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Identification of Differentially Expressed Genes in Yak Preimplantation Embryos Derived from *in vitro* Fertilization

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Abstract: The purpose of this study was to uncover differential patterns of mRNA expression for genes related to different development stages of yak (*Bos grunniens*) oocytes and preimplantation embryos. Researchers identified differentially expressed bands by mRNA differential display technology and evaluated the relative expression level of differentially expressed genes in oocytes, two cells, four cells and eight cells embryos and in morulae and blastulae. Researchers identified four differentially expressed bands of which one gene that was expressed during morula stage showed no homology to any other known genes in GenBank. Three other genes identified showed high identity with zinc finger MYND11 (ZMYND11), Nucleosome Assembly Protein 1-Like 1 (NAP1L1) and Ribosomal Protein L27A (RPL27A), respectively. The expression of ZMYND11 increased in mature oocytes, two cells and eight cells embryos and in the blastula with no significant differences between these stages ($p>0.05$). NAP1L1 was expressed at a relatively low level in mature oocytes, two cells embryos and in the blastula with a sharp increase in eight cells embryos ($p<0.05$). The expression of RPL27A decreased during development from mature oocytes to two cells embryos and then to eight cells stage embryos and increased significantly in cells of the blastula ($p<0.05$). ZMYND11, RPL27A and NAP1L1, the mRNA expression levels of which showed differences in different development stages of yak oocytes and early embryos might be related to normal development of early embryos and may be involved in maintaining normal reproductive function in yak.

Key words: Yak, preimplantation embryo, embryo development, gene expression, oocytes

INTRODUCTION

Sperm-egg fusion triggers the development of the mammalian early embryo. The pre-implantation embryo develops through the following stages: activation of the embryonic genome, compaction and the morula and blastula stages, accompanied by gene expression in a specific spatial-temporal pattern (Kepkova *et al*., 2011). Maternal mRNAs play an important physiological role in the early development of the embryo. Many mRNAs and proteins, synthesized during the formation and maturation of oocytes, regulate the early development of the embryo, a process that is referred to as maternal regulation (Simonelig, 2012). As the fertilized egg develops, the gradual degradation of maternal mRNA and expression of zygotic genes begins. The development process depends entirely on the transcription driven by the activation of the embryonic genome itself and is regulated by zygotic genes. The development and differentiation of the early embryo are controlled by one or more genes, the quantities of which are different at different stages. Differential Display Reverse-Transcription Polymerase Chain Reaction (DDRT-PCR) is frequently used in the analysis of differentially expressed genes (Alves *et al*., 1998; Kim *et al*., 2007; Wang *et al*., 2009). With this method, differentially expressed genes in cells are identified by efficient amplification through PCR following the reverse transcription of cDNA with total cellular RNA as template as well as the design of primers (Liang and Pardee, 1992). Extensive research, focused on gene expression patterns of the early embryo has been conducted with DDRT-PCR in murine (Lee *et al*., 2001), rabbit (Kietz and Fischer, 2003), goat (Li *et al*., 2006a, b), bovine (Balasubramanian *et al*., 2007; Kanka *et al*., 2009) and human (Li *et al*., 2006a, b) models while there have been no reports of the application of this method in yak (*Bos grunniens*).

Yaks are seasonal breeders. In natural-breeding yak populations, the reproduction rate is between 60 and 75%

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while the survival ratio is between 45 and 75% (Zi, 2003). The low reproduction rate is related to environmental conditions, nutritional state as well as the development potential of yak embryos. Embryo transfer (Yu *et al*., 2007) and *In Vitro* Fertilization (IVF) (Zi *et al*., 2008) have yielded some success in improving the reproduction rates; however there are also some issues related to the low efficiency of embryo production. Consequently, cloning genes related to the development of *in vitro* fertilized yak early embryos and comparison of the differences in gene expression between oocytes and the preimplantation embryo can not only help elucidate the regulatory mechanisms driving gene expression changes in the yak early embryo but also provide theoretical and practical insights of the factors that affect yak early embryonic development. A better understanding of the mechanisms and factors involved in early embryonic development, especially those that impact the success of IVF are also of great importance to reproduction research.

MATERIALS AND METHODS

Collection of oocytes and early embryos: Yak oocytes were obtained from designated abattoirs in the Tianzhu Tibetan Autonomous County in China. Ovaries were removed within 30 min after slaughter, placed into preserving fluid and transferred to the laboratory within 3 h. Cumulus-oocyte complexes were collected by careful aspiration. The *in vitro* maturation and fertilization of the oocytes and the *in vitro* culture of the embryos were carried out according to the method of Guo *et al*. (2012). Two, four and eight cells embryos and morulae and blastulae were collected at different developmental stages into RNase-free microcentrifuge tubes.

