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Differentially Expressed Genes in Bovine Embryonic Stem Cell-Like Cells with Different Clone Morphologies and Passages

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Abstract: In this study, we screened the possible differentially expressed genes in bovine Embryonic Stem Cell (ESC)-like cells with different clone morphologies and passages via Differential Display Reverse Transcription PCR (DDRT-PCR) technique in combination with sequence analysis and GenBank BLAST. The results showed that among the bovine ESC-like cells with different clone morphologies, six differentially expressed fragments were identified highly homological with six known genes, namely: *RPL9*, *LOC100850994*, *AMP*, *RPL31*, *Erbb2ip* and *CLIP1*. Real-time quantitative PCR (qRT-PCR) results revealed that the expression levels of *RPL9*, *LOC100850994* and *RPL31* genes were significantly different among the bovine ESC-like cells with slate, block and bubble clone morphologies and Western blotting data showed that the expression levels of RPL9 were significantly different among the cells with the three clone morphologies. From the bovine ESC-like cells of different passages, seven differentially expressed fragments were identified highly homological with seven other known genes, namely: *IK*, *TKDP1*, *BZW*, *PRL9*, *RPL31*, *RP42* and *IGBP1*. qRT-PCR results revealed that the expression levels of IK, TKDP1, BZW, PRL9, RP42 and IGBP1 were significantly different among the bovine ESC-like cells of 1st and 10th passages. Western blotting data showed that protein expression levels of all these genes were significantly higher in passage 1 bovine ESC-like cells than passage 10 cells.

Key words: Genes, Western blotting, ESC-like, cells, bovine

INTRODUCTION

Embryonic Stem Cells (ESCs) are primarily and highly undifferentiated cells originated from blastocyst inner cell mass at the blastocyst stage developed from fertile egg division and primordial germ cells via *in vitro* culturing and cloning. ESC cells are featured by their highly original and pluripotent characteristics. Evans and Kaufman (1981) for the first time established the mouse embryonic stem cell lines. Thereafter, many investigators tried to perform the ESC cell lines of heavy livestock. Until know, there is some progress in the culturing of bovine ES cells. It has been reported that bovine ESC-like cells have been primarily acquired from *in vitro* and *in vivo* fertilised bovine embryos, inner cell mass of the nucleus transplanted embryos and original fetal bovine genital sex ridge (Sims and First, 1994; Saito *et al*., 2004; Gjorret and Maddox-Hyttel, 2005; Roach *et al*., 2006; Munoz *et al*., 2008; Cao *et al*., 2009). However, these bovine ESC-like cells can not support long-term passaging and do not migrate into sex ridge, hence, they are not real ESCs. It is very difficult to hard to successfully culture the bovine ESCs via similar protocols as those of the mouse ESC line establishment which may be due to the difference of the development patterns of early embryos among different species. It is becoming necessary to overcome the problems existing in the establishment of bovine ES cell lines and provide suitable environment for the growth and proliferation of bovine ES cells.

The bovine ESC lines have not been successfully established for long (Rexroad and Powell, 1997). The choice of culture medium and feeders, the approaches to acquire the inner cell mass and other multiple factors affect the growth of the bovine ESCs. Meanwhile, a suitable differentiation inhibitor is the key to the acquisition of a valid bovine ESC line. However, randomly trying the differentiation inhibitors among all the growth factors not only increase the workload but also make it very difficult to explore the true key factors affecting the differentiation and proliferation of bovine ESCs. This study uses the mRNA Differential Display Reverse Transcription PCR (DDRT-PCR) Method (Liang and Pardee, 1992) to compare the expression differences of the genes among different clone morphologies and passages of bovine embryonic stem cell-like cells, in order to screen out the genes closely related to the ESC proliferation and differentiation whereby to adjust the culturing systems of bovine ESCs and isolate and culture the true bovine ESCs *in vitro*.

MATERIALS AND METHODS

The acquisition of *in vitro* fertilised bovine eggs: Bovine ovary was washed with Phosphate Buffer Saline (PBS) at

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37°C. The ovary follicular content (in diameter of 2-8 mm) was extracted from the surface of ovary with a syringe containing a gauge -18 needle. The collected follicular fluid was placed on the sterile dish to detect the oocytes. The oocytes were cultured for 22-24 h until maturation in the culturing medium containing TCM199, 10 mmol L^{-1} HEPES, 1 μL mL^{-1} E2 and 0.1 mg mL^{-1} FSH. Frozen semenprovided from Inner Mongolia livestock improving station (Cat. XM 9621) was thawed in water bath at 37°C, followed by diluting with BO buffer containing 10 mmol L^{-1} caffeine (Brackett and Oliphant, 1975). After twice wash up with centrifugation at 3500 and 3000 rpm, respectively for 5 min, the floating living sperms were diluted with equal volume of buffer consisting of BO buffer, 7 μL mL^{-1} heparin and 20 mg mL^{-1} BSA to adjust the sperm concentration to 10^7/mL. The matured oocytes were put into the semen to co-incubate for 6~8 h. The fertilised embryos were moved to developing culture media containing SOFaa (Tervit *et al*., 1972) and 10% PVA. Observation was started from 7th day, 8-10 days later, blastocysts were observed to be hatched.

Preparation of mouse embryonic fibroblast feeder: Female and male mice were mixed, the next morning and vaginal suppository was checked and recorded as day 0.5. Body trunk of mice fetus of D 12.5-13.5 pregnant female mice was used. The body trunk of fetus was cut into pieces of <1 mm in diameter and the pieces were placed in centrifugal tubes to stabilise for 5 min. After the upper liquid was discarded, 1 mL 0.05% trypsin was added and gently blowed for 30 sec and then equal volume of culturing medium was added to terminate the trypsin digestion. After stabilising for 5 min, the upper cell suspension was collected. The process was repeated for two more times to discard the remained debris. The collected cell suspension was then centrifuged for 5 min at 1500 rpm. The supernatant was discarded and pellet was re-suspended in culturing medium, plated in culturing dishes and cultured at 37°C, 5% CO_2 and saturated humidity. The obtained mouse embryonic fibro blasts were trypsinised and passaged after reaching 100% confluence. Passage 2-3 cells were frozen for future usage.

