Effect of *in vitro* fertilization on gene expression and development of mouse preimplantation embryos

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Abstract

In vitro culture (IVC) of preimplantation mouse embryos is associated with changes in gene expression. It is however, not known if the method of fertilization affects the global pattern of gene expression. We compared gene expression and development of mouse blastocysts produced by *in vitro* fertilization (IVF) versus blastocysts fertilized *in vivo* and cultured *in vitro* from the zygote stage (IVC) versus control blastocysts flushed out of the uterus on post coital day 3.5. The global pattern of gene expression was assessed using the Affymetrix 430 2.0 chip. It appears that each method of fertilization has a unique pattern of gene expression and development. Embryos cultured *in vitro* had a reduction in the number of trophoblastic cells (IVF 33.5 cells, IVC 39.9 cells, and 49.6 cells in the *in vivo* group) and, to a lesser degree, of inner cell mass cells (12.8, 11.7, and 13.8 respectively). The inner cell mass nuclei were larger after culture *in vitro* (140 μm^2 , 113 μm^2 , and 86 μm^2 respectively). Although a high number of genes (1912) was statistically different in the IVF cohort when compared with the *in vivo* control embryos, the magnitude of the changes in gene expression were low and only a minority of genes (29 genes) was changed more than fourfold. Surprisingly, IVF embryos were different from IVC embryos (3058 genes were statistically different, but only three changed more than fourfold). Proliferation, apoptosis, and morphogenetic pathways are the most common pathways altered after IVC. Overall, IVF and embryo culture have a profound effect on gene expression pattern and phenotype of mouse preimplantation embryos.

Reproduction (2007) 134 63–72

Introduction

It is estimated that 1% of children in the western world are born with the help of assisted reproductive techniques, *in vitro* fertilization (IVF), and intracytoplasmic sperm injection (ICSI). While these techniques are thought to be safe (Olivennes *et al.* 2002), new evidence suggests an increased risk of complications associated with their use, such as cerebral palsy and delayed development (Stromberg *et al.* 2002), low birth weight, increased birth defects (Anthony *et al.* 2002, Hansen *et al.* 2002, Schieve *et al.* 2002), and epigenetic disorders such as Angelman and Beckwith–Wiedemann Syndromes (Cox *et al.* 2002, DeBaun *et al.* 2003).

While it is possible that infertility, *per se*, could increase these risks (Kovalevsky *et al.* 2003), studies in animal models have demonstrated that *in vitro* culture (IVC) is suboptimal and represents an additional stress to gametes and embryos. IVC of preimplantation embryos alters their global gene expression patterns (Rinaudo &

Schultz 2004, Rinaudo *et al.* 2006) and affects the behavior of mice born after such procedures (Ecker *et al.* 2004, Fernandez-Gonzalez *et al.* 2004). Unfortunately, these studies have only examined the effect of IVC on outcome and have not explored the effect of gamete manipulation by IVF or ICSI.

In order to investigate the effect that IVF has on development and expand upon our prior IVC studies (Rinaudo & Schultz 2004, Rinaudo *et al.* 2006) we carried out additional experiments utilizing IVF. Specifically, morphologic and gene expression studies were performed on blastocysts fertilized *in vivo* but grown *in vitro* (IVC group) and those fertilized and grown *in vitro* (IVF group) when compared with *in vivo* controls (*in vivo* group). Therefore, any comparisons between the IVC and IVF groups would tease out the effects of gamete manipulation. We refer readers to our past publication for analysis of differences between IVC and *in vivo* embryos (Rinaudo & Schultz 2004, Rinaudo *et al.* 2006).