Total RNA extraction: Total RNA was extracted from the collected yak oocytes and early embryos (20 each at every stage) with cell lysis buffer (RNAiso Reagent) and dissolved in 20 μL RNase-free water. The ratio of the absorbances at 260 and 280 nm (OD_{260}/OD_{280}) of the total RNA was measured with a spectrophotometer. The quality of RNA was ascertained by electrophoresis of the extracted RNA on 2% agarose gels followed by visualization under UV light after ethidium bromide staining.

Reverse transcription and PCR amplification: First-strand of the cDNA was synthesized at 37°C for 15 min using the PrimeScript RT-PCR kit (Perfect Real Time, Takara) and the transcriptase was inactivated at 85°C for 5 sec in a 10 μL Reverse Transcription Reaction System according to manufacturer's instructions.

The product of reverse transcription was used as the template for cDNA synthesis with an anchor primer HT (15) C (5'-AAGCTTTTTTTTTTTTTTCC-3') as well as random primers Hap1 (5'-AAGCTTGCACCAT-3'), Hap2 (5'-AAGCTTTTACCGC-3') and Hap3 (5'-AAGCTTA CGATGC-3') (Takara, Dalian). The reaction system was as follows: 12.5 μL of the 2×Master Mix (KT201, Takara), 3 μL of cDNA, 1 μL each of HT (15) C (10 μM), Hap1 (10 μM), Hap2 (10 μM) and Hap3 (10 μM) and 5.5 μL of ddH_2O. Each reaction was subjected to PCR amplification with the following parameters: 94°C for 1 min; 20 cycles of 94°C for 30 sec, 40°C for 90 sec and 72°C for 1 min and a final extension at 72°C for 10 min.

Electrophoresis and DNA band recovery: After PCR amplification, 10 μL of the reaction was mixed with 2 μL loading buffer and subjected to 6% non-denaturing polyacrylamide gel electrophoresis (200 V, 3.5 h). After silver staining and photography, differentially expressed bands were excised, washed with water, stamped with a pipette tip and boiled. After electrophoresis through 1.5% agarose gels, the DNA was recovered from the supernatant with a DNA gel purification kit (Tiangen, Beijing) and the purity of the recovered DNA was assessed by electrophoresis on a 1% agarose gel, ethidium bromide staining and UV illumination.

Second PCR amplification and product recovery: Recovered DNA was used as template for second PCR amplification as described in reverse transcription and PCR amplification. Amplified DNA was subjected to 1% agarose gel electrophoresis, excised under a long-wave UV light, transferred into a 1.5 mL microcentrifuge tube and recovered according to user's manual of a DNA gel purification kit (Promega).

Sequencing and sequence analysis: Recombinant plasmids were prepared by ligation of the gel-purified DNA and appropriately-digested pMD19-T vector at 16°C overnight and the ligation mixture was transferred into *E. coli* DH5α cells using the heat-shock method. Following transformation, *E. coli* cells were incubated at 37°C for 12 h on Luria-Bertani (LB, Sigma) agar containing ampicillin (Amp, Sigma), 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal, Takara) and Isopropyl β-D-1-Thiogalactopyranoside (IPTG, Sigma-Aldrich) for blue/white screening of transformants. White colonies were isolated and cultured at 37°C in LB liquid media containing Amp at 200 rpm overnight. Positive colonies identified by colony PCR were used for plasmid extraction and further validated by digestion with EcoR I and Hind III to confirm the presence of the inserts followed by sequencing (Takara, Dalian). Sequence identity analysis and comparison to sequences in the GenBank database (http://www.ncbi.nlm.nih. gov/genbank/) was carried out using BLAST (http://blast. ncbi.nlm.nih.gov/).

Table 1: Primer sequences

Genes	Primer sequence	Product size (bp)	Annaling temperature (°C)
GAPDH (NM_001034034)	F:5'-CCACGAGAAGTATAACAACACC-3'	120	60.0
	R:5'-GTCATAAGTCCCTCCACGAT-3'		
ZMYND11 (NM_001045940)	F:5'-CGGCCAAAGTCATGCAGAA-3'	347	63.5
	R:5'-TAACATGCAGCCGGTGAACA-3'		
RPL27A (NM_001024471)	F:5'-CAGAGTTTCTGCCCGACTGT-3'	185	63.0
	R:5'-AATTTGGCCTTCACGATGAC-3'		
NAP1L1 (BM285439)	F:5'-CCTCATCCGCTTCTTCACCTT-3'	146	63.3
	R:5'-TTCTGAAGCTATCCTCGCTGC-3'		

Analysis of mRNA expression by RT-PCR: Control primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Sigma) and gene-specific primers designed according to the corresponding mRNA sequences (Table 1) were synthesized at Takara. The expression levels of mRNA were detected by real-time RT-PCR in triplicate (Bio-Rad, USA). The reaction conditions were as follows: 12.5 μL of 2×SYBR® Premix Ex TaqTM, 1 μL each of the primers (10 mmol L^{-1}), 9.5 μL ddH_2O, 1 μL of cDNA (from two cells, four cells and eight cells embryos and morulae and blastulae, respectively). Each reaction mixture was subjected to PCR amplification with the following parameters: 95°C for 30 sec; 20 cycles of 95°C for 5 sec and 64.5°C for 30 sec.

