TÜBİTAK

Research Article

Turk. J. Vet. Anim. Sci.
2011; 35(1): 15-19

doi:10.3906/vet-0808-19

Stage-specific developmental gene expression of goat preimplantation embryos produced in vitro

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Received: 11.08.2008

Abstract: Single preimplantation embryo mRNA differential display was used to study the gene expression of 2-cell,4-cell, and 8-16-cell stage goat embryos produced in vitro, and 16 different stage specific bands in total were screened. In these specific bands, 5 were in 2-cell stage, 4 were in 4-cell stage, and 7 were in 8-16-cell stage, respectively. The sequencing and alignment results suggested that 4 differential expression bands were homologous with human petidylarginine deiminase (PAD), bovine insulin-like growth factor binding protein-3 (IGFBP3), bovine NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4 (NDUFA4), and dog cyclin B3 (CCNB3) genes, respectively. All these 4 genes had the functions on physiological regulation and were important factors in the process of goat preimplantation embryo development.

Key words: Single preimplantation embryo mRNA differential display, goat, embryo, gene

Introduction

The development of mammal preimplantation embryo is a complex process, and precise expression of genes in time and space pattern is the premise of the normal process (1). By studying different stage-specific genes expression patterns, we can obtain information on genes and also analyze and explain the functions of these stage-specific genes in the process of embryo development as well.

Since the mRNA differential display technique was established (2), it has been widely used in studies of genes related to the mammal preimplantation embryo development and many results have been obtained (3). Based on this technique, single preimplantation embryo differential display (SPEDD) was further established in 1997 (4), which improved study precise of genes expression and was more effective compared to the traditional methods. SPEDD has been mainly used in the studies of genes expression in mouse, rabbit, and human preimplantation embryos development in recent years (5-7). However, to date, we have noted that little was known about developmental genes expression of goat preimplantation embryos. Using SPEDD, we can establish and understand stage-specific gene expression patterns, and explain the molecular mechanism controlling the process of goat preimplantion embryo development. Therefore,

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in the present study, we used an improved SPEDD technique (8) to study goat 2-cell, 4-cell, and 8-16-cell embryos cultured in vitro and to screen the genes controlling and influencing the goat preimplantation embryo development.

Materials and methods

Embryo samples

In the present study 2-cell, 4-cell, and 8-16-cell stage goat embryos were obtained through the process of oocytes matured in vitro (IVM), fertilized in vitro (IVF), and cultured in vitro (IVC). All embryos were placed in acid tyrode solution (pH 2.0-2.5) and observed under a dissection microscope until the zonae pellucidae had just dissolved. The embryos then were washed carefully 3 times in PBS to remove attached cells. A single embryo in 0.5 μL of PBS was added to 1.5 μL of lysis buffer (20 mM DTT (Merck), 0.5% NP-40 (Fluka), 1 U/μL of RNasin (Promega)), and snap frozen in liquid nitrogen before storage at -80 °C. Before being used, embryo samples were heated to 65 °C for 5 min to denature the mRNA and transferred immediately to ice before addition of reverse transcription PCR amplification reagents.

Reverse transcription PCR and PCR amplification

The reverse transcription and PCR amplification were carried out by using the One-Step RT Kit (Qiagen) according to the manufacturer's instruction. The reaction mixture was in a total volume of 25 μL comprising 5 μL of 5 × RT PCR Buffer, 1 μL of 10 mM dNTP Mix, 1 μL of Enzyme Mix, 0.6 μM of each primer (see Table 1 for primer sequences), and 20 U of RNasin. Reverse transcription was carried out at 50 °C for 30 min, and the PCR amplification reaction was performed with the following thermal profiles: initial denaturation step at 94 °C for 45 s, annealing at 40 °C for 1 min, and elongation at 72 °C for 1 min, with a final elongation step at 72 °C for 10 min.

