLA on controling early folliculogenesis and promoting bovine early embryo development. Moreover, continual expression of FIGLA throughout embryogenesis suggests that it is required to sustain transcription of its target genes that may be critical both in folliculogenesis and early embryogenesis.

The cell nuclear transfer is generally successful but its overall efficiency is very low. Cloned embryos die at various stages of development including after birth due to a variety of developmental defects. Therefore if methods can be devised for prior selection of NT embryos with the potential to develop into normal offspring, this technology would be of enormous benefit for animal production and conservation practices. The NT embryos developed from adult female Granulosa cell Nuclear Transferred (GNT) oocytes have a lower incidence of perinatal abnormalities than skin Fibroblast cell Nuclear Transferred (FNT) oocytes do which suggests that reprogramming of GNT may be more complete and stable in gene expression than FNT (Kato *et al*., 2000).

Researchers thought that gene expression analysis from GNT might offer credible insight into the difference of gene expression in embryos derived from IVF, PA and NT. We found no difference in mRNA levels for *FIGLA* gene among bovine parthenogenetic *in vitro* fertilized and nuclear transfer blastocysts (Fig. 6).

Incomplete epigenetic regulation is suspected to be the cause of the abnormalities and the low efficiency associated with nuclear transfer (Suteevun *et al*., 2006). The aberrant expression pattern in nuclear transfer embryos was found for transcription factors thought to be involved in stress adaptation, trophoblastic function, DNA methylation and histone acetylation during preimplantation development.

These early deviations in gene expression patterns seen in nuclear transfer derived embryos warrant further investigations in postimplantation and neonatal development to understand the causative mechanism of the abnormalities and elevated mortality after transfer of nuclear transfer and activation derived embryos (Daniels *et al*., 2001; Wrenzycki *et al*., 2001; Humpherys *et al*., 2002). The change of these genes mRNA directly affect the quality of embryos (Rizos *et al*., 2002; Ruddock *et al*., 2004).

However, it was no difference in mRNA levels for *FIGLA* gene among different type blastocysts in the study. It suggests that transcription and expression of *FIGLA* gene are not affected easily during bovine early embryo development.

## CONCLUSION

Researchers have demonstrated the expression of the transcription factor FIGLA in the bovine ovary and in mature adult oocytes but not in a range of other tissues. Expression of FIGLA rises across early embryos. By using SMART and RT-PCR from amplified cDNA pools in conjunction with cloning and sequencing, the corrected gene structure of FIGLA was presented. By tissue *in situ* hybridization, researchers have been able to demonstrate thus far expression of the *FIGLA* gene to the bovine female germline. With the availability of full sequence information, further characterization of the bovine FIGLA transcription factor and its potential role in female infertility are now possible. The persistence of FIGLA in adult cow suggests that it may regulate additional pathways that are essential for bovine ovarian and embryonic development.

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