Materials and Methods

Collection of mature oocytes and preimplantation mouse embryos

Oocytes and embryos were isolated from super-ovulated dams as previously described (Rinaudo & Schultz 2004). Briefly, CF-1 female mice were injected with 5 IU pregnant mares serum gonadotrophin and 42–46 h later with 5 IU human chorionic gonadotropin (hCG); some of the females were then bred to B6D2F1/J males overnight. The following morning, oocytes and zygotes were obtained from the ampullae. The zygotes were cultured in Whitten Medium (WM) to the blastocyst stage under 5% CO₂ in humidified air at 37 °C. The oocytes were incubated in WM containing 15 mg/ml BSA and sperm obtained from the cauda epididymis of male B6D2F1/J mice for 4 h. The fertilized oocytes were washed and cultured in WM to the blastocyst stage under 5% CO₂ in humidified air at 37 °C. Only late-cavitating blastocysts of similar morphology were selected. To collect blastocysts of identical morphology, the collection had to be performed at different times: the IVF and IVC groups were harvested at ~106–112 h post-hCG, the control *in vivo* group 96–100 h post-hCG. Each treatment was repeated four times. Each time, for the IVF group, sperm from one male was used to fertilize oocytes from two to three females. For the IVC and *in vivo* groups, eggs were collected from two to three females after overnight breeding (one male per female). All animal experiments were approved by the Institutional Animal Care and Use Committee.

Differential embryo staining

A modified dual nuclear staining method described by Thouas *et al.* (2001) was performed to differentially stain inner cell mass (ICM) and trophoblast cell nuclei of fully developed blastocysts. Briefly, the blastocysts were exposed to 1% Triton X100 (Sigma–Aldrich Co.) in minimum essential medium (MEM; Invitrogen Corporation) for 3–5 s, washed three to five times in MEM + polyvinylpyrrolidone (PVP; Sigma–Aldrich Co.) and incubated in MEM + PVP containing 100 µg/ml propidium iodide (Sigma–Aldrich) for 30 s. The embryos were then washed three to five times in MEM + PVP and fixed overnight in 100% ethanol containing 25 µg/ml of bizbenzamide (Sigma–Aldrich Co.). The embryos were mounted on a clean glass slide using glycerol and kept in a dark chamber until observed under fluorescence light using a standard Leica microscope (Model DMRB, Leica Microsystems AG, Wetzlar, Germany).

The embryos were observed under fluorescence microscopy and the numbers of ICM (blue) and trophoblast (TB, red) nuclei were counted and photographed. The staining procedure was repeated at least five times per treatment group with different sets of blastocysts ($n \geq 45$). The area of all the ICM cell nuclei in each

blastocyst in the whole mount preparation was calculated measuring two perpendicular diameters using image processing software (Adobe Photoshop). All the ICM cell nuclei of at least 20 representative blastocysts per treatment group were measured. Margins of overlapping nuclei were carefully drawn using the color contrast of the overlapping and non-overlapping area.

RNA extraction and amplification

For every replicate total RNA was extracted from pools of 10 embryos using PicoPure RNA Isolation Kit (Arcturus, Sunnyvale, CA, USA) according to the manufacturer's instructions. A total of four replicates were performed for each experimental group. In each pool, five embryo equivalents of total RNA was used for RT followed by linear amplification of antisense cDNA strand, fragmentation, and biotin labeling by using NuGEN Ovation Biotin System (NuGEN Technologies Inc., San Carlos, CA, USA). Briefly, RNA is converted to cDNA with a unique DNA/RNA heteroduplex at one end. A linear isothermal DNA amplification process was conducted using DNA/RNA chimeric primer, DNA polymerase and RNase H in a homogeneous isothermal assay that provides highly efficient amplification of DNA sequences. The amplified cDNA strands were subjected to an enzymatic fragmentation and the fragmented product was then labeled with biotin. RNA and cDNA mass and size distribution were determined before and after amplification, and after fragmentation using the Agilent Bioanalyzer (Palo Alto, CA, USA). The cDNA yield before fragmentation and labeling was 12–15 µg. Final yield of fragmented and biotinylated cDNA was 4–4.5 µg, of which 2.2 µg were used for microarray analysis.

Microarray preparation

Fragmented and biotin labeled cDNA samples were submitted to the Genomic Core Facility of University of California San Francisco for GeneChip hybridization. The samples were hybridized to mouse Affymetrix 430 2.0 GeneChips, then washed and stained on fluidics stations and scanned at 3 µm resolution according to the manufacturer's instructions (GeneChip Analysis Technical Manual, www.affymetrix.com). Four replicates per each experimental group were performed, for a total of 12 independent experiments.

cDNA preparation for real-time PCR analysis

RT was accomplished by utilizing the commercially available first strand cDNA synthesis kit (iScript cDNA Synthesis Kit, Bio-Rad Laboratories). The RT reactions were performed by following the kit manufacturer's

protocol. For each treatment group the RT was repeated four times with different sets of blastocyst stage embryos.

