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Gene expression profiles of *in vivo* derived mouse blastocysts after slow-freezing

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1. ABSTRACT

The goals of our study were to analyze change of global gene expression profile of mouse blastocysts after slow-freezing and to explore molecular mechanism underlying the decreased pregnancy rate caused by cryopreservation. The results showed that superovulation differentially regulated the expression of 288 genes with at least 2.0-fold change considerations. Among which, 275 genes were down-regulated and the remainder up-regulated in the cryopreserved group. The independent analysis with real-time PCR fully confirmed the results of microarray. These differential genes were classified into eighteen functional groups belonging to biological process, molecular function, and cellular component. Twenty nine genes could be categorized into one or more of KEGG pathways. The pathways of mitogen-activated protein kinase, Wnt and cell cycle were the most predominantly affected. Thus, the expression pattern reflected a broad spectrum of consequences of slow-freezing on the blastocysts, with most effects on stress-related and cell cycle-related genes.

2. INTRODUCTION

The first human embryos were frozen in the late 1970s, with the first pregnancy reported in 1983 in Australia and a birth occurred the following year (1). Slow-cooled freezing methods have been in existence since 1972 and are considered the "gold standard" for cryopreserving embryos by many cryobiologists (2). Although vitrification is now on the rise, most human ART centers routinely use slow-cooling methods for freezing embryos (3).

Clinics trend to choose cryopreservation at the blastocyst stage, although there is debate as to the developmental stage at which human embryos are best cryopreserved (4, 5). Blastocysts are preimplantation embryos that have successfully passed the critical step of genomic activation and so have a high developmental potential (6). Blastocysts also have the advantage of containing numerous small cells. Loss of some cells during freezing and thawing is probably less harmful for the further development of the embryo (7).

Despite a certain success, cryopreservation techniques cause significant morphological, biochemical alterations and lower the pregnancy rate. During the cryopreservation, embryos are subjected to physical and chemical alterations, including polyploid formations (8), the destruction of the cell membrane integrity, ionic disturbances (9), cellular organelle dysfunctions (10, 11) and increased reactive oxygen species (ROS) productions (10). Such detrimental effects may trigger the apoptotic cascade leading to a decrease in implantational and developmental capabilities of embryos (10, 12).

Moreover, in a long-term study including senescence, Dulioust *et. al.* compared slow-frozen and control mice for several quantitative traits. They found significant differences in morphophysiological and behavioral features, some of them appearing in elderly subjects. Thus, apart from its immediate toxicity, slow-freezing, without being severely detrimental, may have delayed effects (13).

The intricacies of the cold-induced changes in embryos that could affect the intracellular and developmental processes need to be known (14). In this paper, we analyzed change of global gene expression profile of mouse blastocysts after slow-cooled freezing.

3. MATERIALS AND METHODS

3.1. Blastocysts collection

Young ICR female mice and male ICR mice (provided by the Center for Experimental Animals, Peking University Health Science Center) were housed in a specific pathogen free facility with 12h light/ 12hr dark photoperiod, at a temperature of 23 degree and relative humidity of 44%. All experiments were conducted in accordance with the standards for the care and use of animals approved by Peking University Health Science Center Ethical Committee. Mice were sacrificed by cervical dislocation to collect embryos. Female ICR mice were superovulation by standard hormone treatments using an *i.p.* injection of 5 IU pregnant mare serum (PMSG, Sigma, USA) followed 48 hr later by an *i.p.* injection of 10 IU human chorionic gonadotrophin (hCG, Sigma, USA). Blastocysts were flushed from the uterine horns 95 hr after the hCG injection.

3.2. Slow freezing and thawing

A slow-rate freezing protocol was employed, as described by Lassalle *et al*. (1985), using a programmable freezer (Cryomagic, Mirae Biotech, Seoul, Korea). Blastocysts were cryopreserved using 9 % glycerol and 0.2M sucrose (Quinn's Advantage™ Blastocyst Freeze kit; SAGE, Biopharma, Trumbull, CT, USA). The embryos are taken from a starting temperature of 37 degree to –6 degree at 2 degree per minute. They are then seeded manually and held at –6 degree for a total of 10 to 15 minutes before being cooled at about 0.3degree per minute to around –35 degree. They are then transferred to a storage tank of liquid nitrogen.

