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Expression of Stage-Specific Genes during Zygotic Gene Activation in Preimplantation Mouse Embryos

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ABSTRACT—The expression of mouse two-cell stage specific genes was studied using the modified DDRT-PCR method, which overcame the paucity of the experimental materials of preimplantation embryos. Embryo tissues equivalent to that of four blastomeres are sufficient for amplification of target genes as visualized using polyacrylamide gel. Sequence analyses and reverse Northern blots indicate that the genes of *ATPase 6* and *Ywhaz* are expressed specifically in two-cell embryos. *ATPase 6* is essential for one-cell to two-cell transition and plays an important role in establishment of oxidative phosphorylation, while *Ywhaz* is related to initiating cellular communication system.

Key words: preimplantation embryo, DDRT-PCR, stage-specific gene expression, reverse Northern blot

INTRODUCTION

In mammals, fertilization produces a one-cell embryo containing a haploid paternal pronucleus derived from the sperm and a haploid maternal pronucleus derived from the oocyte. Each pronucleus undergoes DNA replication before undergoing mitosis to produce a two-cell embryo containing two diploid “zygotic” nuclei, each with a set of paternal and a set of maternal chromosomes (Wang and Latham, 2000; Henery *et al.*, 1995). Little is known about molecular events during this early stage of development. According to previous studies preimplantation development of mouse embryos is marked by many critical and unique events, such as the maternal-to-zygotic transition which initiates at the early two-cell stage (Flash *et al.*, 1982; Schultz *et al.*, 1993; Nothias *et al.*, 1995), the establishment of cellular communications at compaction during the 8- to 16-cell stage; and the appearance of the first differentiated cells at the blastocyst stage (Ko *et al.*, 2000). The onset of zygotic gene activation (ZGA) in the mouse embryo, as evidenced by the expression of a number of polypeptides whose synthesis is inhibited by α -

amanitin, RNA polymerase II inhibitor, clearly occurs during G₁ of the two-cell stage (Bolton *et al.*, 1984; Ram and Schultz, 1993). ZGA replaces maternal transcripts that are lost during the two-cell stage and common to both the oocyte and preimplantation embryo, as well as generating novel ones that are required for embryogenesis. In this regard, it should be noted that cleavage to the two-cell stage does not require transcription, whereas subsequent cleavages do require transcription (Bolton *et al.*, 1984; Pouev-mirou and Schultz, 1989). Thus, ZGA in the two-cell embryo is a prerequisite for further development (Worrad *et al.*, 1994). Therefore, by comparing differential gene expression during early mouse embryonic development (e.g. MII stage unfertilized egg, fertilized egg, two-cell and four-cell embryo), development-related genes may be found. Nowadays, there are many ways to clone novel genes, such as subtractive hybridization (Zimmerman *et al.*, 1980; St. John and Davis, 1979) and mRNA differential display reverse transcription polymerase chain reaction (DDRT-PCR, Liang and Pardee, 1992; Lonway *et al.*, 1995). Comparatively, DDRT-PCR is one of the most effective methods to validate novel expressive genes during early embryonic development so far (Li and Han Wa, 1997). But it is hampered by the paucity of starting biological materials for early mamma-

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lian embryos. In this study, we introduced modification to the DDRT-PCR, and showed that only a small number of embryos are sufficient for detecting gene expression. We also report cloning of early mouse embryonic development-related genes using the improved DDRT-PCR.

MATERIALS AND METHODS

Treatment of animals and collection of eggs and embryos

Kunming mice were provided by the experimental animal center, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Six-week Kunming female mice were super-ovulated with PMSG (HuaFu high-biotech, Tianjin, China) and hCG (Biochemical company No.1, Shanghai, China), and were divided into group A and group B. The female mice of group B were mated with cognate male mice and were checked p.c.post coitus next morning to confirm female mice pregnant. Fifty of MII-stage unfertilized eggs were obtained from female of group A (without mating). Fifty of fertilized eggstweny five of two-cell embryos and thirteen of four-cell embryos were collected respectively from pregnant mice in group B at 1232and 48 hr after fertilization. All embryos and unfertilized eggs were collected by flushing the oviduct and uterus, and were inspected under stereomicroscope (Capco *et al.*, 1995).

