Molecular and ultrastructural changes of rat pre-implantation embryos during two-cell developmental arrest

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Abstract

Background Rat pre-implantation embryos often suffer 2-cell stage developmental arrest and fail to progress further under in-vitro conditions.

Objective In order to understand underlying mechanism leading to 2-cell arrest, we investigated the molecular changes, culture conditions and subcellular changes.

Methods Gene expression in in-vivo developed 2-cell embryos (in-vivo), in-vitro developed 2-cell embryos (in-vitro), and in-vitro 2-cell arrested embryos (arrested) were investigated using microarrays and real-time PCR. Ultra-structural changes were determined using electron microscopy.

Results Gene expression was similar between in-vivo and in-vitro embryos. Over 2400 genes changed in arrested embryos compared to in-vivo and in-vitro embryos. The mRNAs encoding proteins involved in translation were elevated in arrested embryos. In-vivo and in-vitro embryos highly expressed genes that were involved in cell cycle, and protein catabolic process compared to arrested embryos. Gene expression data suggested subcellular changes associated with 2-cell block. Transmission electron microscopy showed that in-vivo embryos had healthy subcellular structure, whereas arrested embryos did not have a nuclear membrane, contained small

mitochondria and autophagic vacuoles. Furthermore, gene expression data was used for the optimization of culture media conditions to obtain better in-vitro embryonic development. Comparison of five and 20 % oxygen in culture resulted in two times more blastocyst formation with 5 % oxygen.

Conclusions These results showed that although all experimental groups appeared morphologically similar, arrested embryos had ultra-structural and molecular changes associated with oxidative stress and apoptosis. In-vitro culture under low oxygen and media additives reduced 2-cell block in rat embryos.

Keywords Rat embryos · 2-cell arrest · Oxidative stress · mRNA expression · Microarray · Electron microscopy · Developmental block · Real time PCR

Introduction

Successful in-vitro culture of fertilized oocytes to blastocyst stage plays an integral role in assisted reproductive technologies. The ability to grow pre-implantation mammalian embryos under in-vitro conditions is crucial for infertility treatment, biomedical research, embryo biotechnologies such as transgenic animal production and animal cloning programs [1]. Although blastocyst stage embryos can be produced successfully via in-vitro culture of zygotes in many mammalian species including rodents, livestock species and humans, there are still inefficiencies. Laboratory rats are among the important species in biomedical research; however, embryonic development under in-vitro conditions is inefficient which is largely due to 2-cell block [2, 3]. In-vitro development of pre-implantation embryos from one-cell to blastocyst has been achieved for most outbred and many inbred mouse strains [4, 5] but, to date there has been challenges with the in-vitro development of rat pre-implantation embryos [6]. In the mouse system, 2-cell block was successfully overcome by

Capsule Although 2-cell arrested rat embryos are morphologically similar to healthy in-vivo and in-vitro developed embryos, large number of mRNAs as well as ultrastructure in arrested embryos were significantly different. Gene expression data obtained in this study was applied to optimize the in-vitro embryo culture conditions.

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decreasing osmotic pressure of the culture media and supplementation of essential and non-essential amino acids [7–9]. Under suboptimal culture conditions, similar developmental block has been observed in pre-implantation embryos of other species at later stages of embryo development: 4- to 8-cell in human, 4-cell in pigs [10], and 8- to 16-cell in cattle and sheep [11]. The embryo development block often coincides with the time of genome activation in these species [12–15]. This indicates that embryos which fail to transition from maternal to zygotic transcription do not develop further. Similarly, activation of embryonic genes in rats during 2- to 4-cell stage [16] coincides with the 2-cell developmental block.

Improvement of water source, the basic culture media components that regulate the ionic stress significantly reduced developmental block for many species except rats [17]. Inefficiencies in rat embryo culture manifest themselves by either low incidence of in-vitro embryonic development due to 2-cell block or low pregnancy establishment following embryo transfer [18]. Rat embryo culture systems such as rat embryo culture medium (R1ECM) are somewhat efficient in outbred Sprague–Dawley and Wistar rats but resulting embryo development is highly variable. Although the exact nature of this developmental block is not fully understood, it has been largely attributed to detrimental effects of glucose, inorganic phosphate, reactive oxygen species (ROS) and proteins in the culture media [19, 20]. It has been reported that adjustment of inorganic phosphate concentration in the culture media based on the embryonic stage significantly increased developmental potential of rat embryos in-vitro [21, 22]. Unfortunately, the success rate significantly varied from laboratory to laboratory and to date there is no culture conditions which prevents 2-cell block completely.

Our current knowledge about underlying molecular and cellular mechanism involved in 2-cell block in many species and particularly laboratory rats is still limited. Thus, identification of key genes involved in pre-implantation embryonic developmental processes and determination of global patterns of gene expression would be important step forward for better understanding the underlying mechanisms that lead to 2-cell block. In this study we compared gene expression in in-vivo developed, in-vitro developed and in-vitro arrested 2-cell rat embryos. Rat pre-implantation embryos reach 2-cell stage at about 48 h in-vivo and become 4-cell at about 72 h post human chorionic gonadotropin (hCG) injection and mating. The in-vitro developed embryos which were cultured for 24 h was an intermediary experimental group and we hypothesized that mRNAs that differ between in-vivo developed and in-vitro developed embryos may be involved in the initial processes that lead to 2-cell arrest.

Data collected from microarrays and deep sequencing can be used for improving culture conditions in-vitro, treatments against diseases or better understanding molecular processes led to certain pathological conditions. Thus in an attempt to apply gene expression data to physiology, we tested various culture conditions to determine whether the highly regulated

genes were indicative of the initial responses of the embryos. Investigating the differentially regulated mRNAs in this study, led us to improve in-vitro embryo culture conditions by adding glutamate, changing media after 24 h of culture and lowering oxygen level in culture environment. In addition, the mRNA levels that changed between in-vivo derived and arrested 2-cell embryos led us to suspect that subcellular structures such as endoplasmic reticulum and mitochondria would be adversely affected in arrested 2-cell embryos. Thus we added electron microscopy analysis of these two groups to determine subcellular changes.

Materials and Methods

Animals

Sprague–Dawley (SD) rats were purchased from Harlan Sprague–Dawley (Indianapolis, IN) and were housed in a controlled lighting environment (10 h dark/14 h light) and provided free access to water and standard pelleted rodent chow. All animal studies were performed in accordance with the University of Missouri's Animal Care and Use Committee guidelines.

Superovulation

Sexually immature 26 d old SD rats were injected with 20 IU equine chorionic gonadotropin (eCG) and 48 h later they were injected with 20 IU hCG and mated with sexually mature male SD rats to generate embryos.

Experimental Design

There were three experimental groups: 1) In-vivo 2-cell embryos were collected 48 h post hCG injection and mating (in-vivo embryos); 2) In-vitro developed 2-cell embryos were obtained by removing pronuclear stage rat embryos from oviduct 24 h post hCG injection and mating followed by 24 h embryo culture (in-vitro embryos); 3) In-vitro 2-cell arrested embryos were produced by removing pronuclear stage embryos from oviduct 24 h after hCG and mating followed by 48 h culture (arrested embryos). Thus the difference between in-vitro embryos and arrested embryos was that the arrested embryos remained in culture media for an additional 24 h (Fig. 1).