The real-time PCR was performed using TaqMan Universal PCR Master Mix, Gene Expression Assays containing gene specific primers and TaqMan probe (Applied Biosystems, Foster City, CA, USA), and 0.1 embryo equivalents of cDNA. The corresponding ABI TaqMan Assay-on-Demand probe/primer sets used were Mm00498012_m1 (Ube2a), Mm00438084_m1 (Ccng1), Mm00599980_m1 (Dpf1), and Mm99999915_g1 (Gapdh). Duplicates were used for each real-time PCR; a minus template served as control. For each treatment group the real-time PCR was repeated four times. The real-time PCR data were analyzed within the log-linear phase of the amplification curve obtained for each probe/primer using the comparative threshold cycle method (Bio-Rad Laboratories).

Data analysis

The percent of embryos that developed to the two-cell stage and to the blastocyst stage and embryo cell number data were analyzed by χ^2 and One-way ANOVA, respectively. Cell numbers were compared using a mean separation procedure when ANOVA showed significant *F* values using Fisher's Least Significant Difference method. Results are reported as the mean values for each set of data $\pm$ S.E.M.

Microarray gene expression data analysis

The Gene Chip arrays were first processed using GCOS Software (Affymetrix Inc., Santa Clara, CA, USA). Then input into GeneSpring version 7.2 software (Agilent Technologies, Santa Clara, CA, USA) for data analysis. The robust multiarray analysis algorithm (GeneSpring; Agilent Technologies) was applied to the data for background adjustment, normalization, and log₂-transformation of perfect match values (Irizarry *et al.* 2003). Subsequently, the data were subjected to per chip and per gene normalization using GeneSpring normalization algorithms. The resulting generated gene lists of differentially expressed genes included genes with a *P* value of ≤ 0.05 by a one-way ANOVA parametric test and a Benjamini-Hochberg multiple testing correction for false discovery rate.

Principal component analysis (PCA)

PCA is an unsupervised clustering method and visualization tool that is used to analyze large data sets derived from gene expression arrays analyses (Jolliffe & Morgan 1992). It reduces the variability in the data set into three dimensions representing the predominant variations in the data. Each dimension represents a component to which a certain percentage of variance in the data is

attributed. The PCA algorithm was applied to all samples using GeneSpring software.

Gene ontologies (GO) classification

Differentially expressed genes among different embryo culture conditions, identified by microarray gene expression analysis, were classified into known GO using GO Tree software (<http://genereg.ornl.gov/gotm/>; Zhang *et al.* 2005). Gene ontology tree machine (GOTM) was used to interpret biological, molecular, and cellular functions of genes identified in response to different embryo culture conditions. GOTM uses GO hierarchies to derive significant biological processes, molecular functions, and cellular components in a gene list. It implements a statistical analysis of the GO categories for the input gene list and suggests biological areas of potential value for further investigation (Ashburner *et al.* 2000). First, the differentially expressed genes are classified by their corresponding GO categories, and the observed number of genes in each of these GO categories is recorded. Genes represented on the Affymetrix 430 2.0 chip comprise the reference gene list. The expected number of genes in each GO category corresponds to the number of genes falling into that GO category in the reference gene list. A given GO category is considered enriched when the observed number of genes in that category is greater than the expected number. The GO classifications for different conditions were derived, taking into account only non-redundant and specific GO classes for each condition.

KEGG pathways

Differentially expressed genes in IVF versus control were classified in pathways. Hypergeometric testing was applied to derive the enriched genes in each pathway with a *P* < 0.05 (Zhang *et al.* 2005). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were derived for each culture condition as well as each K-means cluster. Only selected pathways and processes are highlighted in the results section.

Results

Effect of IVF and embryo culture on embryo development and cell number

Expanded mouse blastocysts are easily recognizable, and therefore offer an excellent endpoint for morphologic assessment. To assess developmental competence we observed how many embryos developed from the zygote stage to two-cell stage and from the two-cell stage to the blastocyst stage (Table 1). A higher number of IVF embryos developed to the blastocyst stage (*P* < 0.01) when compared with the IVC group.