Total RNA isolation and purification

All solutions were prepared using water that had been treated with 0.1% diethyl pyrocarbonate (DEPC). RNeasy Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instruction of menu to isolate total RNAs. Total RNAs were treated with RNase-free DNase-I (Promega, Madison, WI, USA) to remove contaminating DNA, extracted with Micro RNA Isolation Kit (Stratagene, La Jolla, CA, USA). RNA was precipitated overnight at -20°C with 120 μl of isopropanol and 2.5 μl of glycogen (20 mg / ml). After centrifugation, RNA was dried and dissolved in 20 μl of RNase-free water (Zimmerman and Schultz, 1994).

Reverse transcription, PCR amplification, and electrophoresis analysis

Negative control (without reverse transcriptase) was designed in order to inspect DNA contaminant. Reverse transcription (RT) was performed using 9.4 μl of total RNAs (equal to twenty five blastomeres) and adding 2 μl (10 μM) of anchored primer HT₍₁₅₎C (5'AAGCTTTTTTTTTTTTTTTT-3', Sangon, Shanghai, China) 68°C , 8 min denatured, and then 40°C , 10 min for annealing, adding 8.6 μl of mixture solution [4 μl of RT 5 $\times$ buffer, 2 μl of 0.1 μM DTT, 1 μl of RNase inhibitor (Promega), 1.6 μl of 250 μM dNTP, 100 units of SuperscriptII (Gibco BRL, GrandIsland, NY, USA) and 100 units of M-MLV (Promega)] 42°C , 50 min, continued 95°C , 5 min to denature the enzyme. Nine microliters of reverse transcription products (equal to twelve blastomeres)was used to perform PCR in 20 μl of reaction system (2.5 μM 3'primer, HT₍₁₅₎C, 0.5 μM 5'primer, HAP₁ (5'-AAGCTTGATTGCC-3', Sangon, Shanghai, China), 2 μM dNTP, 2.5 μM MgCl₂, 1 $\times$ PCR buffer, 0.5 μCi [α -³²P]dATP (Yahui, Beijing, China), 2.5 units Taq DNA polymerase (Promega), Polymerase chain reaction was carried out using Mastercycler (Eppendorf, Barkhausenweg, Hamburg, Germany) with the parameters: denature at 94°C for 10 min, annealing at 40°C for 5min, and extension at 72°C for 5 min; followed repeated 30 cycles at 94°C , 30 sec; 40°C , 2 min; 72°C , 90 sec; at last, 72°C , 5 min. The PCR products were divided into three aliquots (each aliquot equal to four blastomeres) to electrophoresize in 6% polyacrylamide gel containing 8 M urea, autoradiography at -20°C , exposed for 3 days (Zimmerman and Schultz, 1994; Minami *et al.*, 2001; Li *et al.*, 2001).

Recovery and reamplification of differential bands

Marked the DD (differential display) bands on x-ray film, and cut the corresponding bands carefully from polyacrylamide gel, put into an 0.5 ml-eppendorf tube, adding 100 μl of DEPC-treated water, immersed at room temperature for 10 min; boiled at 100°C for 15 min; centrifuged at 17000 $\times$ g for 2 min. Remove the supernatant to a fresh tube, adding 8 μl of 3 M KAc, 300 μl of 100% ethanol, and 2.5 μl of glycogen (20 mg /ml) co-precipitated overnight at -20°C , centrifuged, washed, dried and dissolved in DEPC-treated water. PCR reaction mixture was made as described previously, PCR parameters: denature at 94°C for 10 min; then repeated 20 cycles at 94°C , 60 sec; 40°C , 90 sec; 72°C , 90 sec; at last cycle, 72°C ,10 min; denature at 94°C for 10 min; then repeated 20 cycles at 94°C , 60 sec; 42°C , 90secs; 72°C , 90 sec; at last cycle, 72°C , 10 min. PCR products for electrophoresis analysis on 1.5% agarose, and extracted with phenol, chloroform: isopentanol, co-precipitated with glycogen in ethanol.