Embryo collection and in-vitro culture

The oviducts of donor females were removed from vaginal plug positive rats and placed in HEPES buffered R1ECM media [2, 3] and cut using 27 g needle to release the presumptive rat zygotes. The zygotes were held in HEPES buffered R1ECM containing 1 mg/ml hyaluronidase, pipetted to remove attached cumulus cells, and washed three times in

Fig. 1 Schematic representation of experimental groups. 26 d old rats injected with eCG, 48 h later they were injected with hCG and mated. In-vivo developed 2-cell embryos: 48 h after mating, 2-cell embryos were collected from oviducts. In-vitro developed 2-cell embryos: 24 h after

mating, 1-cell embryos containing sperm tail and pronuclei were harvested from oviduct and cultured for 24 h. Arrested 2-cell embryos: 24 h after mating, 1-cell embryos containing sperm tail and pronuclei were harvested from oviduct and cultured for 48 h

R1ECM before in-vitro culture. To confirm fertilization, embryos were checked for presence of pronuclei and sperm tail in cytoplasm under an inverted microscope using a 20X objective. To obtain 2-cell in-vitro embryos and 2-cell arrested embryos, zygotes were transferred into R1ECM and cultured for 24 and 48 h, respectively. The R1ECM contained 76.7 mM NaCl, 3.2 mM KCl, 2 mM CaCl₂, 0.5 mM MgCl₂, 10 mM sodium lactate, 0.5 mM sodium pyruvate, 7.5 mM glucose, 25 mM NaHCO₃, 1.0 mg/ml polyvinylalcohol, 0.1 mM glutamine, 2 % (v:v) Minimal Essential Medium (MEM) essential amino acid solution (EAA; Life Technologies, Grand Island, NY), and 1 % (v:v) MEM nonessential amino acid solution (NEAA; Life Technologies), 1 % (v:v) penicillin-streptomycin solution (Life Technologies). In this particular study, none of the embryos that were cultured for 48 h developed further than 2-cell; thus we assumed that the embryos cultured for 24 h would not have developed to 4-cell. For collection of in-vivo 2-cell embryos, donor female rats were sacrificed 48 h post hCG and mating. Two-cell embryos were released from oviduct, washed three times in HEPES buffered R1ECM and transferred into R1ECM containing polyvinyl alcohol. Four pools (28 to 50 embryos per pool) of 2-cell embryos from each treatment group were collected and snap frozen in liquid nitrogen until RNA isolation.

Effects of oxygen tension during embryo culture

In-vitro embryo cultures were performed either under atmospheric oxygen (20 %) with 5 % CO₂ or under 5 % O₂, 5%CO₂ and 90 % N₂. One-cell embryos having sperm tail and two pronuclei were either cultured in R1ECM for at least 48 h or mR1ECM containing 110 mM NaCl, 3.2 mM KCl, 2 mM CaCl₂, 0.5 mM MgCl₂, 10 mM sodium lactate, 0.5 mM sodium pyruvate, 7.5 mM glucose, 25 mM NaHCO₃, 0.05 % (v:v) GlutaMAX (Life Technologies), 2 % (v:v) MEM EAA, 1 % (v:v) MEM NEAA, 100 U/ml Penicillin G potassium salt and 36 IU/ml streptomycin sulfate for 24 h followed by transfer of 2-cell embryos to mR1ECM media containing 76.7 mM NaCl 3.2 mM KCl, 2 mM CaCl₂, 0.5 mM MgCl₂, 10 mM sodium lactate, 0.5 mM sodium pyruvate, 7.5 mM glucose, 25 mM NaHCO₃, 1.0 mg/ml polyvinylalcohol, 0.05 % (v:v) GlutaMAX, 2 % (v:v) MEM EAA, 1 % (v:v) MEM NEAA, 100 U/ml Penicillin G potassium salt and 36 IU/ml streptomycin sulfate. Effect of oxygen level was determined using four

sets of embryos for each group and embryos which developed to 4-cell were followed up to blastocysts stage for 96 h. Development of embryos was assessed under stereomicroscope.

RNA isolation amplification and microarrays

Total RNA from each pool of 2-cell embryos was isolated using Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, Carlsbad, California) as previously described [23]. During RNA isolation, genomic DNA was digested using DNaseI. Half of the isolated total RNA was amplified by using Ovation Pico WTA System (Nugen, San Carlos, CA) which produces cDNA as the end product. Amplified cDNA (2 ug) was biotin labeled by using Encore BiotinIL Module (Nugen, San Carlos, CA). Biotinylated cDNA target (750 ng) was hybridized to the RatRef-12 Expression BeadChip arrays (Illumina, San Diego, CA) containing 21,910 probes. There were four biological replicates and two technical replicates per experimental group. Thus each experimental group (in-vivo, in-vitro and arrested 2-cell embryos) was represented by eight samples using a total of 24 microarrays. Hybridization of microarrays was done according to manufacturer's suggestions except the hybridization temperature was reduced to 48 °C due to hybridization of cDNA. BeadStudio (Illumina, San Diego, CA) expression analysis software was used to determine the expression level of mRNAs. GeneSpring 7.3 software (Agilent Technologies, Santa Clara, CA) was used to determine the genes that were differentially regulated. The data was log transformed and Welch *t*-test and Benjamini and Hochberg multiple testing correction with 5 % false discovery rate were performed to determine the genes whose expression differentially regulated. Fold changes in gene expression between the treatments were also determined using GeneSpring software. Gene expression in in-vivo embryos were compared to that of in-vitro and arrested 2-cell embryos. In addition gene expression in in-vitro 2-cell embryos were compared to arrested 2-cell embryos.

DAVID Functional Annotation Tool [24, 25] was used to analyze the genes that were differentially expressed among in-vivo, in-vitro and arrested 2-cell embryos. DAVID Functional Annotation Tool determined the co-occurrence probability and provided gene-gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis to highlight the most relevant GO terms or KEGG

pathways associated with a given gene list. The statistical significance of co-occurrence of genes was determined by a modified Fisher Exact test.

Real-time PCR

Remaining total RNA from each sample was amplified by using Ovation PicoSL WTA System V2 (Nugen, San Carlos, CA). The cDNA products of these reactions were used in real-time PCR using Power SYBR Green PCR Master Mix (Life Technologies Corporation, Carlsbad, California) and cDNA samples were PCR amplified in duplicates. Expression of eukaryotic translation initiation factor 2, subunit 2 beta (Eif2s2) was similar among all the samples in microarray analysis and Eif2s2 was used as a housekeeping gene in real-time PCR analysis. Four sets of mRNAs were chosen to validate microarray results by using real time PCR. The first set included mRNAs that were elevated in in-vivo embryos compared to 2-cell arrested embryos. These mRNAs were proteasome (prosome, macropain) subunit beta type 2 (Psm2), nudE neurodevelopment protein 1 (Nde1), tubulin, alpha-like 3 (Tubal3), origin recognition complex, subunit 5

(Orc5), hypoxia-inducible factor 1 alpha subunit (Hif1a), cyclin B2 (Ccnb2), p21 protein (Cdc42/Rac)-activated kinase 1 (Pak1), Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6 (Arhgef6), actin related protein 2/3 complex, subunit 1B (Arpc1b), actin, beta (Actb), and cyclin-dependent kinase 1 (Cdk1). The second set of mRNAs was chosen from the elevated mRNAs in arrested embryos compared to in-vivo embryos. These mRNAs were insulin-like growth factor 2 mRNA binding protein 1 (Igf2bp1), polymerase (RNA) II (DNA directed) polypeptide E (Polr2e), protein phosphatase 1, regulatory subunit 15A (Ppp1r15a), protein kinase, AMP-activated, alpha 1 catalytic subunit (Prkaa1), and cyclin-dependent kinase inhibitor 1A (Cdkn1a). The third set of mRNAs included the mRNAs that were elevated in in-vivo embryos compared to in-vitro 2-cell embryos: hemoglobin alpha, adult chain 2 (Hba-a2), hemoglobin, beta (Hbb), cysteine dioxygenase type 1 (Cdo1), cysteine dioxygenase type 1 (Ptgis). The fourth set included the mRNAs that were elevated in in-vitro embryos compared to in-vivo 2-cell embryos: tafazzin (Taz), succinate dehydrogenase complex subunit D integral membrane protein (Sdhb), NADH dehydrogenase 1 subcomplex unknown 2 (Ndufc2), and ubiquinol-cytochrome