RESULTS AND DISCUSSION

Screening of differentially expressed genes: After amplification by RT-PCR and 6% non-denaturing polyacrylamide gel electrophoresis, DNA isolated from yak embryos at different stages of development was subjected to silver staining for detection of differentially expressed bands (indicated by squares in Fig. 1).

Sequence analysis of differentially expressed bands: Bands corresponding to the differentially expressed genes were gel-purified, cloned and sequenced. The obtained sequences were then compared to sequences in the Expressed Sequence Tag (EST) and Non-Redundant (NR) data in GenBank using the BLAST search and analysis tool (Table 2).

Analysis of the expression of differentially transcribed genes in oocytes and early embryos of yak: Differentially expressed bands were amplified by PCR using cDNA from oocytes and embryos at different stages of development and primers were designed for RT-PCR analysis of the differentially expressed genes. The relative expression of Zinc Finger, MYND Domain-containing 11 (ZMYND11) (Fig. 2), Nucleosome Assembly Protein 1-Like 1 (NAP1L1) (Fig. 3) and Ribosomal Protein L27a (RPL27A) are shown in Fig. 4.

As an important stage in embryo development, the maternal to zygotic transition is accompanied by changes in molecular structure and cell cycle (Han *et al*., 1996). One characteristic of this transition is the degradation of

Table 2: Blast analysis of differentially expressed bands and comparison of the sequences with the data in GenBank

Band origin	Genes	Size (bp)	Score	Identity (%)	Accession No.
Two cell embryo	*RPL27A*	462	843	99	NM_001024471
Eight cell embryo	*NAP1L1*	229	401	96	NM_139207
Morulae	*YME*	722	-	-	-
Blastula	*ZMYND11*	749	1356	97	NM_001045940

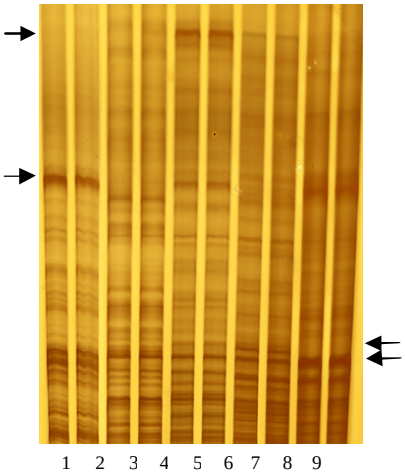

Fig. 1: Identification of differentially expressed bands in yak oocytes, two, four and eight cells embryos and morulae and blastulae; 1, 2: two cells embryos; 3, 4: four cells embryos; 5, 6: eight cells embryos; 7, 8: Morula; 9, 10: blastocysts

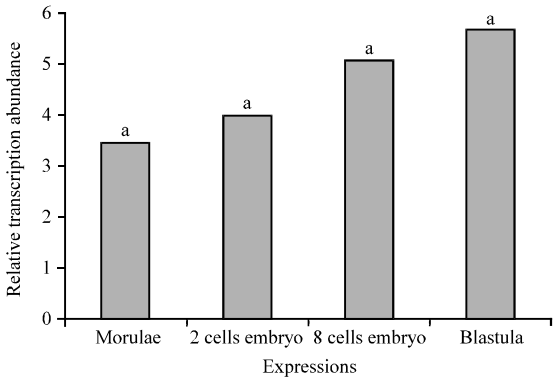

Fig. 2: Expression of ZMYND11 mRNA in yak oocytes and early embryos as detected by RT-PCR for different development stage, same letter indicate no difference(p>0.05)

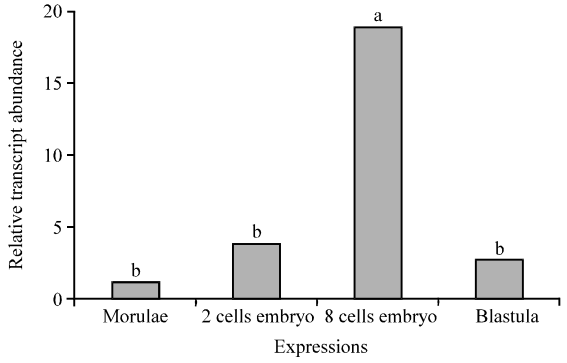

Fig. 3: Expression of NAP1L1 mRNA in yak oocytes and early embryos as detected by RT-PCR for different development stage, different letter indicate significant difference ($p<0.05$) and same letter indicate no difference ($p>0.05$)