Cloning of amplicons and sequence analysis

PCR products were ligated with pGEM-T vector (Promega) and then transformed into *E. coli* JM109 according to the manufacturers instructions. A randomly selected white clone was transferred into 3 ml LB liquid culture medium (without ampicillin), and then shaken at 37°C , overnight. Took 1.5 ml of JM109 culture to extract recombinant plasmid for PCR product analysis, and the rest of 1.5 ml for DNA sequencing. DNA sequences obtained were analyzed on homology, BLASTN program was used to search NCBI-nr, the EST database, and the Genbank non-redundant protein database.

Reverse Northern blot hybridization

The sequenced fragment was labeled with Random Primer DNA Labeling Kit (TaKaRa, DaLian, China) as probe for reverse Northern hybridization. cDNA pools derived from single unfertilized egg, fertilized egg, two-cell and four-cell stage embryos were used for electrophoresis on 1.5% agarose, for 2–3 hr, photographed and transferred on Hybond membrane (Pharmacia, Little Chalfont, Buckinghamshire, England). Prehybridization (1% BSA, 1mM EDTA, 0.5 mM sodium phosphate, 7%SDS pH 7.7) at 65°C for 10 min; adding denatured DNA probe hybridized at 65°C overnight. The membrane was washed three times with a interval of 10 min using solution I0.5% BSA, 1mM EDTA, 40 mM sodium phosphate, pH 7.2, 5% SDS. Then washed three times with interval of 20 min using solution II (1 mM EDTA, 20 mM sodium phosphate, pH 7.2, 1% SDS). Dried the membrane, autoradiography at -20°C for five days (Zegeouti *et al.*, 1997; Adjaye *et al.*, 1998).

RESULTS

Messenger RNA differential display analysis

Several modifications of the method of DDRT-PCR were necessitated due to the limitations of the number of preimplantation embryos and the small amount of mRNA that is present in these embryos (i.e., picogram amounts). Total RNA was prepared from mouse preimplantation embryos (from M-egg to 4-cell stage embryos) using RNeasy Mini Kit coupled with Micro RNA Isolated Kit. Reverse transcription and PCR were performed under optimized conditions using anchored primers (as described above). A portion of a differential display gel comparing M-egg, 1-cell, 2-cell, and 4-cell-embryos mRNA is shown in Fig. 1. A clear X-ray film was obtained. Comparison of banding patterns revealed several discernable fragmentswhich are differentially expressed in different stages. At least two bands were

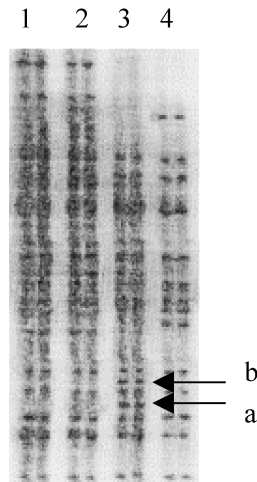


Fig. 1. Portion of an autoradiogram of amplicons obtained from mRNA differential display using Megg's and preimplantation mouse embryos. 1. M-stage unfertilized eggs; 2. fertilized eggs; 3. Two-cell embryo; 4. Four-cell embryo; Arrowheads with the a and b represent *ATPase 6* and *Ywhaz*.

present in the 2-cell stage (Fig. 1, lane 3, arrows with a and b) that were not observed in other stages.

Amplification and sequencing of positive clones

These bands were recovered from polyacrylamide gel and subjected to PCR, using the same primers as before. A white plaque was randomly selected and proliferated in 3ml

LB liquid culture medium. PCR were performed with the template extracted from cultural medium and the primers used as the same as THE previous RT-PCR. PCR products were resolved on 1.5% agarose gel. Two fragments were amplified and analyzed. The DNA sequencing analysis revealed that one fragment corresponded to mitochondrial gene, which was highly similar to *ATPase 6* ($p=2e-47$) by translating and searching the Genbank protein database using BLAST (Fig. 2, fragment a). The other is the *Ywhaz* gene *mus musculus* tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide, accession number NM-011704, 99% identity in aligned region, $p=e-163$, (Fig. 2 fragment b).