Table 1 Real time PCR primer sequences and amplification product sizes

mRNA	Forward primer	Reverse primer	Product (bp)
Actb	TTGCTGACAGGATGCAGAAGGAGA	ACTCCTGCTTGCTGATCCACATCT	159
Arhgef6	TGCCTTGAAGGATGAGGTCAAGGA	ATCGGTTTGCTTCAACAGCTTCCG	127
Arpc1b	TGTGAAGAGCTTGAGTCAGCCTT	AGCATTACAGCAAAGCAGCCCTTAG	130
Ccnb2	CACATGGCCAAGAAGCTCGTGAAA	GCCTGCTGCTGGCATATTTGTTCT	82
Cdk1	TCCCACGTCAAGAACCTGGATGAA	ACAGTAAACGCCACGATCTTCCCT	196
Cdkn1a	TTCTGTAACACCCTGAGCTGGACT	ACACGTGGGAGGTTTACAATCCGA	99
Cdo1	TTGGATAAGAGAGGCCACCCATCA	ACAGAAGAAGTGGGAGCAAGGTGA	177
Eif2s2	TCGATGCTCTGTGGCCAGTATCAA	TCTGATGGTAACCTGTCCAGCCAA	147
Hba-a2	TGCGTGTGGATCCTGTCAACTTCA	ACAGTGCTCACAGAGGCAAGGAAT	133
Hbb	ATGGCCTGAAACACTTGGACAACC	TGGTGGCCCAACACAATCACAATC	133
Hif1a	GGCCTATGGAATTGTTAAGCCTGG	GAGCCGGTAAAGTAAAGAAACACC	196
Igf2bp1	TTCCCAGCTTCTTCTAGTGCCGTT	TGTTTGATGTGCTGGCCCTTCTTG	164
Nde1	TTGCGTTGGACTGGCCTTTGTTAC	TGGCATTGTAGAGCCTGTGCTGAT	164
Ndufc2	ATGAAAGCAGGTTTGCATCGCCAG	ACATGTCATGGTCCCTCACAGCAT	112
Orc5	TCCCAACACAAGGCAGCTTGTTT	TGGTGGTTAACGAGAGTGACCAGT	98
Pak1	TAAACACTCGTTTCGGGAACCTCCG	TAGTCATCCGGTTGCGGTTCTCTT	139
Polr2e	AGCGAGGGCAGGTGTGAAGATTA	TCCCAACACCGAAGAGTCCACAAA	163
Ppp1r15a	AAGACCAAGGGATGTGGAGAAGCA	GGCTTTGAAAGATCTGAGCCGCTT	138
Prkaa1	ATTTGCGTGTGCGAAGGAAGAACC	AGCAGTCCCTGATTGGCTTCTGT	143
Psm2	TATGTGTTGGAGAGGCTGGAGACA	GCCAGGTTTCGGCGTGTGAAATTA	133
Ptgis	TCCCGAGTTTGACCTTAGCAGAT	TGGACACAGCATTCTGGAGGAACA	157
Sdhb	GGAGCTCTCCTTTGTAGAAGGGTT	TGGACAGCTCATTCCTGCACAAGT	93
Taz	TTGATAGGCTACCACTTGCCCACT	ATCTACAGGCCACCCAAGGACAAA	112
Tubal3	AGCAATTGCAGCCATGAAGTCGAG	TTGCTCAACATACACAGCACGC	150
Uqcrrf1	TGATGCTTCTGGCAGGATCAGGAA	AAGAAACCTAGGCCCTGAGTCCAA	125

c reductase Rieske iron-sulfur polypeptide 1 (Uqcrrf1). Table 1 shows the sequences of PCR primers and product sizes. The threshold cycle (C_t) for the Eif2s2 was subtracted from the C_t for the test mRNA to obtain the change (Δ) in C_t (ΔC_t). The amount of each mRNA (relative to Eif2s2) was calculated by using the equation: relative expression = $2^{-\Delta C_t}$. Statistical analysis of real-time PCR results were performed by using general linear models (SAS version 9.3, SAS Institute, Cary, NC). Separation of means was assessed by using the Duncan Multiple Range test.

Transmission electron microscopy

All reagents were purchased from Electron Microscopy Sciences and all specimen preparation was performed at the Electron Microscopy Core Facility, University of Missouri. In-vivo and arrested 2-cell embryos were obtained as described above. For transmission electron microscopy sample preparation, primary fixation of embryos was performed immediately post-harvest in 2 % paraformaldehyde, 2 % glutaraldehyde, in 100 mM sodium cacodylate buffer, pH 7.35. Fixed embryos were surrounded in histogel to keep them together for chemical exchanges. Then they were rinsed with 100 mM sodium cacodylate buffer, pH 7.35 containing 10 mM 2-mercaptoethanol and 130 mM sucrose. Using a Pelco Biowave (Ted Pella, Inc. Redding, California), secondary fixation was performed using 1 % osmium tetroxide in 100 mM sodium cacodylate, pH 7.35 and followed by rinsing with distilled water. Using the Pelco Biowave, a graded dehydration series was performed using acetone. Dehydrated tissues were then infiltrated with Epon/Spurr's resin and polymerized at 60°C overnight. Sections were cut to a thickness of 85 nm using an ultramicrotome (Ultracut UCT, Leica Microsystems, Germany) and a diamond knife (Diatome, Hatfield PA). These sections were stained using Sato's triple lead solution stain and 5 % aqueous uranyl acetate [26]. Images were acquired at 80 kV on the JEOL JEM 1400 transmission electron microscope (JEOL, Peabody, MA) equipped with an Ultrascan 1000 CCD (Gatan, Inc. Pleasanton, CA).

Results

Gene expression profile of arrested 2-cell embryos was greatly different from in-vivo and in-vitro 2-cell embryos. Levels of 3759 and 5123 mRNAs were significantly ($P < 0.05$) different between arrested embryos compared to in-vivo and in-vitro 2-cell embryos, respectively. In order to increase stringency of analysis, we used the mRNAs that were regulated both significantly and changed at least two fold for further analysis to

determine the relevant GOs and KEGG pathways that were changed in experimental groups. There were 939 and 1,012

Table 2 Elevated biological processes in in-vivo developed and in-vitro developed embryos compared to arrested two cell embryos