Eight weeks later, blastocysts were thawed rapidly by removing cryovials from storage, exposure to air for 30 seconds and immersion in a water bath at 30 degree for 45 seconds. Cryoprotectant was removed by exposing the embryos to decreasing concentrations of cryoprotectant (Quinn's Advantage Thaw Kit, SAGE In-Vitro Fertilization, Inc., USA). Post-warming, viable blastocysts re-expand and were allowed up to six hours of incubation to regain their vitality. The expanded blastocysts were either transferred into uteri of psuedopregnant mice or washed in RNAse free water, pooled in 150 ml of TRIzol (Invitrogen, Carlsbad, CA) per group and stored at -80 degree until RNA extraction.

3.3. Embryo Transfer

Female mice were mated with vasectomized males to generate pseudopregnant recipients. The following morning, plug-positive females were selected. Frozen blastocysts were transferred into one uterine horn and control blastocysts transferred into the other one of the same recipient pseudopregnant mouse. On 5th day after transfer, the number of uterine implantation sites was recorded. On 18th day, the number of live fetuses was counted

3.4. RNA extraction and RNA amplification

Two independent biological replicates of fresh and 8-week cryopreserved blastocysts pool were used for microarray experiments. Total RNA was extracted from pools of 150 fresh or cryopreserved blastocysts in either control or superovulation group with use of Trizol reagent (Invitrogen, Life Technologies, USA) according to the manufacturer's instructions. Total RNA yield was 80-110ng per pool. Eighty nanograms of total RNA was used for linear, two-round amplification by *in vitro* transcription using MessageAmp^{TM} II aRNA Amplification Kit (Ambion, Huntingdon, UK). Final yield of biotinylated cRNA was 80-120 microgram. The quality and purity of cRNA were determined by RNA integrity examined by electrophoresis on 1 % formaldehyde denaturing gel. These samples were used for the subsequent hybridization.

3.5. Hybridization and slide processing

A total of 15μg labeled cRNA was fragmented and hybridized to the Mouse Genome 430 2.0 array (Affymetrix Santa Clara, CA), which contains 45,000 probe sets analyzing the expression level of over 39,000 transcripts and variants from over 34,000 well characterized mouse genes (Two-cycle Target Labeling Assays). The GeneChip arrays were washed and then stained (streptavidin–phycoerythrin) on an Affymetrix FluidicsStation 450 followed by scanning on a GeneChip Scanner 3000. The procedures were repeated for replicate experiment with independent hybridization and processing.

3.6. Scanning and result analyses

The hybridization data were analyzed using GeneChip Operating software (GCOS 1.4). The scanned images were first assessed by visual inspection then analyzed to generate raw data files saved as CEL files using the default setting of GCOS 1.4. A global scaling procedure was performed to normalize the different arrays using dChip software.

In a comparison analysis, we applied a two class unpaired method in the Significant Analysis of Microarray