Fetal fibroblasts of 80~90% confluence were treated with 30 ng mL^{-1} mitomycin C in serum-free DMEM medium for 2.5~3 house at 37°C. The cells were then washed with Ca^{2+} and Mg^{2+}-free PBS for 5 times, followed by trypsin digestion and centrifugation. The cells were then re-suspended and seeded at the density of 1.25×10^5 mL^{-1} inside 24-well plate coated with 0.1% gelatin. The plate was incubated at 37°C, 5% CO_2 and saturated humidity overnight.

Culture of bovine ESC-like cells: Referred to the protocols mentioned by Wang *et al*. (2012). The feeder cellswere replaced the medium by the stem cell culturing medium containing DMEM/F12, 15%FBS, 0.1 mmol L^{-1} β-mercaptoethanol, 0.1 mmol L^{-1} non-essential amino acid, antibiotics, 10 ng mL^{-1} LIF and 10 ng mL^{-1} bFGF. Feeder cells were balanced in incubator for 2 h and then the *in vitro* fertilised and hatched blastocysts were moved. The medium was changed daily and the cells were passaged every 3-5 days. Bovine ESC-like cells at 1st, 5th and 10th passagesand those with clone morphological features of slate, block and bubble were collected in RNase free centrifugal tubes and stored in liquid nitrogen for future use.

Alkaline Phosphatise Activity test: Bovine ESC-like cell colonies of different passages were selected and fixed in 4% paraformaldehyde for 30 min at room temperature or overnight at 4°C. Samples were then added with buffer premixed with AP, NBT and BCP at a ratio of 100:1:1 and incubated in dark for 40 min and imaged. MEF feeder layer around the stem cells was used as negative control.

Stem cell surface markers (SSEA-1, SSEA-4,TRA-1-60 and OCT-4) test: The fifth passage of bovine ESC-like cell colonies were picked out and fixed with 4% paraformaldehyde for 30 min at room temperature. Samples were then permeabilized in PBS with 0.1% TritonX-100 for 20 min and washed with PBS for three times, each time for 5 min. Primary antibody against SSEA-1, SSEA-4, TRA-1-60 or OCT-4 (Sigma Aldrich, USA) was added and incubated at 4°C overnight and washedwith PBS3 times, each time for 10 min. Secondary antibody was added in dark at room temperature and incubated for 1 h, followed by three times of wash with each for 10 min. The Propidium Iodide (PI) was added to stain for 15 min. Imaging procedures and photography were performed subsequently. The experimental procedures for the negative control did not include the addition of primary antibody but involved all other steps identical to other samples.

Total RNA extraction and reverse transcription: Total RNA was extracted according to the operation manual of RNAiso Plus reagent (Takara Co., Ltd. China). Reverse transcriptions to synthesize the first strand of cDNA were carried out per the manual of PrimeScript® RT reagent kit Perfect Real Time (Takara Co., Ltd. China).

Differential display of mRNA: PCR reaction system 10 μL) contained 5 μL Premix Ex Taq enzyme, 0.5 μL Anchorprimer, 0.5 μL Random primer, 1.0 μL cDNA template and 3.0 μL double distilled H_2O.

PCR reaction programme was 94°C, 60 sec; 21 cycles of (94°C, 30 sec; 40°C 90 sec; 72°C 60 sec) repeat; 72°C 600 sec; 94°C, 60 sec; 21 cycles of (94°C, 30 sec; 42°C, 90 sec; 72°C, 60 sec) repeat; 72°C 600 sec finished.

Table 1: Anchorand random primers

Tag name	Anchor primer sequences	Tag name	Random primer sequences
H-T15G (A)	5'-AAGCTTTTTTTTTTTTTTTG-3'	1	5'-AAGCTTGATTGCC-3'
H-T15A (B)	5'-AAGCTTTTTTTTTTTTTTTA-3'	2	5'-AAGCTTCGACTGT-3'
H-T15C (C)	5'-AAGCTTTTTTTTTTTTTTTC-3'	3	5'-AAGCTTTGCTCAG-3'
-	-	4	5'-AAGCTTCTCAACG-3'
-	-	5	5'-AAGCTTAGTAGGC-3'
-	-	6	5'-AAGCTTGCACCAT-3'
-	-	7	5'-AAGCTTAACGAGG-3'
-	-	8	5'-AAGCTTTTACCGC-3'

The anchor primers (20 bp) and random primers (13 bp) for PCR reaction shown in Table 1 were synthesised by Dalian TAKARA Biotech Co., Ltd. Sequences are as follows:

Silver staining, gel retrieval and re-amplification: The 10.0 μL RT-PCR products mixed with 2.0 μL loading bufferwere performed electrophoresis on 5% un-denatured paraformaldehyde (100 V constant voltage, electrophoresis for 220 min). The gel underwent silver staining and the differentiation bands were retrieved with EZNA Poly-Gel DNA Extraction kit (OMEGA). The retrieved products were applied as template for the secondary PCR amplification in a similar way to the afore mentioned method. The final products were identified by 2.0% agarose gel electrophoresis. The aimed PCR bands were separated under UV image, stored in 1.5 mL centrifugal tubes and retrieved according to the manual for AxyPrep DNA gel DNA retrieval.

Sequencing and sequence comparison: The retrieved DNA products were cloned in pMD®19T vector (Takara Co., Ltd. China) and heat shock-transformed in *E. coli* DH5α. White clones were picked up and cultured for enrichment and the extracted DNA was sent to Invitrogen Co. (Invitrogen Co., Ltd. USA) for sequencing. The sequencing products were analysed against NCBIGenBank database for gene identity search and similar gene information.