Biological process	Vv vs Ar		Vt vs Ar	
	N	P	N	P
Cell cycle	69	3E-10	99	2E-15
Cellular protein catabolic process	50	1E-8	67	1E-10
Modification-dependent protein catabolic process	44	3E-7	60	2E-9
Cell division	29	3E-7	39	4E-9
Protein localization	78	6E-7	112	2E-10
Regulation of protein ubiquitination	21	9E-7	22	4E-5
DNA metabolic process	49	1E-6	70	2E-9
Protein transport	67	2E-6	94	6E-9
Cellular response to stress	56	3E-6	76	1E-7
Response to DNA damage stimulus	38	7E-6	50	1E-6
Regulation of organelle organization	29	8E-6	37	3E-6
Nuclear division	21	9E-6	26	4E-6
Organelle fission	21	2E-5	26	1E-5
Amyloid precursor protein metabolic process	6	2E-5		
Intracellular transport	58	2E-5	83	1E-7
Biopolymer methylation	16	4E-5		
DNA repair	28	8E-5	41	3E-7
Regulation of binding	23	1E-4		
Cell morphogenesis	42	1E-4		
Cytoskeleton organization	40	1E-4	49	5E-4
Protein heterooligomerization	17	2E-4		
Vesicle-mediated transport	50	2E-4	69	2E-5
Regulation of cellular protein metabolic process	48	2E-4		
Regulation of ligase activity	14	4E-4		
Negative regulation of molecular function	36	4E-4	48	1E-4
Hemopoietic or lymphoid organ development	32	6E-4	41	5E-4
Immune system development	33	6E-4		
Positive regulation of molecular function	54	6E-4		
Establishment or maintenance of cell polarity	9	9E-4		
Death	46	1E-3	60	9E-4
Macromolecular complex assembly			80	4E-5
Golgi vesicle transport			21	7E-5
Protein complex biogenesis			63	1E-4
Phosphate metabolic process			109	4E-4
Regulation of actin filament length			13	5E-4
RNA elongation			13	1E-3
Transcription			89	1E-3

Vv vs Ar indicates biological processes that were upregulated in in-vivo developed embryos compared to arrested embryos; Vt vs Ar indicates biological processes that were upregulated in in-vitro developed embryos compared to arrested embryos; N number of elevated mRNAs; P P-value

Table 3 Elevated mRNAs involved in cell cycle and protein catabolism in in-vivo developed and in-vitro developed embryos compared to arrested embryos. In order to avoid duplication, the mRNAs that belong to both biological processes, were listed only under one biological process

Cell cycle

Anaphase promoting complex subunit 4 (Anapc4)	Anaphase-promoting complex subunit 5 (Anapc5)
Arginine vasopressin-induced 1 (Avpi1)	Biorientation of chromosomes in cell division 1 (Bod1)
Budding uninhibited by benzimidazoles 1 homolog, beta (Bub1b)	Calcium/calmodulin-dependent protein kinase II gamma (Camk2g)
Calmodulin 1 (Calm1)	CDC28 protein kinase regulatory subunit 1B Cks1b (Cks1b)
Cell division cycle 25 homolog A (S. Pombe) (Cdc25a)	Cell division cycle 25 homolog B (S. Pombe) (Cdc25b)
Cell division cycle associated 3 (Cdca3)	Centrin 2 (Cetn2)
Centromere protein F (Cenpf)	Centromere protein J (Cenpj)
Centrosomal protein 55 kda (Cep55)	Citron (rho-interacting, serine/threonine kinase 21) (Cit)
CSRP2 binding protein (Csrp2bp)	Cullin 1 (Cul1)
Cyclin B2 (Ccnb2)	Cyclin D binding myb-like transcription factor 1 (Dmtf1)
Cyclin D-type binding-protein 1 (Cndbp1)	Cyclin-dependent kinase inhibitor 1B (Cdkn1b)
Cyclin-dependent kinase inhibitor 2C (Cdkn2c)	Cytoplasmic linker associated protein 2 (Clasp2)
Cytoplasmic polyadenylation element binding protein 1 (Cpeb1)	Dnaj (Hsp40) homolog, subfamily C, member 2 (Dnajc2)
Dynactin 2 (Dctn2)	Endoplasmic reticulum to nucleus signaling 1 (Ern1)
Increased sodium tolerance 1 homolog (yeast) (Ist1)	Interferon gamma (Ifng)
Katanin p60 (atpase-containing) subunit A1 (Katna1)	Ligase I, DNA, ATP-dependent (Lig1)
Macrophage erythroblast attacher (Maea)	MAD2 (mitotic arrest deficient, homolog)-like 1 (Mad2l1)
Mdm2 p53 binding protein homolog (mouse) (Mdm2)	Microtubule-associated protein, RP/EB family, member 1 (Mapre1)
Microtubule-associated protein, RP/EB family, member 2 (Mapre2)	Mitochondrial tumor suppressor 1 (Mts1)
Mitofusin 2 (Mfn2)	Mitogen activated protein kinase kinase 1 (Map2k1)
Mitogen-activated protein kinase 6 (Mapk6)	Multiple endocrine neoplasia 1 (Men1)
Myeloid leukemia factor 1 (Mlf1)	Myosin, heavy chain 10, non-muscle (Myh10)
NDC80 homolog, kinetochore complex component (Ndc80)	NEDD8 activating enzyme E1 subunit 1 (Nae1)
Nicotinamide phosphoribosyltransferase (Nampt)	Non-SMC condensin II complex, subunit D3 (Ncapd3)
Nuclear mitotic apparatus protein 1 (Numa1)	Nucleolar and spindle associated protein 1 (Nusap1)
Nude nuclear distribution gene E homolog 1 (A. Nidulans) (Nde1)	NUF2, NDC80 kinetochore complex component, homolog (nuf2)
Par-3 (partitioning defective 3) homolog (Pard3)	PEST proteolytic signal containing nuclear protein (Pcnp)
PIN2-interacting protein 1 (Pinx1)	Piwi-like 2 (Drosophila) (Piwi2)
Podoplanin (Pdpn)	Polo-like kinase 1 (Drosophila) (Plk1)
Polo-like kinase 1 substrate 1 (Plk1s1)	Polymerase (DNA directed), alpha 1 (Pola1)
Proline rich 5 (renal) (Prr5)	Proteasome 26S subunit, atpase 3, interacting protein (Psmc3ip)
Proteasome 26S subunit, non-ATPase, 2 (Psmc2)	Proteasome 26S subunit, non-ATPase, 3 (Psmc3)
Proteasome 26S subunit, non-ATPase, 4 (Psmc4)	Proteasome subunit, alpha type 1 (Psm1)
Proteasome subunit, alpha type 2 (Psm2)	Proteasome subunit, alpha type 4 (Psm4)
Proteasome subunit, alpha type 6 (Psm6)	Proteasome subunit, alpha type 7 (Psm7)
Proteasome subunit, beta type 2 (Psmb2)	Proteasome subunit, beta type 3 (Psmb3)
Protein phosphatase 1, catalytic subunit, alpha isoform (Ppp1ca)	RalA binding protein 1 (Ralbp1)
Ras association (RalGDS/AF-6) domain family member 2 (Rassf2)	Regulator of G-protein signaling 2 (Rgs2)
Retinoblastoma 1 (Rb1)	Septin 9 (Sept9)
Seven in absentia 2 (Siah2)	Shugoshin-like 1 (S. pombe) (Sgo1)
Shwachman-Bodian-Diamond syndrome homolog (Sbds)	Similar to transcription factor (MGC112830)
SKI-like oncogene (Skil)	Spastin (spast)
SPC25, NDC80 kinetochore complex component, homolog (Spc25)	SPECC1-like (Specc1l)
S-phase kinase-associated protein 1 (Skp1)	Spindle and kinetochore associated complex subunit 1 (Ska1)
Structural maintenance of chromosomes 1B (Smc1b)	TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor (Taf10)
Thioredoxin interacting protein (Txnip)	Timeless homolog (Drosophila) (Timeless)
Transforming growth factor, beta 2 (Tgfb2)	Transforming, acidic coiled-coil containing protein 2 (Tacc2)
Tubulin, beta 5 (Tubb5)	Tumor protein p53 inducible nuclear protein 1 (Trp53inp1)