Table 1 Effect of fertilization method on preimplantation development.

Treatment	Number of zygotes	Number of two-cell embryos	Number of blastocysts	Zygote to two-cell (%)	Two-cell to blastocyst (%)	Zygote to blastocyst (%)
IVF	215	186	167	86.5	89.8*	77.7*
IVC	553	458	340	82.8	74.2 [†]	61.4 [†]

*[†]Values with different superscripts in each column differ significantly ($P < 0.01$).

In order to investigate the effect of the method of fertilization and IVC on cell division and cell allocation, we counted the number of ICM and TE cell in expanded blastocysts. It is important to notice that only late cavitating blastocysts with similar morphology as observed with a stereomicroscope were used for analysis. *In vivo* embryos had on average one additional ICM cell when compared with IVF embryos ($P = 0.053$, non significant) and two additional cells when compared with IVC embryos ($P < 0.01$). In addition, the area of the ICM nuclei in the IVF group was larger than the area of the ICM nuclei in the IVC group and the *in vivo* group ($P < 0.01$). The IVF embryos had reduced number of TE cells when compared with the IVC embryos and to the *in vivo* control embryos ($P < 0.01$; Table 2).

Effect of IVF and embryo culture on global gene expression

To analyze the global pattern of gene expression we used the new mouse Affymetrix 430 2.0 microarray chip, which is believed to represent the complete mouse genome. Out of 45 101 probe sets, by using a cut-off value of 50 (Baum *et al.* 2003) in the raw expression level, 11 037 (24.4%), 10 570 (23.4%), and 11 076 (24.5%) transcripts were considered detected in IVF, IVC, and *in vivo* blastocysts respectively. These results are consistent with data reported in the literature (Zeng & Schultz 2005).

Published evidence confirms that microarray data are robust and reliable (Zeng & Schultz 2005). Nevertheless, we conducted an additional analysis of a number of genes using real-time RT PCR to confirm the microarray results obtained in this study (Fig. 1). Results of this analysis indicated that the three genes tested (Ube2a, Ccgn1, and Tlk1) confirm the microarray changes. We then performed pairwise comparison to observe how many genes are statistically different between the three

groups of embryos as demonstrated in Fig. 2. A significant number of genes are statistically misregulated following IVF (1912 transcripts) or IVC (4660 transcripts) when compared with *in vivo* control embryos. As expected, the magnitude of change in gene expression for most of the genes was small (Rinaudo & Schultz 2004), as only 18% (357/1912) and 12% (555/4660) of the mis-expressed genes showed more than twofold change in IVF versus *in vivo* and IVC versus *in vivo* embryos respectively. In addition, only 27 genes from the IVF group and 35 from the IVC group genes showed more than a fourfold change. Unexpectedly, the comparison of IVC versus *in vivo* embryos showed a nearly double number of mis-expressed genes when compared with the IVF versus *in vivo* group. The direct statistical comparison of IVF versus IVC revealed that 3058 genes were differently expressed. The list of significantly different genes after each pairwise comparison with the relative expression profiles is summarized in supplemental Tables 1 (IVF versus *in vivo*), 2 (IVF versus IVC) and 3 (IVC versus *in vivo*) (which can be viewed online at www.reproduction-online.org/supplemental/).

Hierarchical clustering analysis and PCA

Hierarchical clustering analysis and PCA of the expression profiles offer the opportunity to assess how similar the different treatments are and to obtain a visual understanding of the relationship between the different experimental components (Fig. 3A and B). The results of these analyses underscore that *in vivo* embryos, IVC and IVF embryos have a very unique and distinctive transcriptome. Hierarchical clustering of samples using differentially expressed gene set resulted in a striking segregation of samples into two major clustering branches (Fig. 3A). All the replicate samples of *in vivo* control group self-cluster into one branch. Both IVF and IVC samples self-cluster into another branch that further

Table 2 Effect of fertilization method on blastocysts cell number and on the nuclear area of the ICM cells.