Fluorescent real time PCR: The gene specific primers were designed to cover the introns according to the GenBank BLAST search on mRNA sequencing fragments (Table 2). For the bovine ESC-like cells with different clone morphologies, 5th passage cells with slate clones were used as adjustment and block and bubble samples were used for the test. For the cells with different passages, cell of the primary passage were used as the adjustment whilst the fifth and tenth passages were tested. GAPDH was chosen as the internal control gene. Real time PCR machine (ABI 7300 Real time PCR System, Applied Biosciences Co.) and SYBR Premix Ex Taq (TaKaRa Co., Ltd. China) were used for the application.

Real-time PCR reaction was carried out per the manual of STAKARA. Reaction system (20 μL) consisted 12.5 μL SYBRPremix Ex Taq (2x), 0.5 μL forward primer, 0.5 μL reverse primer, 2.0 μL cDNA template and 9.5 μL double distilled H_2O. PCR reaction programme was 95°C (pre-denaturation) 30 sec; (95°C, 15 sec; 60°C 31 sec) repeat of 40 cycles. The Ct values acquired from the RT-PCR assay were normalised against those of passage 5 cells with slate clones and the primary passage of cells, respectively. Equation ratio = $2^{-\Delta\Delta C_T}$ was used for comparison of the gene expression among samples and the data were statistically analysed using SPSS stats package.

Table 2: Primers used in real time PCR tests

Genes	Direction	Primer sequence (5'-3')
RPL9	Forward	TACTGCGAGAATGAAGACCA
	Reverse	GATGTGATTGAAGTCCCTCC
RPL31	Forward	AAATCCGGAAGTTTGCCATGAA
	Reverse	TGGTGACGGGCACGTAGGTA
AMP	Forward	TATGCTTTATGCGTGGTGT
	Reverse	CCCTATGTCAGCGGAAACT
LOC100850994	Forward	ACAAAGCATCGCGAAGGC
	Reverse	GCACTGGGCAGAAATCACATC
ERBB2IP	Forward	AGACTCGCTTTGGTTTCC
	Reverse	TGTGAGAAGATTTTGGAAGG
CLIP1	Forward	GTCCACTTCGGGAATCAG
	Reverse	CTTCGTGCCACCAACCAG
IK	Forward	AAATTCTTGGGTGGTGACATGGA
	Reverse	TGCTGGCAATTTCAGCTCGTA
TKDP1	Forward	TGAATACTCAGCACTCAACGAGG
	Reverse	GGCCGTTCTGGGCATTGTAG
BZW	Forward	CTCTTTCCTCCTCCTCCTCCT
	Reverse	TTGGCTTTTGCTGCTTTTGA
RP42	Forward	GCCACTGGAGCAGCTAATGAC
	Reverse	GCCGAAGAATCCTGACAATACAA
IGBP1	Forward	CCAAACCTACAGAACACACAGCA
	Reverse	CCACAACTGATGAACCTATTATCCA

Western blotting: Passage 1, 5 and 10 bovine ESC-like cells and passage 5 cells with clone morphologies of slate, block and bubble were collected. Cells were put in centrifugation tubes with lysis buffer and stored in liquid nitrogen. Cells were lysed and the lysate was diluted by equal volume of 2× sample buffer and denatured by heating at 95°C for 4 min followed by chilling on ice top for 2 min. After a brief centrifugation at 12000 rpm, the supernatant was applied for the electrophoresis with 5% stacking gel and 15% separating gel. The separated proteins on the gels were transferred to PVDF membrane (Amresco Ohio Inc. USA). Primary antibodies used include anti-RPL9 (Sigma Aldrich, USA), anti-RPL31 (Sigma Aldrich, USA), anti-DCUN1D1 (RP42) (Sigma Aldrich, USA), anti-IGBP1 (Sigma Aldrich, USA),

anti-BZW1 (Sigma Aldrich, USA), anti-AMPK (Sigma Aldrich, USA) and anti-tubulin (Sigma Aldrich, USA). Secondary antibody was HRP conjugated goat anti-rabbit at 1:2500 (GE Sciences Co., UK). All antibodies were incubated for 1 h. Images were developed in ECL system and analysed by Image J electrophoresis software package.

RESULTS

Characteristic testsof bovine ESC-like cells: Alkaline phosphatase staining results showed that the bovine ESC-like cellsat all the threepassages and those with the three clone morphologies were alkaline phosphatase positive (Fig. 1 and 2). In addition, immunostaining showed the four embryonic stem cell specific markers including OCT-4, SSEA-1, SSEA-4 and TRA-1-61 were positive either in the passage 5 cells (Fig. 3). Collectly, these results revealed the ESC characteristics of the bovine ESC-like cells.

DDRT-PCR: DDRT-PCR was carried out on bovine ESC-like cells with different clone morphologies and those with different passages. Figure 4 showed in the gel

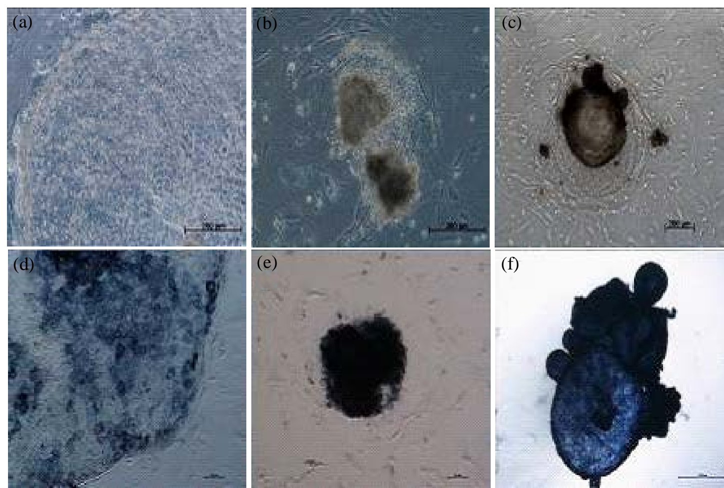

Fig. 1: Alkaline phosphatase staining of the bovine ESC-like cells with differentclone morphologies; a) slate clone morphology (unstained); b) block clone morphology (unstained); c) bubble clone morphology (unstained); d) slate clone morphology (stained); e) block clone morphology (stained) and f) bubble clone morphology (stained)