Table 1. Primers used for real-time quantitative RT-PCR

Gene	GB.accession	Primer Sequence 5'→3'		Amplification Size (bp)
Jun	NM_010591	Forward Primer	GCTGGAAGAAGGGCTGTTG	196
		Reverse Primer	CAGATTCAAAGTTGGAAGGAGAC	
Rasa2	NM_053268	Forward Primer	TCCTATGCTGTGCTCTTGCG	228
		Reverse Primer	ACTGTGCTAAGTTTCCTCCTATCA	
Jund	NM_010592	Forward Primer	ACTCTTTCGAGACTCGGTGC	246
		Reverse Primer	GAAACATAAATAGGGCGGAATC	
Gadd45b	NM_008655	Forward Primer	CGTTCTGCTGCGACAATGAC	270
		Reverse Primer	TTGCTTTAGATGTTTGGAGTGG	
Casp2	NM_007610	Forward Primer	TGAACCTTATCGGGCATAACT	245
		Reverse Primer	AAGTGGCAAGAAACATCCTACA	
Fos	NM_010234	Forward Primer	GGGAGGACCTTACCTGTTCG	275
		Reverse Primer	AATTCCAATAATGAACCCAACA	
Hspb1	NM_013560	Forward Primer	AGTGAAGACCAAGGAAGGCG	150
		Reverse Primer	TCAGGGGATAGGGAAGAGGA	
Ppp3r1	NM_024459	Forward Primer	CACATCATGGGCTCTGTCTG	237
		Reverse Primer	CAAACCACTGGCTGGGATT	
Nfkbia	NM_010907	Forward Primer	TGGCAATCATCCACGAAGAG	256
		Reverse Primer	TGCGTCAAGACTGCTACACTG	
Csnk1a1	NM_146087	Forward Primer	GCAGCAGAGCAGACGATTG	123
		Reverse Primer	ACCGAAATGAAGTTCTTTACACC	
Fzd5	NM_022721	Forward Primer	GGGAAACCTAACAAGACAGTAAAG	272
		Reverse Primer	CCAACCCGTATCAAAAGTCACTA	
Sox17	NM_011441	Forward Primer	AGCTCAGCGGTCTACTATTGC	203
		Reverse Primer	ATCTCGTGTAGCCCCTCAAC	
Ctnnb1	NM_007614	Forward Primer	GCCACCAAACAGATACATACCT	174
		Reverse Primer	CACAATGCACGTTCAGACACT	
App	NM_007471	Forward Primer	TATCACATACATAGCCCCTTAGC	127
		Reverse Primer	GCTAGACGCCCACGTTCAC	
Tcf12	NM_011544	Forward Primer	GAAGAGCCAGATTAGCACCG	193
		Reverse Primer	TTACTTAGCCAAGACCATCACG	
Gsc	NM_010351	Forward Primer	GCACCAGATGCTGCCCTAC	295
		Reverse Primer	CTCGGCGTTTTCTGACTCC	
Actin		Forward Primer	GAGATTACTGCTCTGGCTCCTA	150
		Reverse Primer	GGACTCATCGTACTCCTGCTTG	

software (SAM) to identify significantly differentially expressed genes between EM and non-EM groups.

3.7. Quantitative real-time RT-RCR□

Gene expression profiles derived from microarray analyses were confirmed quantitatively in 3 pooled samples by RT-PCR analysis. The selected genes for RT-PCR included following 16 genes. Three replicates were used for each real-time PCR reaction. The primers for selected genes are listed in Table 1. The housekeeping gene, beta-actin, was chosen as the internal control for sample normalization.

Two microgram of total amplified RNA from blastocysts was reverse transcribed into cDNA using SuperScript II (Invitrogen, CA, USA). All qPCR assays were performed with QuantiTect^{TM} SYBR Green PCR kit (Qiagen, Hilden, Germany) as indicated. The 2-delta delta CT relative quantification method was performed to analyze the data from the qPCR experiment as previously described (15)

3.8. Statistical analysis

All statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls method. A probability of $p<0.05$ was used to indicate a significant difference.

4. RESULTS

4.1 Development potential of cryopreserved blastocysts

We analyzed the impact of cryopreservation on the embryo implantation potential *in vivo* (Table 2). The results showed that on 5.5dpc, the implantation rates (implantation sites/blastocysts transfered) of cryopreserved embryos were significantly lower than the fresh embryos (38.75% vs. 72.50%, $p<0.01$). In each group, eighty fetuses were allowed to develop to 18.5dpc. there were significantly less viable fetuses developed from cryopreserved blastocysts (20/80 vs. 47/80, $p<0.01$) (Table 2)

4.2. The differential expression profile of cryopreserved blastocysts detected by microarray

After SAM analysis of microarray data from the 4 subjects consisting of 2 pooled samples of fresh blastocysts and 2 pooled samples of cryopreserved blastocysts, 288 genes were determined to be significantly differentially expressed with a selection threshold of false discovery rate, FDR = 8.06% and fold change >2.0 in the SAM output result (see supplementary data). The SAM result was visualized in Figure 1 using SAM software and visualized with TreeView tools after unsupervised hierarchical clustering. Totally, 217 out of the 288 genes exhibited down-regulation and the remainder were up-regulated in the cryopreserved group. Sixteen differential genes or transcripts were selected to confirm their expression differences in three samples with qRT-PCR. Among them, nine genes were down-regulated and seven genes up-regulated (Figure 2).