Validation of mRNA differential display for mouse preimplantation embryos

Even though the samples were treated with DNase prior to RT, it is necessary to design a negative control (omitting reverse transcriptase) to confirm a lack of DNA contamination. No bands were observed on negative controls (data not shown). To validate the specific expression of *ATPase 6* and *Ywhaz* in the 2-cell stage embryo, an experiment based on reverse Northern blot hybridization was designed. A radiolabeled *ATPase 6* and *Ywhaz* cDNA probes hybridized to cDNA pools from different stages that is strongly expressed in 2-cell embryo, and did not hybridize to any mRNA expressed in other stages (Fig.3). This indicates that the two amplicons, which represent *ATPase 6* and *Ywhaz*, are specifically expressed in two-cell embryo.

Fragment a

ANTGGGCCCCGACGTCGCATGCTCCCGGCCGCCTGGCCGCGGGATTAAGCTTTTTTTTTT
TTTTTTTCTGTATTATCATGTAGATATAGGCTTACTAGGAGGGTGAATACGTAGGCTTGAA
TTAATGCTACTGCAAATTCTAGAAATTGTGAGTAGAAGTAAATAATAATGTAATGGTAG
CTGTTGGTGGGCTAATATTTATTAATACTAGAGTAGCTCCTCCGATTAGGTGTATTAATAA
GTGTCCTGCAGTAATGTTAGCTGTAAGCCGGACTGCTAANGCCATTGGTTGAATAAATA
GGCTAATTGTTTCAATAATAAAGTATTGGAATTAGTGAAATTGGAGTTCCTTGTGGAA
GGAAGTGGGCAATCAAGCTTAATCACTAGTGCGGCCGCCTGCAGGTCGACCATAT

Fragment b

GANTGGGCCCCGACGTCGCATGCTCCCGGCCGCCTGGCCGCGGGATTAAGCTTTTTTTTTT
TTTTTTTTTCTCTGATAAACTCTGCATTCAAAATTGGTAAAAGCCCATTAGCCGCCAAG
AGGGAATAAGGAGACAATTTCTTTGGAGAAGTCTCAAAACCAAAGACTAATTTACA
TGAAAAGCTGTAGAGAAAAGTAGTTGAAAAGTCCATTCAAAAACCTTTATTCCACTTA
CATGAACCTTAATACATGTGTTCTTAACAATTATGCTTGGATTGTTTCATGAAAATTTCTTA
AGACATTAAAACAAAGCTAGCCGTCATCTCAAGTTATTTCCCTGTTAACCATTTTACA
GCACATGCATGTTAGGCAAGTATCAAAAAGAAAAAAAAAAAAAAAAAGGCTTAATCAC
TAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATG

Fig. 2. The sequences of cDNA fragment of *ATPase 6* and *Ywhaz* (Zeta polypeptide). Fragment a, 336 bp, *ATPase 6*; Fragment b, 359bp, *Ywhaz*. The sequences underscored indicate primers used in RT-PCR, the thickened sequence represents T vector sequence.

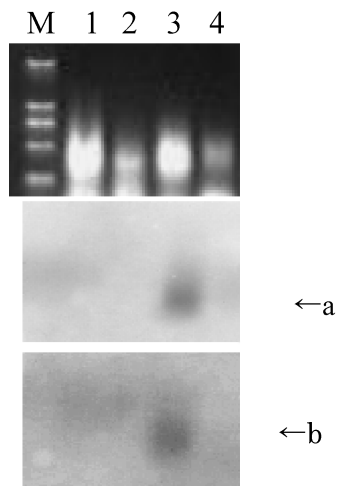


Fig. 3. Stage-specific expression of *ATPase 6* and *Ywhaz* Reverse Northern Blot analysis. 1. M-stage unfertilized eggs; 2. fertilized eggs; 3. Two-cell embryo; 4. Four-cell embryo. The blots marked by arrowhead with a and b represent *ATPase 6* and *Ywhaz* respectively.