Table 3 (continued)

Ubiquitin-conjugating enzyme E2I (Ube2i)	Ubiquitin-like modifier activating enzyme 3 (Uba3)
Ubiquitin-like with PHD and ring finger domains 1 (Uhrf1)	Vacuolar protein sorting 24 homolog (Vps24)
Zinc finger protein 655 (Zfp655)	Zinc finger protein 830 (zfn830)
Protein catabolic process	
Aminoacylase 1 (Acy1)	Amplified in osteosarcoma (Os9)
Anterior pharynx defective 1 homolog A (<i>C. elegans</i>) (Aph1a)	ATG7 autophagy related 7 homolog (<i>S. cerevisiae</i>) (Atg7)
Carboxypeptidase A1 (Cpa1)	Cas-Br-M ecotropic retroviral transforming sequence b (Cblb)
Ceroid-lipofuscinosis, neuronal 8 (Cln8)	Damage-specific DNA binding protein 1 (Ddb1)
F-box protein 11 (Fbxo11)	F-box protein 39 (Fbxo39)
F-box protein 46 (Fbxo46)	F-box protein 7 (Fbxo7)
Hect domain and RLD 4 (Herc4)	Kelch-like 12 (<i>Drosophila</i>) (Klhl12)
Membrane-associated ring finger (C3HC4) 7 (March7)	Midline 1 (Mid1)
Neural precursor cell expressed, developmentally down-regulated 8 (Nedd8)	Non-SMC element 2, MMS21 homolog (Nsmce2)
OTU domain containing 5 (Otu5)	Presenilin 1 (Psen1)
Presenilin 2 (Psen2)	Presenilin enhancer 2 homolog (<i>C. elegans</i>) (Psenen)
Protein inhibitor of activated STAT, 2 (Pias2)	Protein phosphatase 2, catalytic subunit, beta isoform (Ppp2cb)
Ring finger protein 1 (Ring1)	Ring finger protein 34 (Rnf34)
Ring-box 1 (Rbx1)	SMT3 suppressor of mif two 3 homolog 3 (Sumo3)
Tnf receptor-associated factor 2 (Traf2)	Tnf receptor-associated factor 6 (Traf6)
Transducin (beta)-like 1 X-linked receptor 1 (Tb11xr1)	Tumor rejection antigen gp96 (Tra1)
Ubiquitin carboxyl-terminal esterase L1 (Uchl1)	Ubiquitin carboxyl-terminal esterase L3 (uchl3)
Ubiquitin carboxyl-terminal hydrolase L5 (Uchl5)	Ubiquitin specific peptidase 11 (usp11)
Ubiquitin specific peptidase 15 (Usp15)	Ubiquitin specific peptidase 19 (usp19)
Ubiquitin specific peptidase 2 (Usp2)	Ubiquitin specific peptidase 33 (usp33)
Ubiquitin specific peptidase 39 (Usp39)	Ubiquitin specific peptidase 46 (usp46)
Ubiquitin specific peptidase 47 (Usp47)	Ubiquitin specific peptidase 7 (usp7)
Ubiquitin specific protease 24 (Usp24)	Ubiquitin specific protease 44 (usp44)
Ubiquitination factor E4B (Ube4b)	Ubiquitin-conjugating enzyme e2c binding protein (ube2cbp)
Ubiquitin-conjugating enzyme E2G 2 (Ube2g2)	V-rel reticuloendotheliosis viral oncogene homolog A (Rela)
Zinc metalloproteinase, STE24 homolog (Zmpste24)	

genes elevated in arrested 2-cell embryos compared to in-vivo and in-vitro embryos, respectively. Furthermore, 1466 and 2061 mRNAs elevated in in-vivo and in-vitro embryos compared to arrested 2-cell embryos, respectively. Since we only compared two experimental groups at a time, elevated mRNAs in one of the groups are the same as reduced mRNAs in the comparison group. Supplementary Table 1 shows the significantly regulated mRNAs that were different at least two fold in in-vivo and in-vitro embryos compared to arrested 2-cell embryos. The regulated mRNAs in in-vivo and in-vitro embryos compared to arrested 2-cell embryos were similar. Thus, in order to reduce redundancy, comparisons of in-vivo and in-vitro embryos to arrested 2-cell embryos were combined.

Elevated mRNAs and biological processes in in-vivo developed and in-vitro developed 2-cell embryos compared to arrested embryos

Analysis of elevated mRNAs using DAVID showed that 63 biological processes were significantly ($p < 0.001$) up-regulated in in-vitro embryos compared to arrested 2-cell embryos. Similarly, 69 biological processes were up-

regulated ($P < 0.001$) in-vivo embryos compared to arrested 2-cell embryos. Table 2 shows the subset of up-regulated biological process in in-vivo and in-vitro embryos compared to arrested 2-cell embryos. Cell cycle and protein catabolic processes were highly enriched by the up-regulated mRNAs in in-vivo and in-vitro embryos and mRNAs encoding proteins in these processes are listed in Table 3.

Elevated mRNAs and biological processes in arrested embryos compared to in-vivo developed and in-vitro developed 2-cell embryos

Biological processes that are involved in translation were highly up-regulated in arrested 2-cell embryos compared to in-vivo and in-vitro embryos. There were 34 and 43 up-regulated ($p < 0.001$) biological processes that were enriched by the elevated mRNAs in arrested embryos compared to in-vivo and in-vitro embryos, respectively. Table 4 shows a subset of the up-regulated biological processes and Fig. 2 shows the significantly elevated mRNAs that encode ribosomal proteins in arrested embryos.

Table 4 Elevated biological processes in arrested 2-cell embryos compared to in-vivo developed and in-vitro developed embryos

Biological process	Ar vs Vv		Ar vs Vt	
	N	P	N	P
Translational elongation	51	5E-43	55	6E-47
Translation	77	9E-27	79	9E-26
RNA processing	66	2E-23	64	5E-20
Ribonucleoprotein complex biogenesis	36	2E-18	35	2E-16
Ribosome biogenesis	28	5E-16	27	4E-14
mRNA metabolic process	39	6E-12	35	1E-8
RNA splicing	32	4E-11	32	3E-10
ncRNA metabolic process	28	1E-8	26	9E-7
rRNA metabolic process	16	6E-8	17	3E-8
RNA splicing, via transesterification reactions	20	1E-6	21	1E-6
Negative regulation of macro-molecule metabolic process	55	3E-6	54	6E-5
Macromolecular complex assembly	45	6E-5	47	1E-4
Transcription	53	6E-5	61	3E-6
Nucleocytoplasmic transport	17	9E-5	18	7E-5
Negative regulation of gene expression	38	1E-4	38	5E-4
Negative regulation of nitrogen compound metabolic process	39	1E-4		
Negative regulation of RNA metabolic process	30	2E-4		
Cellular macromolecular complex assembly	24	3E-4	25	4E-4
Transcription, DNA-dependent	17	4E-4		
RNA localization	11	5E-4	13	6E-5
RNA biosynthetic process	17	5E-4		
Transcription from RNA polymerase II promoter	13	9E-4		
Establishment of RNA localization			12	2E-4
Nucleic acid transport			12	2E-4
Nucleobase, nucleoside, and nucleic acid transport			13	2E-4
Male gamete generation			26	3E-4
Modification-dependent protein catabolic process			28	5E-4
Protein ubiquitination			13	5E-4
Multicellular organism reproduction			38	6E-4
Reproductive process in a multicellular organism			38	6E-4
Proteolysis involved in cellular protein catabolic process			29	8E-4
Ubiquitin-dependent protein catabolic process			19	9E-4
Cellular protein catabolic process			29	1E-3