4.3. Gene ontologies (GO) classification of differential genes

All 288 differentially expressed genes were analyzed the association with particular function from the

Table 2. Development potential of fresh and cryopreserved blastocysts

Group	No. of thawed blastocysts	No. of surviving blastocysts	No. of implantation sites	No. of live fetuses
Fresh	-	160	58/80	47/80
Frozen-thawed	167	160	31/80 [a]	20/80[a]

[a] Significant difference with the fresh group (p<0.01)

Table 3. Significant pathways involved in the influence of cryopreservation on mouse blastocysts

Pathway Name	Total	Gene	p-value
MAPK signaling pathway	9	Jun, Rasa2, Gadd45b, Taok1, Fos, Pdgfra, Hspb1, Ppp3r1, Map3k2	0.000
Wnt signaling pathway	7	Csnk1a1, Jun, Fzd5, Sox17, Dkk1, Ctnnb1, Ppp3r1	0.000
Cell cycle	5	Rbl1, Cdc14a, Cdk2, Gadd45b, Chek1	0.000
B cell receptor signaling pathway	4	Jun, Nfkbia, Fos, Ppp3r	0.000
T cell receptor signaling pathway	4	Jun, Nfkbia, Fos, Ppp3r1	0.000
mTOR signaling pathway	2	Pdpk1, Ddit4	0.000
Calcium signaling pathway	4	Adcy9, Pdgfra, Phka2, Ppp3r1	0.002
Alzheimer's disease	2	Lpl, App	0.002
Apoptosis	3	Nfkbia, Ppp3r1, Dffb	0.003
Gap junction	3	Adcy9, Pdgfra, Map3k2	0.003
Toll-like receptor signaling pathway	3	Jun, Fos, Nfkbia	0.003
Focal adhesion	4	Jun, Pdpk1, Pdgfra, Ctnnb1	0.004
VEGF signaling pathway	2	Hspb1, Ppp3r1	0.021
Regulation of actin cytoskeleton	3	Gna13, Pip5k1a, Pdgfra	0.023

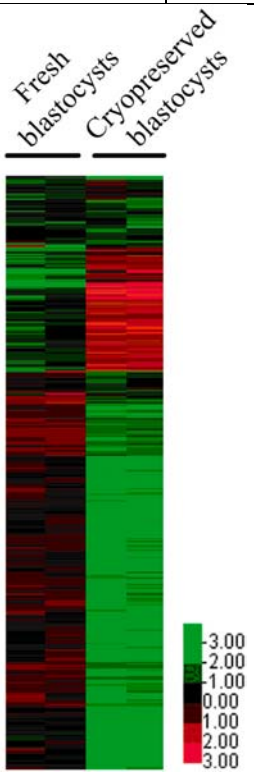

Figure 1. Clustering display of microarray data after SAM analysis of 2 pools of slow-freezing following warming blastocysts pools and 2 control blastocysts pools. Red: up-regulated; Green: down-regulated. The differentially expressed genes between slow-freezing blastocysts and control were determined using SAM software and visualized with TreeView tools after hierarchical clustering.

Gene Ontology classification using a free web-based Molecular Annotation System 2.0 (MAS 2.0, www.capitalbio.com). The data were categorized based on three independent GO terms with p<0.05: biological process, molecular function, and cellular component. All the differentially expressed gene probes were classified into eighteen functional groups. The first five categories accounted for approximately 41.5% of the total up- and down- regulated genes indentified. Results are summarized in Figure 3.

4.4. Pathway analysis of differential genes

The GO database does not cover every aspect of biology. The differentially expressed genes were also analyzed by MAS, which integrates three different open source pathway resources—KEGG, BioCarta and GenMAPP. The pathways are ranked with statistical significance by calculating their p-values based on hypergeometric distribution (16, 17). Among the 288 genes differentially expressed, 29 of them could be categorized into one or more of KEGG pathways listed in Table 3 (p<0.05). Among these, the pathways of mitogen-activated protein kinase (MAPK), Wnt and cell cycle were the most predominant.

5. DISCUSSION

In this study, we demonstrated for the first time the global transcript variations between cryopreserved blastocysts and the control groups. We showed the alterations of the genes to different important biological process, molecular function, cellular component and signaling pathways, to enable detailed understandings of the effects of slow-freezing on gene functions after warming. Cold stress affects cellular physiological by inhibiting transcription and translation and by increasing the denaturation and misaggregation of proteins (18). Cold stress also slows progression through the cell cycle, with phase G1 typically being the most sensitive (19, 20). Such reactions are evolutionary conserved among species (21) and expected in mammals. Besides the very few molecular level cold response studies available in mammalian cells, this study provided embryonic evidence to support the conservation of cold response and also revealed the difference between reactions of blastocyst stage embryo and mammalian cells to cold response.