Treatment	# ICM cells (mean $\pm$ S.E.M.)	Area of ICM nuclei (μm^2) (mean $\pm$ S.E.M.) ^a	# TE cells (mean $\pm$ S.E.M.)	# Total cells (mean $\pm$ S.E.M.)
<i>In vivo</i> ($n = 46$)	13.80 $\pm$ 0.50*	86.01 $\pm$ 1.48 [†]	49.67 $\pm$ 1.50*	63.48 $\pm$ 1.70*
IVC ($n = 70$)	11.77 $\pm$ 0.40 [†]	113.65 $\pm$ 2.16 [†]	39.99 $\pm$ 1.22 [†]	51.76 $\pm$ 1.38 [†]
IVF ($n = 63$)	12.84 $\pm$ 0.42* [†]	140.20 $\pm$ 5.89*	33.51 $\pm$ 1.28 [†]	46.35 $\pm$ 1.45 [†]

*[†],[‡]Values with different superscripts in each column differ significantly ($P < 0.01$). At least five replicates per treatment group were performed.

^aArea of the ICM nuclei is the average of all the ICM cell nuclei area (range 279–313 nuclei measured) from at least 20 representative embryos per group. n , number of embryos examined.

Figure 1 Real-time PCR verification of gene expression data. Expression profile of cyclin G1 (*Ccng1*), ubiquitin-conjugating enzyme E2A (*Ube2a*) and tousled-like kinase 2 (*Tlk2*) genes in murine blastocysts produced by *in vivo* fertilization and culture (*in vivo*), *in vitro* fertilization and *in vitro* culture (IVC), and *in vitro* fertilization and culture (IVF) using both (A) microarray and (B) real-time PCR techniques.

divides into two major sub-branches and into which all the replicates from IVF and IVC self-cluster.

PCA distributes samples into a two dimensional space based on the variance in gene expressions, and samples that have similar trends in their gene expression profiles will cluster together in the PCA plot. This analysis, using the whole probe set on the chip, resulted in a clear segregation of the 12 replicated samples into three clusters, corresponding to IVF, IVC, and *in vivo* (Fig. 3B).

Analysis of classes of genes and gene pathways altered in the experimental groups IVF versus *in vivo* control

Results of gene ontology in IVF embryos when compared with control embryos are summarized in Table 3. IVF embryos show an up-regulation of cell cycle, apoptosis, RNA metabolism and phosphoenolpyruvate-dependent sugar phosphotransferase system gene categories. Embryonic development genes and morphogenesis genes were down-regulated and this can explain a reduced

Figure 2 Number of transcripts differentially regulated according to the fertilization method. The number of genes either down- or up-regulated is the number that is statistically different after direct comparison of the described experimental group. The exact number of genes is specified in the inferior portion of the graph.

performance of the IVF embryos. Genes involved in heart development, forebrain development, bone mineralization, and segmentation are among the family of morphogenetic genes mis-expressed. Additional classes of down-regulated genes consist of amino acid transport and metabolism genes, fat cell differentiation, fluid transport, and genes involved in intracellular signaling cascade. Of particular interest is the down-regulation of pathways important in early development, such as the cell cycle, apoptosis, and Wnt signaling pathways.

IVF versus IVC

This comparison allows us to observe and isolate the effect of the conditions at fertilization (*in vitro* versus *in vivo*), since, the culture conditions from the zygote stage to the blastocyst stage were exactly the same in the two groups.

Surprisingly, IVF embryos developed few hours faster from the two-cell stage to the blastocyst stage than IVC embryos. On the other hand, IVF embryos had a reduced number of TB cells ($P < 0.01$) and an equal number of ICM cells. The average area of the ICM cell nucleus was larger in the IVF group.

Gene expression was remarkably different: more than 3000 genes were statistically different between the two groups (3058 transcripts, Fig. 2). Eighty-eight percent (2696/3058) of the IVF transcripts were down-regulated; however, the intensity of change was small and only 98 genes (0.03%, 98/3058) were modified more than twofold.