Ar vs Vv indicates biological processes that were elevated in arrested embryos compared to in-vivo developed embryos; *Ar vs Vt* indicates biological processes that were elevated in arrested embryos compared to in-vitro developed embryos; *N* number of elevated mRNAs in arrested embryos; *P* *P*-value

Comparison of in-vivo developed embryos to in-vitro developed 2-cell embryos

All of the embryos cultured for 48 h were arrested, thus it is very likely that all the embryos that were cultured 24 h would have been arrested if they were kept in culture for 48 h. There were no significant gene expression differences between in-vivo and in-vitro embryos. Thus, we further analyzed the mRNAs that changed at least two fold between these groups (Supplementary Table 2). There were 148 mRNAs that were up-regulated in in-vivo embryos compared to in-vitro embryos and 190 mRNAs were up-regulated in in-vitro

embryos compared to in-vivo embryos. Table 5 shows the mRNAs that were different at least 2.5 fold between these groups. Interestingly, hemoglobin alpha adult chain 2 and hemoglobin beta were among the highly up-regulated mRNAs in in-vivo embryos. In addition, transportin 2 which is important in mRNA export from nucleus [27] was elevated in in-vivo embryos.

Real time PCR

Table 6 shows the fold change comparison of real-time PCR and microarray analysis. Four sets of mRNAs were chosen to validate microarray results by using real time

Fig. 2 Majority of the mRNAs encoding ribosomal proteins were elevated in arrested 2-cell embryos compared to in-vivo developed and in-vitro 2-cell embryos. The elevated ribosomal proteins (RP) were Rpl10; Rpl10a; Rpl12; Rpl13; Rpl13a; Rpl14; RPL15; Rpl17; Rpl18; Rpl18a; Rpl19; Rpl221; Rpl23; Rpl23a; Rpl26; Rpl27; Rpl27a; Rpl27a; Rpl3; Rpl30; Rpl34; Rpl35; Rpl35a; Rpl36; Rpl36a; Rpl37; Rpl37a-ps1; Rpl4;

Rpl41; Rpl6; Rpl7; Rpl8; Rplp1; Rplp2; Rps10; Rps13; Rps15; Rps17; Rps18; Rps19; Rps20; Rps21; Rps23; Rps24; Rps25; Rps26; Rps27; Rps29; Rps3; Rps3a; Rps5; Rps6; Rps7; Rps8; Rps9; Rpsa; Rsl24d1; acidic ribosomal phosphoprotein P0 (Arbp); Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed (Fau); similar to 40S ribosomal protein S2 (RGD1562399)

PCR. The first set included mRNAs that were up-regulated in-vivo embryos compared to arrested 2-cell embryos. Real time PCR results were similar to microarray results and fold change comparison for most of the mRNAs were greater for real time PCR compared to microarrays. The second set of mRNAs for real time PCR validation were chosen from the up-regulated mRNAs in arrested embryos compared to in-vivo embryos. Microarray and real time PCR results were similar for the mRNAs tested with the exception of Prkaa1 which showed no change in the real time PCR analysis. The third set of mRNAs included the mRNAs that were up-regulated in in-vivo embryos compared to in-vitro embryos. Real time PCR results were similar to microarray results, but the fold change of the two hemoglobin mRNAs were much greater for real time PCR analysis:

Hba-a2 increased 382 fold and Hbb increased 40 fold. The fourth set included the mRNAs that were up-regulated in in-vitro embryos compared to in-vivo embryos. The mRNAs tested were Taz, Sdh, Ndufc2, and Uqcrl1 and contrary to microarray results, real time PCR did not show any change in levels of these mRNAs.

In-vitro embryo culture under 5 or 20 % oxygen

None of the embryos cultured in R1ECM developed further than 2-cell regardless of the oxygen level and 5 % oxygen did not overcome 2-cell block when used in combination with R1ECM. Thus we used modified R1ECM which enabled embryo development further than 2-cell. When mR1ECM was used, 26 out of 52 one-cell embryos became blastocyst

Table 5 mRNAs that changed at least 2.5 fold between in-vivo developed and in-vitro developed 2-cell embryos

mRNA (gene symbol)	FC	mRNA (gene symbol)	FC
Elevated mRNAs in in-vivo collected 2-cell embryos			
Hemoglobin alpha, adult chain 2 (Hba1)	8.3	Transportin 2 (Tnp2)	5.2
Hemoglobin, beta (Hbb)	3.8	Cathepsin L1 (Ctsl1)	3.6
ABI family, member 3 (Abi3)	3.3	DDB1 and CUL4 associated factor 11 (Dcaf11)	3.3
Zinc finger CCH domain-containing protein 4-like	3.2	Acyl-CoA thioesterase 8 (Acot8)	3.2
All-trans retinoic acid-induced differentiation factor (Atraid)	3.1	Fc gamma receptor II beta (LOC498276)	3.1
FAT tumor suppressor homolog 1 (Fat1)	3.1	Epithelial cell adhesion molecule (Epcam)	3.0
Cytochrome c-1 (Cyc1)	3.0	Transferrin (Tf)	3.0
Transient receptor potential cation channel, subfamily V, member 4 (Trpv4)	3.0	Coiled-coil-helix-coiled-coil-helix domain containing 10 (Chchd10)	2.8
Methyl cpG binding protein 2 (Mecp2)	2.9	Kruppel-like factor 5 (Klf5)	2.9
Caspase 2 (Casp2)	2.9	Cysteine dioxygenase, type I (Cdo1)	2.9
Kinesin light chain 4 (Kns18)	2.8	Complement component 1, s subcomponent (C1s)	2.9
Zinc finger CCH type (Zc3hav1)	2.8	Ribosomal protein S20 (Rps20)	2.8
SMEK homolog 1 (Smek1)	2.7	MAD2L1 binding protein (Mad2l1bp)	2.7
MAP kinase-interacting serine/threonine kinase 2 (Mknk2)	2.7	Vacuolar protein sorting 37 homolog A (Vps37a)	2.7
Cell adhesion molecule 1 (Cadm1)	2.6	ADAM metalloproteinase domain 17 (Adam17)	2.6
Fatty acid binding protein 3, muscle and heart (Fabp3)	2.6	Nucleoside phosphorylase (Np)	2.6
Stromal interaction molecule 1 (Stim1)	2.6	Cholinergic receptor, nicotinic, epsilon (Chrne)	2.6
RNA binding motif protein 25 (Rbm25)	2.6	LIM homeobox 6 (Lhx6)	2.5
Gastrulation brain homeobox 2 (Gbx2)	2.5	Filamin, alpha (Flna)	2.5
Nucleoporin 205 (Nup205)	2.5	C-terminal binding protein 1 (Ctbp1)	2.5
Similar to Acidic ribosomal phosphoprotein P0	2.5	Trafficking protein particle complex subunit 2-like	2.5
Elevated mRNAs in in-vitro developed 2-cell embryos			
Ring finger protein 219 (Rnf219)	4.8	Sulfatase modifying factor 2 (Sumf2)	4.3
Lin-28 homolog A (Lin28a)	3.3	Ribose 5-phosphate isomerase A (Rpia)	3.3
RAB13, member RAS oncogene family (Rab13)	3.2	Tafazzin (Taz)	3.3
Mitogen activated protein kinase kinase kinase 4 (Map3k4)	3.3	Myeloid/lymphoid or mixed-lineage leukemia translocated to, 11 (Mllt11)	3.2
Sulfotransferase family, cytosolic, 1C, member 2 (Sult1c2)	3.0	Coenzyme Q10 homolog A (Coq10a)	3.0
Ankyrin repeat domain 37 (Ankrd37)	2.9	Myotubularin related protein 4 (Mtmr4)	2.9
Growth inhibition and differentiation-related protein 88 homolog	2.9	Transmembrane protein 17 (Tmem17)	2.9
Glycogen synthase 2 (Gys2)	2.8	Similar to zinc finger protein 341 (LOC296300)	2.8
Similar to divalent cation tolerant protein CUTA	2.7	Family with sequence similarity 184, B (Fam184b)	2.8
Loc362793 (rgd1307315)	2.8	Breast carcinoma amplified sequence 3 (Bcas3)	2.7
Rcg35079-like, transcript variant 1 (LOC100364984)	2.7	Solute carrier family 20, member 1 (Slc20a1)	2.7
Solute carrier family 41, member 1 (Slc41a1)	2.7	Coiled-coil domain containing 90A (Ccdc90a)	2.7
High mobility group protein B4-like (RGD1561558)	2.6	CSRP2 binding protein (Csrp2bp)	2.6
Zinc finger, FYVE domain containing 26 (Zfyve26)	2.6	Phosphodiesterase 7A (Pde7a)	2.6
Ubiquitin specific peptidase 45 (Usp45)	2.5	Transmembrane protein 175 (Tmem175)	2.5
Dynein cytoplasmic 1 intermediate chain 1 (Dncic1)	2.5	RELT-like 1 (Rel11)	2.5
Nuclear receptor subfamily 1, group D, member 1 (Nr1d1)	2.5	Iroquois homeobox 3 (Irx3)	2.5
Putative uncharacterized protein ZNRD1-AS1-like	2.5	Membrane-spanning 4-domains, subfamily A,3 (Ms4a3)	2.5
Solute carrier organic anion transporter family, member 1a5 (Slco1a5)	2.5	Natriuretic peptide receptor B/guanylate cyclase B (Npr2)	2.5
Zinc finger protein 524 (Zfp524)	2.5	Transcription elongation regulator 1-like (Tcerg11)	2.5