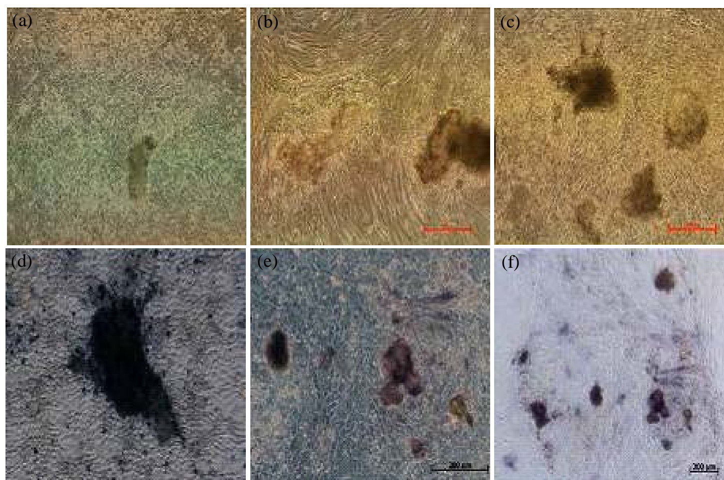

Fig. 2: Alkaline phosphatase staining of the bovine ESC-like cellsatdifferent passages; a) primary passage (unstained); b) fifth passage (unstained); c) tenth passage (unstained); d) primary passage (stained); e) fifth passage (stained) and f) tenth passage (stained)

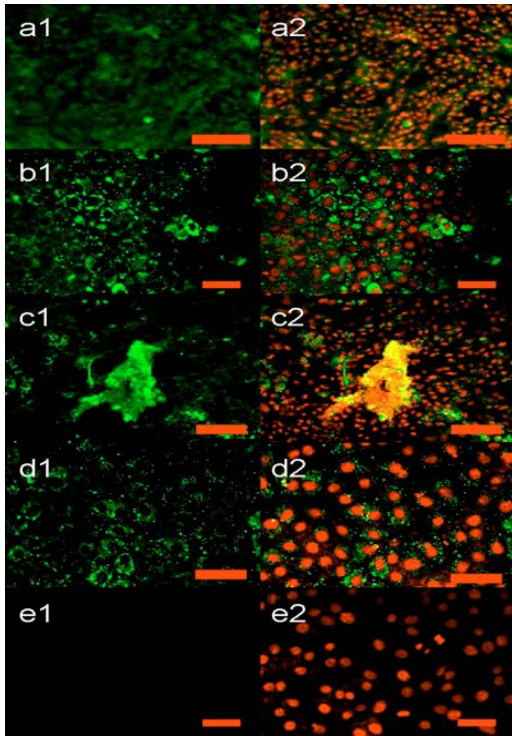

Fig. 3: Immuno histo chemistry; a1) anti-OCT-4 immunostaining; a2) anti-OCT-4 immunostaining overlapping with Propidium Iodide (PI) staining; b1) anti-SSEA-1 immunostaining; b2) anti-SSEA-1 overlapping with PI staining; c1) anti-SSEA-4 immunostaining; c2) anti-SSEA-4 overlapping with PI staining; d1) anti-TRA-1-61 immunostaining; d2) Anti-TRA-1-61 overlapping with PI staining; e1) no primary antibody stainingand e2) no primary antibody staining overlapping with PI staining

electrophoresis map of bovine ESC-like cells with different clone morphologies, 6 differentially expressed fragments were detected which were respectively numbered as No. 1-6 and pointed by arrows (Fig. 4). Figure 5 indicated the gel electrophoresis map of bovine ESC-like cells with different passages where the sites of 7 differentially expressed fragments (namely No. 7-13) were pointed by arrows (Fig. 5).

Sequence analysis of differentially expressed genes: Sequence analysis results were compared to those in GenBankvia BLAST software (Table 3). The 13 genes were screened out highly homological with known sequences. From the bovine ESC-like cells with different clone morphologies, six locinamely RPL9, RPL31, AMPK, ERBB2IP, CLIP1 and LOC100850994 were identified highly homological with known genes. In addition, from the cells

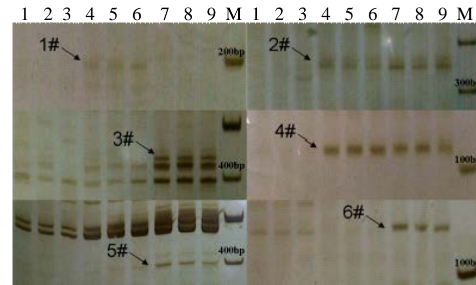

Fig. 4: Differentially expressed fragments in bovine ESC-like cellswith different clone morphologies determined by DDRT-PCR. 1~6# indicated bands with differential brightness on non-denaturing polyacrylamide gels corresponding to the cells with different clone morphologies. Lane 1-3: bovine ESC-like cells with slate clone morphology; lane 4-6: bovine ESC-like cells with block clone morphology; lane 7-12: bovine ESC-like cells with bubble clone morphology, lane M: DNA marker. Arrows showed the bands with differential brightness

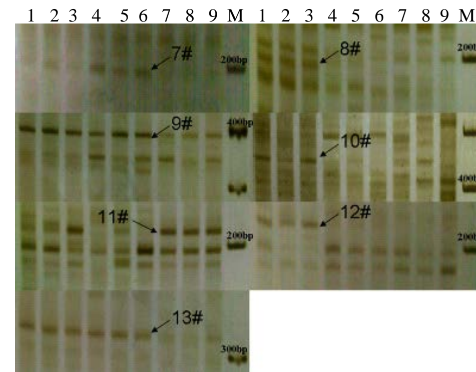

Fig. 5: Differentially expressed fragments in bovine ESC-like cells of different passages determined by DDRT-PCR. 7~13# indicated bands with differential brightness on non-denaturing polyacrylamide gels corresponding to cells at different passages. Lane 1-3: bovine ESC-like cellsfrom primary passage; lane 4-6: bovine ESC-like cells from 5th passage; lane 7-12: bovine ESC-like cellsfrom 10th passage; lane M: DNA marker. Arrows showed the bands with differential brightness

with different passages, seven loci, namely IK, TKDP1, BZW, PRL9, RPL31, RP42 and IGBP1 were identified highly homological with known genes. Among these genes, *RPL9* and *RPL31* were identified in both screening groups.