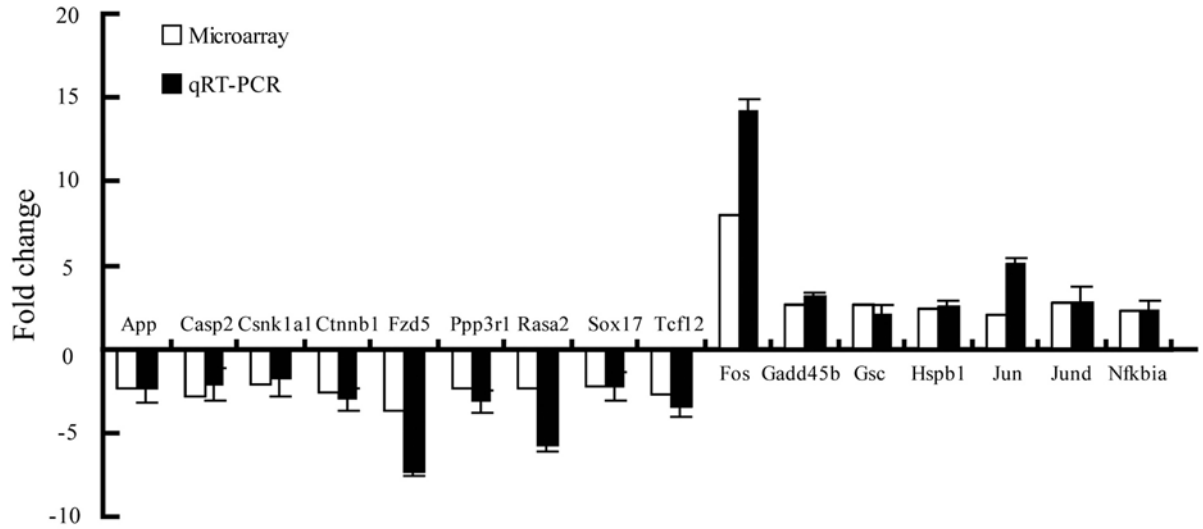

Figure 2. Results from the real-time PCR verification of microarray data using the RNA from individual samples. Despite the variations in scale, the trend remains the same with full confirmation for all.