Analysis of common processes and gene pathways revealed that overall IVF embryos displayed up-regulation of protein biosynthesis and protein amino acid

Figure 3 Hierarchical clustering and PCA analysis. (A) Hierarchical clustering analysis of all samples from different developmental stages or treatments. Unsupervised clustering in GeneSpring was used to analyze similarities among replicate samples across all treatment groups tested. Colors correspond to relative RNA abundance for the transcripts detected; each is represented by a horizontal bar in the heat-map. Red indicates up-regulation, blue denotes down-regulation and yellow no change in expression. Note the appropriate clustering of data from the four samples derived from IVF-generated embryos (brown lines/bars), the four samples from IVC embryos (green lines) and the four samples derived from embryos that developed *in vivo* (black lines/bars). *In vivo*, IVF and IVC refer to blastocysts developed *in vivo*, cultured *in vitro* after fertilization *in vitro* and cultured *in vitro* after fertilization *in vivo*, respectively. (B) Principal component analysis of gene expression of all groups as in the hierarchical clustering analysis.

glycosylation genes and down-regulation of steroid and lipid metabolism and transport genes (Table 4). It is particularly interesting to note that heart development genes and genes involved in axon guidance are down-regulated in the IVF group.

Discussion

This study establishes that IVF has a discrete and distinctive effect on the phenotype and gene expression pattern of mouse preimplantation embryos. The differences between IVF and *in vivo* embryos are quite remarkable. The most unanticipated findings are the net reduction of TE cells and increase in sizes of ICM cell nuclei in the IVF group when compared with the *in vivo* control. The gene expression results paralleled the morphologic results and showed that genes involved in development, cell division, and apoptosis were mis-regulated following IVF. Interestingly, although we expected the IVF and IVC groups to be similar, we found remarkable differences between these two groups both in gene expression and morphology.

The increase in size of the nucleus in the IVF embryos was surprising and unexpected and its significance is unclear. Because we only stained the nucleus and did not visualize the cytoplasm, we cannot infer that the nucleocytoplasmic ratio is altered. Only ultrastructural examination will help clarify the issue. Other authors have observed changes in the morphology of preimplantation embryos after culture. Crosier *et al.* (2001) analyzed the ultrastructural morphometry of bovine blastocysts produced *in vivo* or *in vitro* using three different culture media and noted that *in vitro* blastocysts had a smaller nucleocytoplasmic ratio. They associated this finding with reduced quality of *in vitro* embryos. However, Petzoldt & Muggleton-Harris (1987) manipulated the nucleocytoplasmic ratio of mouse preimplantation embryos by removing or injecting cytoplasm and concluded that gene stage specific gene activity was independent of this ratio.

The different morphology in cultured embryos suggest that a number of fundamental processes are altered by the culture condition; i.e., cell proliferation, apoptosis, and/or allocation of cells to the ICM or TE lineages are different depending upon whether fertilization occurs *in vivo* or *in vitro*. Together, these findings suggest that blastocysts produced by IVF may be at a significant developmental disadvantage when compared with those that develop *in vivo*. In agreement with our findings, Trounson & Bongso (1996) reported that human IVF embryos often show abnormal or delayed cell division. Hence, we postulated that the observed embryo development and number of cells present in the ICM and TE layers might significantly be associated with differences in gene expression in IVF embryos.

Indeed, the gene expression results confirm the morphologic studies. The IVF embryos have a very

Table 3 Gene ontology of family of genes statistically different between *in vitro* fertilization (IVF) and *in vivo* generated control blastocysts.