The mRNA expression of differentially expressed genes in bovine ESC-like cells with different clone morphology and cells at different passages: Real time quantitative PCRassay showed that RPL9mRNA expression in the bovine ESC-like cells with block and bubble clone morphologies was significantly different ($p<0.01$) from that of cells with slate clone morphology by 67% and 146% times, respectively. LOC100850994 expression in the cells with block and bubble clone morphologies was 33 and 273% times the expression in the cells with slate clone morphology ($p<0.01$). *RPL31* gene expression showed similar results. The expression of RPL31 in the cells with block and bubble clone morphologies was significantly different from thatof cells with slate clone morphology by 71% ($p<0.05$) and 158% ($p<0.01$) times, respectively. AMP, Erbb2ip and CLIP1expression did not exhibit significant difference between cells with slate and block clone morphologies ($p>0.05$) whilst these genes expressed significantly higher in the cells with bubble clone morphology than in the cells with slate clone morphology by 211, 143 and 161% times, respectively ($p<0.05$) (Fig. 6a).

Table 3: Sequence analysed on GenBank homology comparison

Size (bp)	Gene	Score	Identity (%)	Accession No.
166	*RPL9*[*]	302.0	100	NM_001024469.2
359	*RPL31*	664.0	100	NM_001025341.2
408	*AMP*	115.0	86	NM_001109802.1
118	*LOC100850994*	152.0	100	XR_139140.1
401	*ERBB2IP*	809.0	99	NM_001206153.1
166	*CLIP1*	206.0	100	NM_001192823.1
192	*IK*	206.0	99	NM_001103349.1
206	*TKDP1*	250.0	100	NM_205776.1
402	*BZW*	715.0	100	NM_001206613.1
394	*RPL31*[*]	649.0	100	NM_001025341.2
220	*RPL9*	298.0	99	NM_001024469.2
186	*RP42*	344.0	100	AC156365.3
334	*IGBP1*	69.4	79	NG_012084.1

[*]Indicates ribosome protein

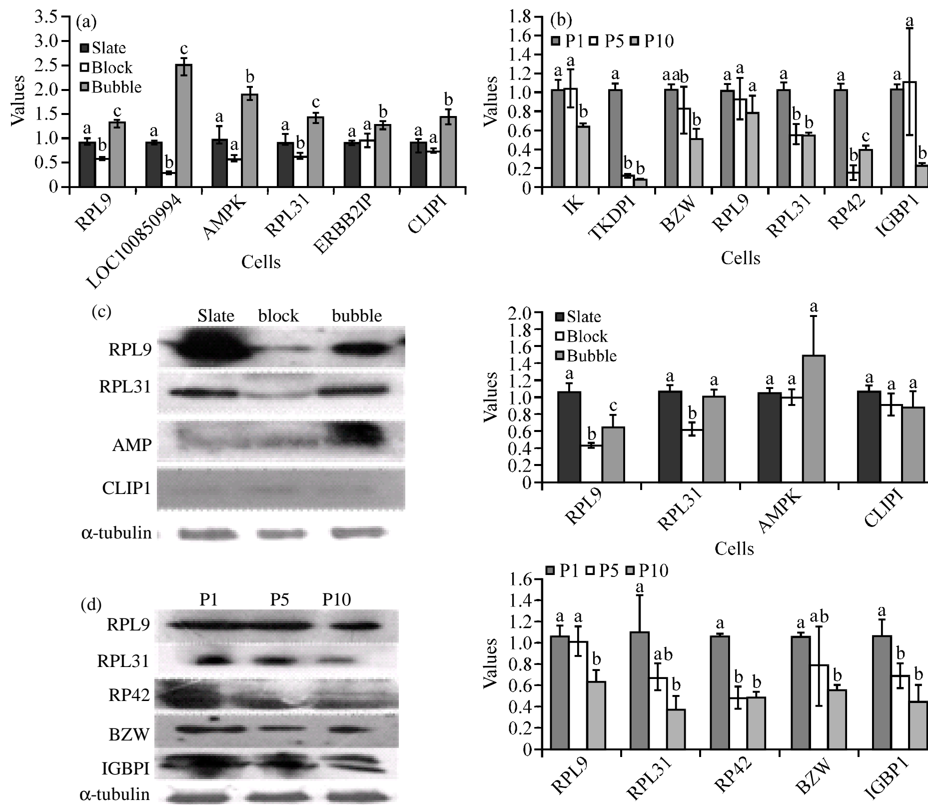

Fig. 6: Quantitative analysis of the mRNA and protein expression of the differentially expressed genes; a) qPCR tests on mRNA level of RPL9, RPL31, AMPK, ERBB2IP, CLIP1 and LOC100850994 in bovine ESC-like cells with slate, block and bubble clone morphologies; b) qPCR tests on mRNA level of IK, TKDP1, BZW, PRL9, RPL31, RP42 and IGBP1 in bovine ESC-like cells at primary, 5th and 10th passages; c) Western bloting assays on the protein levels of differentially expressedgenes in the bovine ESC-like cells with slate, block and bubble clone morphologies and d) Western bloting assays on protein level of the differentially expressed genes in the bovine ESC-like cells at primary, 5th and 10th passages

Real time quantitative PCRassay also revealed that the expression levels of IK, TKDP1, BZW, RPL9, RP42, and IGBP1 were significantly lower in the passage 10 bovine ESC-like cells than those in the primary passage cells ($p<0.05$) whilst RPL31 showed no significant difference ($p>0.05$) among passages (Fig. 6b).

The protein expression of differentially expressed genes in bovine ESC-like cells with different clone morphology and cells at different passages: To confirm the data from real time quantitative PCR, Western blotting assays examined the protein levels of the genes in the bovine ESC-like cells with different clone morphologies and cells at different passages. The results were normalized against the cells with slate clone morphology or primary passage cells. The α-tubulin was used as internal control for band densitometry normalization.