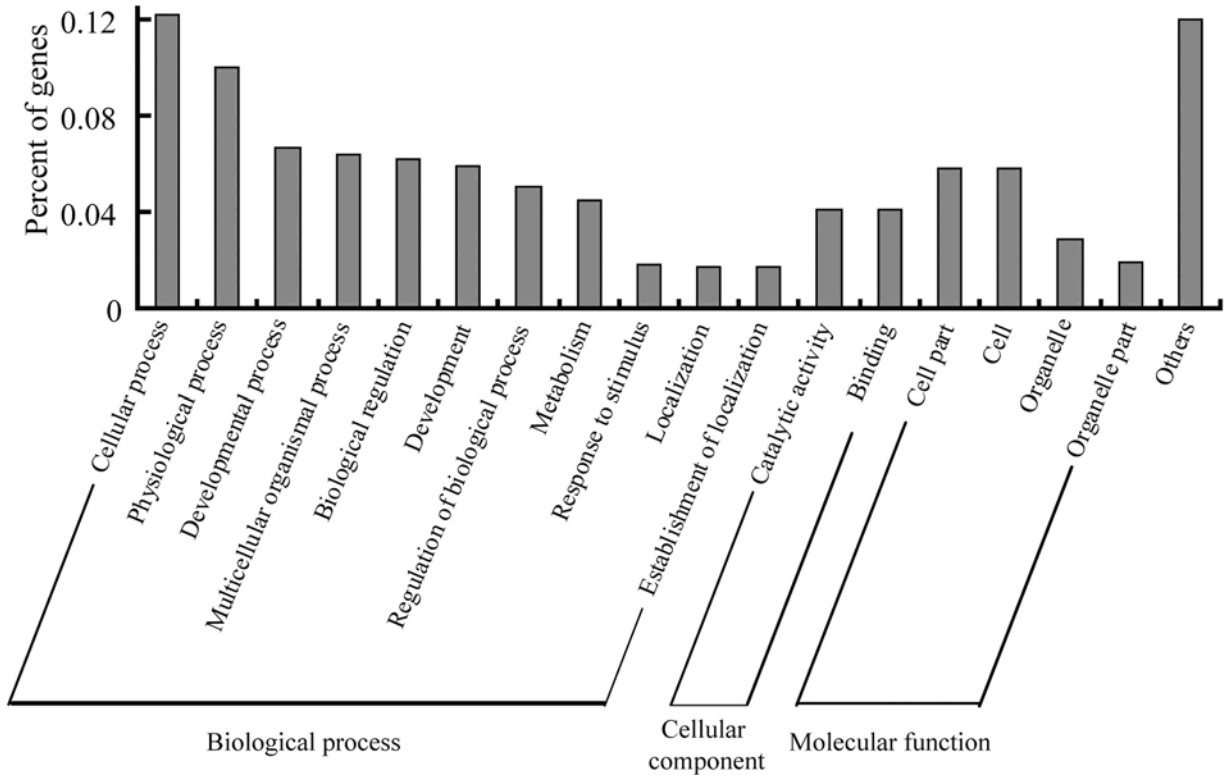

Figure 3. Gene ontology categories for differentially expression genes in cryopreserved blastocysts compared with the control blastocysts.

Our findings suggested that 3% (9/288) differential genes belong to MAPK signaling pathway, they changed dramatically upon cold stress to blastocysts. MAPK signaling promotes trophectoderm formation from mouse embryos (22). It is also served as a target of anandamide, as well as agonists that stimulate trophoblast differentiation through intracellular calcium signaling (23). Our data are consistent with the previous study which also showed 3% differential genes of MAPK cascades in 8-cell mouse embryos after vitrification (24). Among these differential genes, growth arrest and DNA-damage-inducible 45 beta (Gadd45b) and heat shock protein 1 (Hspb1) were both up-regulated in vitrified 8-cell embryos and slow-frozen blastocysts. These two genes are highly

conserved and induced by stresses. They have been implicated in cell cycle arrest (25-27).

Cells cultivated under hypothermic conditions undergo cell cycle arrest, predominantly in G1, but also in G2/M (28). It has recently been suggested that this is in part due to the expression of the RNA-binding cold shock proteins (Cirp) and RNA binding motif 3 (Rbm3), as their overexpression under normothermic conditions can lead to cell cycle arrest (29). In this study, several cell cycle genes changed, including Rbl1, Cdc14a, Cdk2, Gadd45b, Chek1. However, we did not detect the change of Cirp or Rbm3. Instead, there were 4-fold and 2.4-fold increase of Gadd45b and transducer of ERBB2, 2 (Tob2) respectively. They are genes induced by stress and their overexpression leads to cell cycle arrest (25, 26) (unpublished data).

Among the 288 differentially-expressed genes, we also focused on the genes encoding secreted proteins (known or predicted). Detection of the secreted proteins in culture medium may help develop diagnostic approach to identify the "best" embryo for transfer. Dickkopf-1 (Dkk1), one of these secreted proteins, was 2.5 folds down-regulated by slow-freezing after thawing. Dkk1 is the most well-characterized member of Dkk family, it inhibits the Wnt signaling pathway (30). As the important antagonist of Wnt, Dkk1 plays roles in blastocyst activation and uterine receptivity during the window of implantation (7). Decreased level of Dkk1 may account for the failure of implantation capacity of cryopreserved embryos.

In summary, the expression pattern reflected a broad spectrum of consequences of slow-freezing on the blastocysts, with most effects on stress-related genes and cell cycle-related genes. As mice are model animals for human embryo development studies (31), this study can fill the knowledge gap for the human model. Moreover, this study allowed the identification of new candidate marker genes to optimize cryopreservation protocol and provide candidates for diagnostic purpose.

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Abbreviations: IVF (*in vitro* fertilization); ROS: reactive oxygen species; GO: Gene ontologies; MAPK: mitogen-activated protein kinase; Gadd45b: DNA-damage-inducible 45 beta; Hspb1: heat shock protein 1; Cirp: cold-inducible RNA-binding protein; Rbm3: RNA binding motif 3; Tob2: transducer of ERBB2, 2; Dkk1: Dickkopf-1

Key Words: Cryopreservation, Microarray, Blastocyst, Gene Expression

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