Differential display and re-amplification

After PCR amplification, 2 μL of the PCR products were electrophoresed on 5% non-denaturing polyacrylamide gel. The stage-specific bands were screened using silver staining and were eluted by boiling. The PCR re-amplification was carried out by using a PCR Kit (Promega). The reaction mixture was in a total volume of 50 μL comprising 5 μL of 10 × PCR Buffer, 3 μL of 25 mM $MgCl_2$, 1 μL of 10 mM dNTP, 2 U of TaqE, and 0.6 μM of each primer with the same set of primers as used for PCR amplification. The re-amplification cycles consisted of an initial denaturation step (94 °C for 10 min) following by 20 cycles of denaturation at 94 °C for 1 min, annealing at 40 °C for 90 s, and elongation at 72 °C for 90 s, with a final elongation step at 72 °C for 10 min. After PCR re-amplification, the reaction products were electrophoresed on 1.5% agarose gels. The products were visualized and photographed under short wavelength UV light.

DNA sequencing

Re-amplification cDNA bands were gel-purified by QIAEXIT Kit (Qiagen) and cloned into the pGEM-T Easy vector according to the manufacturer's instructions and sequenced using T3 and SP6 primers.

Result

Differential display RT-PCR result

After RT-PCR, non-denaturing polyacrylamide gel electrophorese and silver staining, we got the electrophoresed pattern of different stage-specific bands (Figure 1). Three single same stage embryo examples were used in the experiment to confirm the repetition in statistics and the reliability of the reaction system. After recovering and re-amplification of the specific bands, most of them reappeared (Figure 2), and 16 stage-specific bands were obtained in total,

Table 1. Sequence of 3 couple primers.

Anchor primers	Random primers
P1 5'-AAGCTTTTTTTTTTTTTTTC-3'	P2 5'-AAGCTTGATTGCC-3'
P3 5'-AAGCTTTTTTTTTTTTTTTG-3'	P4 5'-AAGCTTTGGTCAG-3
P5 5'-AAGCTTTTTTTTTTTTTTTA-3'	P6 5'-AAGCTTAACGAGG-3'

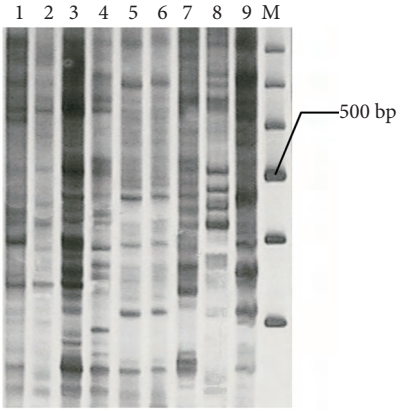

Figure 1. The partial pattern of goat 2-cell, 4-cell, and 8-16-cell embryo in silver PAGE.
1 ~ 3: 8-16- cell embryo, 4 ~ 6:4 -cell embryo, 7 ~ 9:2 -cell embryo, M is 100 bp DNA Ladder.

finally (Figure 3). The sequencing and alignment results suggested that the sequence length of these bands were between 200 bp and 700 bp, and 4 of differential expression bands were homologous to function genes or regulatory genes already known from the GenBank, whereas other 12 differential expression bands were unknown genes (Tables 2 and 3).

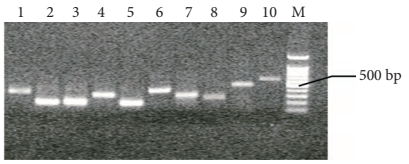

Figure 2. Agarose gel electrophoresis result of partial second PCR products for different stage of goat embryos.
1 ~ 4: Specific bands of 2-cell stage, 5 ~ 6: specific bands of 4-cell stage, 7 ~ 10: specific bands of 8-16-cell stage; M is 100 bp DNA Ladder.

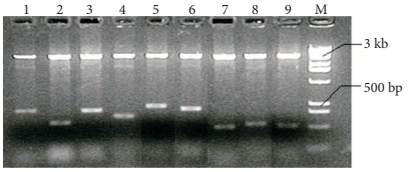

Figure 3. The results of partial plasmid DNA digested by EcoR I
1 ~ 3: Specific bands of 8-16-cell stage, 4 ~ 6: specific bands of 4-cell stage, 7 ~ 9: specific bands of 8-16-cell stage; M is 1 kb molecular weight marker.

Discussion

The development of mammalian preimplantation embryo is the process of gene differential expression in time and space pattern. A very precise regulatory mechanism of developmental genes expression was required. Thus, we needed an effective method to do the research in this field. The establishment and utilization of SPEDD technique could help surmount the restrictions such as scarcity of study materials, unsynchronism of embryo development (3), which were vital in doing such research work, and could facilitate large scale system research in developmental genes expression of preimplantation embryos as well. The study results in this field all confirm effective and important function about the technique of SPEDD.