Assay results showed that RPL9 protein was expressed at its highest level in bovine ESC-like cells with slate clone morphology, followed by bubble clone morphology and the cells with block clone morphology had the lowest RPL9 expression ($p<0.05$). RPL31 expression level were observed insignificant in difference among the cells with slate and bubble clone morphologies ($p>0.05$) whilst it was significantly lower ($p<0.05$) in cells with block clone morphology. As to AMP and CLIP1 proteins, the expression levels revealed by protein densitometry did not exhibit significant difference among the cells with the three clone morphologies ($p>0.05$) (Fig. 6c). Although, the mRNA level of AMPK in cell with bubble clone was 211% of the level in cells with slate clone ($p<0.05$) the protein level was just vaguely changed ($p>0.05$). Similarly, CLIP1mRNA expression in cell with bubble clone was 161% of that in cells with slate clone ($p<0.05$) while no significant difference was observed in CLIP1 protein expression in between ($p>0.05$).

As shown in Fig 6d, the 10th passage cells presented significantly lower protein expression of IK, TKDP1, BZW, PRL9, RPL31, RP42 and IGBP1 than the primary passage cells ($p<0.05$).

DISCUSSION

In this study, we screened the possible differentially expressed genes in the bovine ESC-like cells with different clone morphologies and cells at different passages via DDRT-PCR technique. From the bovine ESC-like cells with different clone morphologies, we identified six loci, namely RPL9, LOC100850994, AMP, RPL31, Erbb2ip and CLIP1 and from the bovine ESC-like cells at different passages, we identified seven loci, namely IK, TKDP1, BZW, PRL9, RPL31, RP42 and IGBP1. Among these genes, *RPL9* and *RPL31* encoding ribosomal proteins were shown to express significantly different in both the bovine ESC-like cells with different clone morphologies and cells at different passages.

Ribosomes are protein synthesisers that are constituted by two subunits named as 40S and 60S according to their sedimentation coefficient. Ribosomes are mainly built up by ribosomal RNA (rRNA) and Ribosomal Protein (RP). RPs are further categorised into RPL and RPS according to their source from the major or minor ribosome subunit. The main function of RPs is protein synthesis. In addition, some Rpsare also involved in other biological processes such as DNA replication, transcription and translational regulations (Walleczek *et al*., 1989). RPL9, encoded by *rpL9* gene, belongs to the L6P ribosome protein family and is a component of 60S subunit (Brimacombe *et al*., 1990). RPL9 protein locates adjacent to L1, L2, L16 and L28 proteins (Nag *et al*., 1991) and exposes on the surface of the major ribosome complex (Agafonov *et al*., 1997). Some studies suggested that RPL9 services as ribosomal scaffolding protein which stabilises the the RNA molecules of the 23S subunit (Hoffman *et al*., 1994; Lillemoen *et al*., 1997). RPL31 (Ribosome Protein L31) is one of the components constituting the binding site for peptidyltRNAtransferase. RPL31 plays important role in the activation of the activity of peptide transferasein the major ribosome subunit (Fabijanski and Pellegrini, 1981). Researchers have identified multiple unique domains among the ribosome proteins. These domains include zinc finger, leucine zipper, nucleus localisation signal, etc. (Wool *et al*., 1995; Durand *et al*., 2003) which created DNA binding domainand regulates gene expression. It canbe thus speculated that the varied expression of RPL31 among the bovine ESC-like cells with different clone morphologies and cells at different passages regulates not only the protein synthesis in the cells but also the gene expression. In present study, both fluorescent real time PCR and Western blotting assay revealed that RPL9 was expressed at its highest level in bovine ESC-like cells with slate clone, followed by cells with bubble clone and the cells with block clone had the lowest RPL9 expression. It is thus indicated that the cells with the slate and bubble clones have high level of protein synthesis activities and the gene replication, transcription as well as translation are also relatively highly active. Cells of different passages further confirmed this view. The passage 10 cells showed significantly lower RPL9 and RPL31 protein expression than the primary ($p<0.05$) indicating that the efficiency of protein synthesis and gene replication is decreasing in the passage 10 bovine ESC-like cells and thus slowed the cell proliferation.

AMPK is a eukaryotic kinase sensitive to the change of cellular energy level. AMPK adjusts the cellular energy level by enhancing catabolism whilst inhibiting anabolism (Hardie, 2007). Recent studies showed that AMPK regulates the differentiation of progenitors of 3T3-L1 lipocytes and MC3T3-E1 osteoblasts (Fulco *et al*., 2008) by specifically inducing the expression of osteoblast related genes whilst inhibiting that of the lipocyte related genes (Habinowski and Witters, 2001; Kanazawa *et al*., 2008). This study showed that AMPK expressed in bovine ESC-like cells with bubble clonewas 211% of the level in cells with slate clone ($p<0.05$) whilst the protein level was just vaguely changed ($p>0.05$). It is known that AMPK mediates the differentiation of stem cells and the result in bovine ESC-like cells implies that the bovine ESC-like cellswith bubble clone has the tendency of differentiation but the AMPK protein expression was not significantly changed. The immunostaing against the specific cell surface markers for the embryonic stem cells showed that the bovine ESC-like cells with bubble clone morphology are still stem cells but have a tendency of differentiation.

CLIP1 (CAP-GLY domain containing Linker Protein 1) is a protein closely involved in cellular nucleic microtubule interaction and dynamics (Lohmann *et al*., 1995). The gene is located at bovine chromosome 5 (Allan *et al*., 2001). Cellular Architecture Protein (CAP) is constituted by three fragments. The first is the glycine-rich spherical N-terminal; the second fragment is the long central sequence speculated as α helices; the third fragment is the relatively short spherical C-terminal. The CAP-GLY structure in CLIP1 is a glycine rich 42-residue conserved region which plays important role in cytoplasmic movement, cell division, signal transduction, energy conversion, metabolism as well as fibroblast morphology (Minami *et al*., 2007). In the present study, the mRNA level of *CLIP1* gene had no significant change ($p>0.05$) between the bovine ESC-lie cells with slate and block clone morphology. The cells formed bubble clone had a significant ($p<0.05$) higher mRNA transcription level of that in cells with slate clone morphology by 161%. However, no protein expression difference was identified in Western blotting assay. This may indicate that cell signalling activities in cells with bubble clone morphology were more frequent than cells with other clone morphological types, leading to more active in cell movement. However, the actual function of CLIP1 protein in bovine ESC-like cells remains to be elucidated.