DISCUSSION

Several modifications of DDRT-PCR method were introduced in order to conquer drawbacks of the limitations on the number of early embryos that are readily isolated. Arbitrary criteria were used for selecting amplicons which will be analyzed further. Usually, the amplicons are shorter in length with the 3'-terminal untranscriptional area, and generate a substantial fraction of false positives (Ma *et al.*, 2001). A primary obstacle that has delayed molecular analysis of this developmental program is the difficulty of collecting and analyzing large numbers of eggs and embryos. Usually, about 30 eggs should be obtained from a superovulated female mouse; and one unfertilized egg contains about 0.43 ng RNA; one fertilized egg contains about 0.35 ng RNA; one two-cell embryo contains about 0.24 ng RNA; while one four-cell embryo only contains about 0.60 ng RNA. Total RNAs are extracted and purified using RNeasy Mini Kit, removing DNA contaminant, and then using MicroRNA Isolation Kit to purify it. Negative control was designed in reverse transcription (without reverse transcriptase). No band was found in negative control lane, indicating no DNA contaminant in total RNAs (data not shown). There is only picogram amounts of mRNAs in these embryos, so, it is difficult to isolate the poly (A)⁺mRNA from total RNA. Therefore, total RNAs were used for DDRT-PCR directly. Two genes were obtained and DNA sequencing analysis showed no homology to rRNAs. The modest number of eggs (not more than 50) and different stage embryos (as the same amount of total RNAs as eggs) were used for DDRT-PCR. The results suggest that using (HT)C as 3'primer and HAP1 as 5'primer work better. Based on experimental comparison, many parameters for performing reverse transcription, PCR and electrophoresis were

improved. In order to obtain cDNAs from lower amount of mRNAs, superscriptII and M-MLV were used coordinately in reverse transcription system. The cDNAs obtained from DDRT-PCR are less qualified and producing high rate of pseudo clones. To identify positive-negative clones is important for availability of DDRT-PCR. For the present studies, a new validation approach was designed based on reverse northern hybridization as mentioned above, meanwhile, combining DNA sequence analysis and RT-PCR detection, and the *ATPase 6* and *Ywhaz* expressed were confirmed during two-cell stage, not at MII stage and four-cell stage. *ATPase 6* is encoded by mitochondrial DNA and is one of the subunits of F₁F₀-ATP synthase which is complex V of the respiratory chain (Michel *et al.*, 2000; Bhat *et al.*, 1991; Mariottini *et al.*, 1983). *ATPase 6* expression is essential for the production of ATP (Ozawa, 1997). Mitochondrial DNA is an independent genetic unit existing in oocyte, zygote cells and somatic cells; but it is not an independent expression unit, which gene expression is controlled largely by the action of products of the nuclear genome (Stepien *et al.*, 1992). So, *ATPase 6* expression is regulated by some elements outside of mitochondrion. In the embryo early developmental period, the DNA modification (Methylation and demethylation) is the critical event for epigenetic reprogramming (Reik *et al.*, 2001). During the reprogramming process, more energy to be needed for DNA modification. This, maybe, is a reason for *ATPase 6* expressed at 2-cell stage. *Ywhaz* (Zeta polypeptide) gene is encoded by nuclear DNA and is activation protein for tyrosine -monooxygenase (TH)/tryptophan 5-monooxygenase (TPH), which is the rate-limiting enzyme in the biosynthesis of serotonin, a sort of neurotransmitter (Mockus *et al.*, 1997; Mockus and Vrana, 1998; Teerawatanasuk *et al.*, 1999). The neurotransmitter gene expression is related to initiate the cellular communication system (Yager *et al.*, 2001).

Major events including fertilization, massive degradation of maternally stored RNAs, initiation of zygotic transcription, compaction, first cell differentiation into inner cell mass (ICM) and trophectoderm, and implantation occur from unfertilized egg to blastocyst (Ko *et al.*, 2000). The most important one among the events is initiation of zygotic transcription. ZGA may comprise a period of minor gene activation in the 1-cell embryo that is followed by a period of major gene activation in the 2-cell embryo (Schultz *et al.*, 1993). The genes expressed differentially during the development of mouse preimplantation embryos should be related to these major events above. So, the finding of *ATPase 6* and *Ywhaz* expressed specifically at two-cell stage is of significance to study the mechanism of preimplantation embryo at molecular level. The data obtained from this study support the inferences (1), that the higher transcriptional activity of these ATP synthase-related genes is essential for preimplantation embryo development. (2), this rapid buildup of the mitochondrial oxidative phosphorylation system is mostly preparatory for postimplantation development. (3), the biosynthesis of neurotransmitter may play a key role for the

establishment of cellular communications at compaction on the beginning of mouse development. So far, the exact functions of these genes are still unclear, the further studies will be continued.

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