We used an improved SPEDD technique to study gene expression in goat 2-cell, 4-cell, and 8-16-cell embryos which were cultured in vitro, and 16 different stage specific bands in total were screened. The sequencing and alignment results suggested that 4 differential expression bands were homologous to function genes or regulatory genes already known from GenBank. The study results indicated that human petidylarginine deiminase (PAD) was a 2-cell stage specific gene, bovine insulin-like growth factor binding protein-3 (IGFBP3) was a 4-cell stage specific gene, and bovine NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4 (NDUFA4) and dog cyclin B3 (CCNB3) were 8-16-cell stage specific genes. It is well known that PAD had the functions on post-translated modification about histone and cytoskeletal protein, and played an important role in the regulation of gene transcription and expression (9,10). IGFBP3 was the regulator of IGFs bioactivity, it can also promote cell division and influence endocrine activation (11-13). NDUFA4 was essential for ATP synthesis to provide energy for cell metabolism, DNA demethylation, and genomic reprogramming. CCNB3 was related with the process of cell division (14-16). For goat, the time of maternal-zygotic transition (MZT) was exactly in the 8-16-cell embryo period. The specific expression of NDUFA4 and CCNB3 genes in 8-16-cell embryo indicated that the activation of embryo genome in this period was so active that it needed more energy materials and more

Table 2. The sequencing and alignment results of differential expression bands.

No.	Differential bands	Patterns of differential display	Homology ID No.	Prediction of function
1	A21	2C	gi|45426857|emb|AJ549502.2|HSA549502	Human petidylarginine deiminase gene (PAD)
2	A22	2C	gi|40255175|ref|NM_173529.3	Human unknown function MGC33382 protein (MGC33382)
3	A23	2C	gi|45488165|gb|CK970191.1	Bovine unknown function cDNA clone
4	B21	2C	gi|50247307|emb|CR552397.1	Bovine unknown function cDNA clone
5	B22	2C	-	Unknown sequence
6	A43	4C	gi|45488379|gb|CK970405.1	Bovine unknown function cDNA clone
7	B41	4C	-	Unknown sequence
8	B42	4C	-	Unknown sequence
9	B43	4C	gi|11095302|gb|AF305712.1|AF305712	Bovine insulin-like growth factor binding protein-3 gene (IGFBP3)
10	A81	8-16C	-	Unknown sequence
11	A82	8-16C	-	Unknown sequence
12	A83	8-16C	-	Unknown sequence
13	A86	8-16C	gi|31343592|ref|NM_175820.2	Bovine NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4 gene (NDUFA4)
14	B81	8-16C	gi|1732687|gb|AA157876.1|	Human unknown function chromosome 3 RP11-64C1 clone,
15	B82	8-16C	-	Unknown sequence
16	B83	8-16C	gi|54114985|ref|NM_001005763.1|	Dog cyclin B3 gene (CCNB3)

Note: From A21 to B22 were 5 specific bands for 2-cell stage of goat embryos; from A43 to B43 were 4 specific bands for 4-cell stage of goat embryos; from A81 to B83 were 7 specific bands for 8-16-cell stage of goat embryos.
A means amplification was used with P1 and P2 primers, B means amplification was used with P3 and P4 primers.

Table 3. Information on 4 stage-specific bands which were homologous to function or regulatory genes.

Differential bands	Size (bp)	E-value	Identity	bp overlap/total bp
A21	332	9e-13	83%	111/133
B43	617	3e-91	88%	280/315
A86	392	0.0	97%	362/370
B83	262	3e-21	82%	160/195

cyclins to participate in the physiological process at this moment. Therefore, we think that these 4 genes had very important functions in goat preimplantation embryo development. Some researchers mentioned above have also studied and analyzed the important functions of these genes in other mammals about preimplantation embryo development (9,12,13,15).

This work was just a preliminary study on genes' specific expression in goat preimplantation embryos produced in vitro, and the results revealed some stage feature of goat preimplantation embryo development. At present, we are continuing our studies about others stage-specific bands to analyze whether or not those bands have specific physiological functions.

Acknowledgements

This work was supported by the fund of National Natural Science Foundation of China (No.30270162).

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