Erbin (erbb2 interacting protein) encoded by *ERBB2IP* gene, belongs to the LAP protein family featured by rich leucine repeats and PDZ domain (Borg *et al*., 2000). The protein has plural functions with multiple binding partners which are mostly related to cellular connection and cellular matrix chemotaxis. Erbin is important for cellular structural integrity, cell proliferation and differentiation, formation of organ morphology and cell signal transduction. In this study, real time quantitative PCR on mRNA revealed that *ERBB2IP* gene showed no significant expression difference ($p>0.05$) between bovine ESC-like cells with block and slate clone morphologies but the mRNA level in cells with bubble clone showed significant difference ($p<0.05$) from that in cells with slate clone morphology by 161%. This may probably attributable to the high proliferation and signal transduction activities of the bovine ESC-like cells with bubble clone which requires a high number of ERBB2IP molecules.

IK is a downstream regulator of MHCII (major Histocompatibility Complex II). The function of T cells is, to a large extent, dependent on the expression level of MHCII molecules. The fine tuning of MHCII molecules is crucial to immunological response regulation. The expression of MHCII family molecules is restricted to Antigen Presenting Cells (APC) such as dendritic cells and B lymphocytes. While in cells that do not expression MHCII family members, multiple types of stimulations, especially Interferon γ (IFNγ) can induced the expression of MHCII. IK protein can inhibit the IFNγ-mediated MHCII expression. It was reported that overexpression of IK protein in dermis T cell lymphoma causedthe failure of IFNγ stimulation and up-regulation of MHCII expression (Willers *et al*., 2001). In this study, IK expression in the bovine ESC-like cells at passage 10 was 61% of that in primary passage cells and the difference was significant ($p<0.05$). The actual function of IK protein in ESCs is not clear and remains for further investigation.

BZW1 (Basic leucine Zipper and W2 domains 1) is a member of the BZW1 transcription factor super family. The gene encodes a protein of 45 kDa and the N-terminal of the protein has a typical leucine zipper domain. The leucine zipper dimer is capable of binding to similar groups to form double helix (Mitra *et al*., 2001). Human BZW1 initiates the expression of histone H4. BZW1, together with other transcription factors, regulates cell cycle. It was reported that BZW1 was over expressed in mucoepidermoid carcinoma (Li *et al*., 2009). It has been revealed in this study that *BZW1* gene demonstrated a lower mRNA and protein synthesis level in passage 10 bovine ESC-like cells than passage 1 cells, implying that BZW1 may be involved in cell proliferation. Maintenance of high *BZW1* gene expression may be helpful for the *in vitro* culture of bovine ESC.

IGBP1 (Immunoglobulin CD79A Binding Protein 1) is highly similar to the rapamycin sensitive TAP42 in Escherichia coli in terms of sequence structure. The gene is located at Xq13.1-13.3 coding a 1.4 kb mRNA (Onda *et al*., 1997). Although the function of IGBP1 is still unclear in culturing bovine ESC-like cells *in vitro*, the present study showed that the mRNA and protein expression *IGBP1* gene was significantly lower in passage 10 bovine ESC-like cells than in primary passage cells (Grech *et al*., 2008) which implies that IGBP1 may also play a role in enhancing cell proliferation.

Three are three more genes, namely: *LOC100850994* (Bostaurus uncharacterised), *RP42* (Bostaurus Clone RP42-18L9) and TKDP1 (Bostaurus Trophoblast Kunitz Domain Protein 1) differentially expressed in bovine ESC-like cells with different clone morphologies and cells at different passages. However, the precise function of these genes in ESCs is unclear and remains further investigation.

CONCLUSION

This study revealed the differently expressed genes in bovine ESC-like cells with different clone morphologies and passages which provided experimental basis for further investigations upon the mechanism underlying the proliferation, differentiation, etc. of bovine ES cells.

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REFERENCES

Agafonov, D.E., V.A. Kolb and A.S. Spirin, 1997. Proteins on ribosome surface: Measurements of protein exposure by hot tritium bombardment technique. Proc. Natl. Acad. Sci., 94: 12892-12897.

Allan, G.J., D.J. Flint and K. Patel, 2001. Insulin-like growth factor axis during embryonic development. Reproduction, 122: 31-39.

Borg, J.P., S. Marchetto, A. Le Bivic, V., Ollendorff and F. Jaulin-Bastard *et al*., 2000. ERBIN: A basolateral PDZ protein that interacts with the mammalian ERBB2/HER2 receptor. Nat. Cell Biol., 2: 407-414.

Brackett, B.G. and G. Oliphant, 1975. Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod., 12: 260-274.

Brimacombe, R., P. Gornicki, B. Greuer, P. Mitchell and M. Osswald *et al*., 1990. The three-dimensional structure and function of *Escherichia coli* ribosomal RNA, as studied by cross-linking techniques. Biochimica Biophysica Acta Gene Struct. Expr., 1050: 8-13.

Cao, S., F. Wang, Z. Chen, Z. Liu and C. Mei *et al*., 2009. Isolation and culture of primary bovine embryonic stem cell colonies by a novel method. J. Exp. Zool. A Ecol. Genet. Physiol., 311: 368-376.

Durand, S., P. Abadie, S. Angeletti and S. Genti-Raimondi, 2003. Identification of multiple differentially expressed messenger RNAs in normal and pathological trophoblast. Placenta, 24: 209-218.

Evans, M.J. and M.H. Kaufman, 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature, 292: 154-156.