FC fold change

Table 6 Real time PCR and microarray comparisons of mRNA levels

	Real time PCR				Microarray			
	Vv/Ar	Ar/Vv	Vv/Vt	Vt/Vv	Vv/Ar	Ar/Vv	Vv/Vt	Vt/Vv
Psmb2	12.6*	0.1	0.9	1.2	8.0*	0.1	1.1	0.9
Nde1	6.5*	0.2	1.1	0.9	7.3*	0.1	0.8	1.3
Tubal3	7.1*	0.1	0.9	1.1	5.4*	0.2	1.1	0.9
Orc5	10.8*	0.1	0.8	1.3	5.1*	0.2	1.0	1.0
Hif1a	2.2*	0.4	0.6	1.8	4.6*	0.2	1.4	0.7
Ccnb2	9.7*	0.1	1.3	0.8	4.1*	0.2	1.0	1.0
Pak1	3.8*	0.3	0.8	1.3	3.0*	0.3	0.9	1.1
Arhgef6	11.7*	0.1	1.1	0.9	2.8*	0.4	0.7	1.4
Arpc1b	26.6*	0.0	1.2	0.8	2.8*	0.4	0.5	2.0
Actb	2.8*	0.4	1.8	0.6	2.3*	0.4	0.9	1.1
Igf2bp1	0.0	24.8*	2.3	0.4	0.0	21.6*	0.8	1.2
Polr2e	0.0	47.5*	2.8	0.4	0.1	8.9*	0.9	1.1
Ppp1r15a	0.5	2.2*	0.7	1.4	0.4	2.3*	1.8	0.6
Prkaa1	0.9	1.1	1.2	0.8	0.5	2.0*	0.7	1.4
Hba-a2	32.9*	0.0	381.7*	0.0	5.6	0.2	8.3	0.1
Hbb	1164.8*	0.0	40.4*	0.0	3.5	0.3	3.8	0.3
Ptgis	44.3*	0.0	1.9	0.5	1.4	0.7	2.4	0.4
Taz	12.6*	0.1	0.9	1.1	1.6	0.6	0.3	3.3*
Sdhd	6.6*	0.2	1.3	0.8	1.7	0.6	0.4	2.2
Ndufc2	1.9	0.5	1.0	1.0	1.3	0.8	0.5	2.2

Bold numbers indicate the fold increase of mRNAs in the intended comparisons

Vv/Ar mRNA level comparison of in-vivo developed embryos to arrested 2-cell embryos; *Ar/Vv* mRNA level comparison of arrested embryos to in-vivo developed 2-cell embryos; *Vv/Vt* mRNA level comparison of in-vivo developed embryos to in-vitro 2-cell embryos; *Vt/Vv* mRNA level comparison of in-vitro embryos to in-vivo developed 2-cell embryos

*Indicates $P < 0.05$.

under atmospheric oxygen ($43 \% \pm 0.41$) and 5 % oxygen in combination with mR1ECM improved blastocyst rate and 108 out of 126 ($84 \% \pm 0.12$) embryos reached to blastocyst stage.

Transmission electron microscopy

In-vivo 2-cell embryos displayed a healthy cell structure with intact nuclear membrane, nucleoplasm containing nucleolus, euchromatin and heterochromatin (Fig. 3). Electron dense material (EDM) surrounded the nucleus and similar EDM surrounded the vesicle clusters in the cytoplasm. Cytoplasmic lattices (CPL) occupied the majority of the cytoplasm. Mitochondria appeared healthy and concentrated around the nucleus and at the side of the cell that contacted the sister blastomere. Furthermore, an RNA particle appeared to be coming out of the nucleus in one of the in-vivo developed embryos.

Arrested 2-cell embryos did not contain a nuclear membrane, heterochromatin or euchromatin (Fig. 3). Nucleoplasm

was distinguishable by the presence of nucleolus and absence of cytoplasmic organelles such as CPL. Mitochondria were smaller and fewer and CPLs were larger compared to in-vivo derived embryos. Furthermore, cells contained numerous autophagic vacuoles containing cytoplasmic organelles and apoptotic bodies.

Discussion

Gene expression profiles of in-vivo and 2-cell arrested embryos were highly different. This is likely due to the fact that the arrested 2-cell embryos aged 24 h more than in-vivo derived and in-vitro developed embryos. Substantial expression similarities between mRNA collected from in-vivo and in-vitro embryos were somewhat unexpected. This may be due to large number of transcripts in 2-cell embryos may be originating from oocyte as opposed to newly established embryo,

Fig. 3 Transmission electron microscopy images of in-vivo developed (**a, b, c**) and arrested (**d, e, f**) 2-cell embryos. In-vivo embryos appeared healthy and contained nuclear membrane, numerous mitochondria and cytoplasmic lattices (**a**). An electron dense martial surrounded the nuclear membrane and vesicles in the cytoplasm (**b**). Panel **c** shows a probable RNA exiting the nucleus. Arrested embryos did not contain a nuclear membrane (**d**). Mitochondria were smaller and CPL were larger

compared to in-vivo derived embryos (**d** and **e**). Large number of vacuoles containing degradation products (**f**) and apoptotic bodies were observed. *Ab* apoptotic bodies, *AC* adjacent cell, *IC* inter cellular space, *Ec* euchromatin, *EDM* electron dense material, *Hc* heterochromatin, *M* Mitochondria, *N* nucleus, *Nc* nucleolus, *NM* nuclear membrane, *CPL* cytoplasmic lattices, *Va* autophagic vacuole, *V* vesicle

where timing of maternal to embryonic transcript transition is during 2- to 4-cell stage in rats [16].