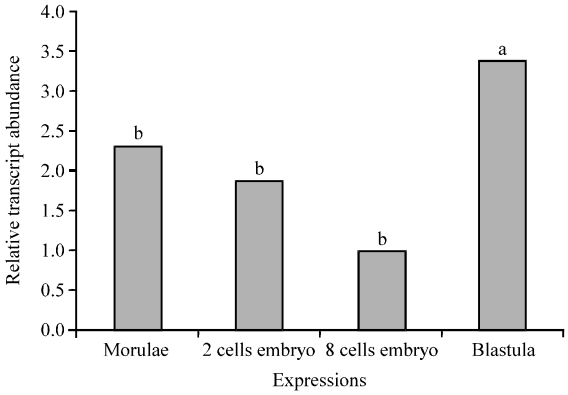

Fig. 4: Expression of RPL27A mRNA in yak oocytes and early embryos as detected by RT-PCR for different development stage, different letter indicate significant difference ($p<0.05$) and same letter indicate no difference ($p>0.05$)

maternal mRNA and increased transcription of the zygotic genome (Telford *et al*., 1990). Numerous differences exist in gene expression during the process of oocyte maturation and maternal to zygotic transition. Accordingly, research focused on genes that are differentially expressed during maternal to zygotic transition may help elucidate the regulatory mechanisms in early embryo development and better understand the developmental processes during the earliest stages of life.

Study of genes that exhibit differential expression in an important step toward delineating the spatial-temporal expression and regulation of genes during early embryonic development (Balasubramanian *et al*., 2007). In this study, researchers successfully used the DDRT-PCR Method to identify four differentially expressed genes at specific stages during yak embryonic development. Based on the analyses, researchers identified the *YMU* gene (Yak Morula Unknow gene) which showed no homology to any other genes in GenBank while three of the other genes researchers identified showed high identity ZMYND11, NAP1L1 and RPL27A, respectively. The discovery of these novel differentially expressed genes indicates that there may exist some functional genes, compared to other species, specific to the regulation of yak early embryonic development. The relationship of these differentially expressed genes to seasonal breeding of yak needs to addressed further in future studies.

ZMYND11 is involved in nucleic acid metabolism and participates in DNA transcription as well as post-transcriptional processing (Zimin *et al*., 2009). The data indicate that ZMYND11 is expressed at all developmental stages of early embryos with increased expression levels in mature oocytes, two cells and eight cells embryos and in the blastula with no significant differences in the levels between these stages ($p>0.05$). Researchers may infer that the ZMYND11 mainly participates in promoting protein translation and inhibiting transcription during yak embryonic development.

As chaperons of histone proteins, the NAP family is involved in the process of transcriptional activation and mitosis, through transport of histone proteins into the nucleus (Ito *et al*., 1996). NAP1 is required for nucleosome assembly *in vitro* and participates in many cellular processes including transcriptional regulation and cell cycle progression (Zlatanova *et al*., 2007). There are at least five NAP1 proteins in humans and mice including NAP1L1 (Attia *et al*., 2011). The results presented here indicate that NAP1L1 is expressed at a relatively low level in mature oocytes, two cells stage embryos and the blastula with a sharp increase in NAP1L1 mRNA levels in eight cells stage embryos ($p<0.05$). The eight cells to sixteen cells stage is a key stage in embryonic development when yak embryo development after IVF stops and most cells degenerate (Meirelles *et al*., 2004). The analysis of the change in the expression levels of the differentially expressed genes indicates that NAP1L1 is significant for development of *in vitro* cultured yak embryos at the eight cells stage and that the eight cells stage is of particular importance in the development of the yak early embryo.

With respect to general expression in embryos, RPL27A is a highly-conserved cytoplasmic ribosomal protein and was inferred to act as a maternal effector gene (Szakonyi and Byrne, 2011). The effect of ribosomal proteins on embryonic development might be due to pleiotropic effects such as abnormal protein synthesis,

however, several developmental deficiencies indicate that ribosomal proteins play specific roles in regulating the expression of genes related to embryonic development (Byrne, 2009). The results show that the expression of RPL27A decreases during development from mature oocytes to the two cells stage and then to the eight cells stage before increasing significantly in cells of the blastula ($p<0.05$). Massive amounts of RPL27A mRNA, accumulated during the oocyte stage are the result of persistent RPL27A expression after fertilization and this accumulation is important for early embryonic development. By extension, a second increase or burst in RPL27A expression at the blastula stage might be significant for subsequent development.

CONCLUSION

The expression of ZMYND11, NAP1L1 and RPL27A mRNA in yak oocytes and early embryos showed temporal differences which might be significant to the normal development of the yak early embryo and may inform future studies of genes involved in maintaining normal reproductive function in yak.

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