Fabijanski, S. and M. Pellegrini, 1981. Identification of proteins at the peptidyl-tRNA binding site of rat liver ribosomes. Mol. Gen. Genet., 184: 551-556.

Fulco, M., Y. Cen, P. Zhao, E.P. Hoffman, M.W. McBurney, A.A. Sauve and V. Sartorelli, 2008. Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt. Dev. Cell, 14: 661-673.

Gjorret, J.O. and P. Maddox-Hyttel, 2005. Attempts towards derivation and establishment of bovine embryonic stem cell-like cultures. Reprod. Fertil. Dev., 17: 113-124.

Grech, G., M. Blazquez-Domingo, A. Kolbus, W.J. Bakker, E.W. Mullner, H. Beug and M. von Lindern, 2008. Igbp1 is part of a positive feedback loop in stem cell factor-dependent, selective mRNA translation initiation inhibiting erythroid differentiation. Blood, 112: 2750-2760.

Habinowski, S.A. and L.A. Witters, 2001. The effects of AICAR on adipocyte differentiation of 3T3-L1 cells. Biochem. Biophys. Res. Commun., 286: 852-856.

Hardie, D.G., 2007. AMP-activated/SNF1 protein kinases: Conserved guardians of cellular energy. Nat. Rev. Mol. Cell Biol., 8: 774-785.

Hoffman, D.W., C. Davies, S.E. Gerchman, J.H. Kycia, S.J. Porter, S.W. White and V. Ramakrishnan, 1994. Crystal structure of prokaryotic ribosomal protein L9: A bi-lobed RNA-binding protein. EMBO J., 13: 205-212.

Kanazawa, I., T. Yamaguchi, S. Yano, M. Yamauchi and T. Sugimoto, 2008. Metformin enhances the differentiation and mineralization of osteoblastic MC3T3-E1 cells via AMP kinase activation as well as eNOS and BMP-2 expression. Biochem. Biophys. Res. Commun., 375: 414-419.

Li, S., Z. Chai, Y. Li, D. Liu and Z. Bai *et al*., 2009. *BZW1*, a novel proliferation regulator that promotes growth of salivary muocepodermoid carcinoma. Cancer Lett., 284: 86-94.

Liang, P. and A.B. Pardee, 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science, 257: 967-971.

Lillemoen, J., C.S. Cameron and D.W. Hoffman, 1997. The stability and dynamics of ribosomal protein L9: Investigations of a molecular strut by amide proton exchange and circular dichroism. J. Mol. Biol., 268: 482-493.

Lohmann, J., H. Schickle and T.C. Bosch, 1995. REN display, a rapid and efficient method for nonradioactive differential display and mRNA isolation. Biotechniques, 18: 200-202.

Minami, N., T. Suzuki and S. Tsukamoto, 2007. Zygotic gene activation and maternal factors in mammals. J. Reprod. Dev., 53: 707-715.

Mitra, P., P.S. Vaughan, J.L. Stein, G.S. Stein and A.J. van Wijnen, 2001. Purification and functional analysis of a novel leucine-zipper/nucleotide-fold protein, BZAP45, stimulating cell cycle regulated histone H4 gene transcription. Biochemistry, 40: 10693-10699.

Munoz, M., C. Diez, J.N. Caamano, A. Jouneau, I. Hue and E. Gomez, 2008. Embryonic stem cells in cattle. Reprod. Domest. Anim., 4: 32-37.

Nag, B., S.S. Akella, P.A. Cann, D.S. Tewari, D.G. Glitz and R.R. Traut, 1991. Monoclonal antibodies to *Escherichia coli* ribosomal proteins L9 and L10. Effects on ribosome function and localization of L9 on the surface of the 50 S ribosomal subunit. J. Biol. Chem., 266: 22129-22135.

Onda, M., S. Inui, K. Maeda, M. Suzuki, E. Takahashi and N. Sakaguchi, 1997. Expression and chromosomal localization of the human alpha 4/IGBP1 gene, the structure of which is closely related to the yeast TAP42 protein of the rapamycin-sensitive signal transduction pathway. Genomics, 46: 373-378.

Rexroad, Jr., C.E. and A.M. Powell, 1997. Culture of blastomeres from *in vitro*-matured, fertilized and cultured bovine embryos. Mol. Reprod. Dev., 48: 238-245.

Roach, M., L. Wang, X. Yang and X.C. Tian, 2006. Bovine embryonic stem cells. Methods Enzymol., 418: 21-37.

Saito, S., B. Liu and K. Yokoyama, 2004. Animal embryonic stem (ES) cells: Self-renewal, pluripotency, transgenesis and nuclear transfer. Hum. Cell, 17: 107-115.

Sims, M. and N.L.M. First, 1994. Production of calves by transfer of nuclei from cultured inner cell mass cells. Proc. Natl. Acad. Sci. USA., 91: 6143-6147.

Tervit, H.R., D.G. Whittingham and L.E.A. Rowson, 1972. Successful culture *in vitro* of sheep and cattle ova. J. Reprod. Fertil., 30: 493-497.

Walleczek, J., B. Redl, M. Stoffler-Meilicke and G. Stoffler, 1989. Protein-protein cross-linking of the 50 S ribosomal subunit of *Escherichia coli* using 2-iminothiolane. Identification of cross-links by immunoblotting techniques. J. Biol. Chem., 264: 4231-4237.

Wang, R., S. Cong, M. Cang, Y. Ma, J. Wen and D. Liu, 2012. Factors affecting the derivation and expansion of bovine embryonic stem cell-like cells. J. Anim. Vet. Adv., 11: 2865-2872.

Willers, J., A. Haffner, K. Zepter, M. Storz, M. Urosevic, G. Burg and R. Dummer, 2001. The interferon inhibiting cytokine IK is overexpressed in cutaneous T cell lymphoma derived tumor cells that fail to upregulate major histocompatibility complex class II upon interferon-γ stimulation. J. Invest. Dermatol., 116: 874-879.

Wool, I.G., Y.L. Chan and A. Gluck, 1995. Structure and evolution of mammalian ribosomal proteins. Biochem. Cell. Biol., 73: 933-947.