Genes encoding proteins that are involved in translation elongation as well as mRNAs encoding ribosomal proteins were highly elevated in arrested 2-cell embryos. Since the arrested 2-cell embryos stop cell cycle, high level of mRNAs encoding proteins involved in translation elongation may indicate RNA degradation. Overrepresentation of mRNAs encoding proteins in translation elongation has been observed when the gene expression of degraded RNA was compared to intact RNA [28]. Similarly, translation initiation, translation elongation, RNA-protein complex assembly, structural constituent of ribosome, and RNA binding were significantly enriched in formalin fixed paraffin embedded samples which yield lower quality RNA compared to fresh-frozen samples [29]. In addition, mRNAs encoding eukaryotic translation initiation factor 2 alpha kinase 1 (*Eif2ak1*) and *Eif2ak4* were up-regulated in in-vivo embryos compared to arrested embryos. The enzymes encoded by these mRNAs phosphorylate eukaryotic translation initiation factor 2 (*eif2*) which leads to reduction in global translation. upregulation of these mRNAs indicates decreased translation in in-vivo 2-cell embryos.

In-vivo and in-vitro 2-cell embryos highly expressed mRNAs involved in cell cycle and protein catabolic process. This was expected since cell cycle and protein degradation through ubiquitin-proteasome pathways work coordinately to

enable rapid production and degradation of proteins that lead to progression of cell cycle [30]. Similar biological processes were also up-regulated in mouse embryos where mRNAs encoding proteins involved in cell cycle and protein catabolism increased in 2-cell mouse embryos [31]. Contrary to our results, Zeng et al. [31] reported significant increase in mRNAs involved RNA processing in 2-cell mouse embryos. This discrepancy is likely due to comparison of gene expression in healthy 2-cell embryos to 1- and 8-cell embryos and blastocysts in that particular study. Our results also showed that ATPase H transportin lysosomal V1 subunit B2 (*Atp6v1b2*) and *Atp6v1f* were elevated 22 and seven fold in in-vivo embryos. The proteins encoded from these mRNAs were involved in acidification of intracellular organelles which is necessary for protein sorting, zymogen activation, receptor-mediated endocytosis, and synaptic vesicle proton gradient generation. Furthermore, our results indicate activation of nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF- κ B) in arrested embryos. NF- κ B is involved in cellular response to stimuli such as stress. The inhibitors of NF- κ B bind to NF- κ B and prevent it from entering the nucleus and activating genes in the nucleus. Arrested embryos had reduced levels of mRNAs encoding inhibitors of NF- κ B (*Nfkbie*, *Nfkbib*, *Nfkbia*). In contrast, mRNA encoding NFKB activating protein was elevated in arrested embryos.

Transmission electron microscopy comparison of in-vivo embryos and arrested embryos showed that ultrastructure of these embryos were very different from one another. In-vivo embryos contained an intact nuclear membrane, healthy looking mitochondria, and numerous cytoplasmic lattices. Cytoplasmic lattices have been shown to store maternally derived mRNA and ribosomes in mammalian oocytes and embryos [32, 33]. Mitochondria and energy production are important in progression of cell division [34]. Furthermore, we were able to locate a probable RNA molecule that was exiting the nucleus. This suggests beginning of the transition from maternal to zygotic transcription. Furthermore, nuclear membrane of in-vivo embryos was surrounded by an electron dense material and this material also surrounded the vesicles in the cytoplasm. Appearance of vesicles and EDM suggest that EDM may be responsible for trafficking in the cell. Nuclear membrane in arrested embryos was completely disintegrated and cytoplasm contained vacuoles and apoptotic bodies. Ultrastructure analysis indicates cessation of transcription in 2-cell arrested embryos. Furthermore, smaller mitochondria in arrested embryos indicate oxidative stress and reduced energy supply.

Small number of genes were different between in-vivo and in-vitro 2-cell embryos and we hypothesized that these genes may be involved in initial reactions that take place before 2-cell arrest of rat embryos. Real-time PCR analysis confirmed the up-regulated mRNAs in in-vivo derived embryos compared to in-vitro embryos. Interestingly, real time PCR comparison of mRNAs that were elevated in in-vitro embryos compared to in-vivo embryos did not show any change for the mRNAs tested. This may be due to inefficient normalization of microarray data. Since we were not able to confirm the microarray results with real-time PCR for the mRNAs that were higher in in-vitro embryos compared to in-vivo embryos, we did not further analyze these genes.

Genes encoding hemoglobins (Hba-a2 and Hbb) were highly up-regulated in in-vivo embryos compared to in-vitro embryos. Furthermore, real time PCR showed much greater and significant increase in levels of the hemoglobin mRNAs in in-vivo embryos. Increase in hemoglobin mRNAs in in-vivo embryos suggest that oxygen level in culture conditions may affect genes that are sensitive to oxygen levels. It has been shown that hemoglobin may be involved in oxygen or nitric oxide sensing [35] and hemoglobin mRNA and protein were up-regulated during hypoxia [36]. Thus, lower levels hemoglobin mRNAs in cultured embryos in our study suggest that decrease in hemoglobin mRNAs may be the initial response against atmospheric oxygen levels in in-vitro culture. In addition, arrested 2-cell embryos had lower levels of hypoxia inducible factor 1a and 1b (Hif1a and Hif1b) compared to in-vivo embryos. Similar to hemoglobins, HIF1a involved in hypoxia response and mediate aerobic respiration, energy metabolism and glycolysis, as well as antioxidant defense [37].

Low oxygen levels mimic the environment that the early embryos develop in-vivo. It has been shown that uterine and oviduct surface oxygen concentrations ranged between 2 and 9 % [38]. In-vitro culture of embryos under low oxygen (5 %) as opposed to atmospheric oxygen (20 %) resulted in higher blastocyst formation rates in rats [39, 40], mice [41, 42], sheep and cattle [43]. Similarly, culture of embryonic or adult stem cells under low oxygen improved their growth and maintained their pluripotency [44]. We also obtained two fold greater blastocyst formation under 5 % oxygen compared to 20 % atmospheric oxygen when mR1ECM was used.

Our experimental design allowed us to compare in-vivo developed embryos to 24 h cultured embryos. In addition, we compared the gene expression in arrested 2-cell embryos which were cultured an additional 24 h. Rat embryos reach 2-cell stage at about 48 h in-vivo and become 4-cell at about 72 h after hCG injection and mating. Thus, in-vivo embryos at 72 h post hCG which would be at 4-cell stage would not be relevant with this study.

In conclusion, gene expression profile of 2-cell arrested embryos were significantly different from the in-vitro developed and in-vivo developed 2-cell embryos and 2-cell arrested embryos had low levels of mRNAs that are required for cell division. Although arrested embryos and in-vivo embryos appeared morphologically similar under light microscope, electron microscopy showed substantial ultra-structural differences and apoptotic changes in arrested embryos. Embryo culture using mR1ECM improved in-vitro blastocyst formation and low oxygen had additive effect on blastocyst formation when combined with mR1ECM. This suggests that media composition, replenishment of nutrients and removal of toxic compounds such as ROS by using mR1ECM is important in elimination of 2-cell block and low oxygen levels further improve the transition from 2-cell stage to blastocyst development. In this study we were able to link the large gene expression data to physiological conditions and showed the relevance of gene expression to physiological problems in 2-cell rat embryo culture and subcellular structures of 2-cell embryos.

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