Gene ontology: up-regulated in IVF when compared with <i>in vivo</i>	
1) Regulation of cell cycle:	Elk3 (1.77), Cops5 (2.23), Dhx16 (2.91), S100a6 (3.39), Ccng1 (5.64)
Negative regulation	Hrasls3 (1.60), Grlf1 (1.64), Hipk2 (1.66), Dst (1.77), Pycard (2.21), Ak1 (2.24), Rb1 (2.54), Cdkn1a (3.11)
2) Apoptosis:	Bak1 (1.63), Ercc5 (1.88), Bcl2l2 (1.99), Phlpp (2.74), Cdkn1a (3.11), Tnfrsf10b (3.19), Eda2r (5.29)
Negative regulation	Birc3 (1.75), Faim (2.05), Snai2 (3.49)
Positive regulation	Hipk2 (1.66), Bcl2l1 (1.69), Itm2b (1.82), Dapk1 (1.84), Pycard (2.21), Trp53inp1 (2.51), Ngfrap1 (2.70), Trim35 (3.74)
3) RNA metabolism:	Mphosph10 (1.60), Hnrpa2b1 (1.71), Scnm1 (1.81), Tarsl2 (3.39)
mRNA processing	Casc3 (1.58), 6530403A03Rik (1.62), Sfrs8 (1.65), Ptbp2 (1.66), 2600011C06Rik (1.80), Pabpn1 (1.81), 2610031L17Rik (1.90), Rnasel (2.25), Zcrb1 (2.78), Dhx16 (2.91)
4) PEP-dependent sugar phospho-transferase system:	Reep5 (1.92), Acacb (2.32)
Gene ontology: down-regulated in IVF when compared with <i>in vivo</i>	
1) Intracellular signaling cascade:	Socs3 (0.05), Arhgef2 (0.21), Blnk (0.21), Sh3bp5 (0.33), Prkch (0.40), Zfp36 (0.48), Arl6 (0.49), Rasa1 (0.49), Tdgf1 (0.49), Trim23 (0.49), Spred2 (0.53), Ndst1 (0.54), Jak1 (0.55), Ptk6 (0.55), Socs6 (0.56), Ppm11 (0.58), Als2 (0.59), Ywhag (0.59), Gna13 (0.60), Rhpn2 (0.60), Rsu1 (0.60), Itm2b (0.61), Pik3c2a (0.61), Sos2 (0.62), Snx9 (0.63), Yes1 (0.64), Pkd2 (0.65), Unc13b (0.65), Dvl2 (0.66), Rhob (0.66)
2) Fat cell differentiation:	Ccnd1 (0.42), Cebpa (0.43), Runx1t1 (0.45), Pparbp (0.63)
3) Apoptosis:	Apaf1 (0.58), Hip1 (0.62), Dffa (0.64), Dffb (0.64)
4) Amino acid and derivative metabolism:	Socs3 (0.05), Slc7a7 (0.39), Khsp4 (0.42), Bmp4 (0.44), Shmt1 (0.46), Sms (0.51), Aldh18a1 (0.54), Ndst1 (0.54), Hibadh (0.57), Pdgrb (0.58), P4ha1 (0.59), Ppap2a (0.62), Pparbp (0.63), Wars (0.66)
5) Glutathione biosynthesis:	Gclm (0.49), Gclc (0.63)
6) PI metabolism:	Pip5k1b (0.52), Pip5k1a (0.63)
7) Organic acid transport:	Slc7a3 (0.23), Slc7a7 (0.39), Slc7a1 (0.46), Slc1a4 (0.51), Slc6a14 (0.51), Slc12a2 (0.56), Slc6a6 (0.62)
8) Water transport:	Aqp3 (0.43), Aqp8 (0.57), Aqp9 (0.65)
9) Embryonic development:	Fgf4 (0.33), Cebpa (0.43), Ube2a (0.47), Eomes (0.48), Pitpnb (0.62)
Heart	Adam19 (0.23), Gata4 (0.43), Bmp4 (0.44), Srf (0.47), Pparbp (0.63), Pofut1 (0.64), Dvl2 (0.66), Pten (0.66)
Forebrain	Bmp4 (0.44), E2f1 (0.52), Ndst1 (0.54), Apaf1 (0.58), Mx1 (0.64)
Bone mineralization	Bmp4 (0.44), Spp1 (0.47), Fgfr2 (0.49)
Segmentation	Tdgf1 (0.49), Axin2 (0.52), Mlt3 (0.61), Pofut1 (0.64), Dvl2 (0.66)

distinct fingerprint when compared with embryos fertilized *in vivo*, as shown by the PCA and the unsupervised hierarchical analysis (Fig. 3). Overall, the IVF embryos have an increased alteration (both up and down-regulation) in the expression of apoptotic genes. This could explain the reduced number of TE cells. The down-regulation of several genes involved in differentiation (fat cell differentiation, heart and forebrain development, bone mineralization and segmentation) are provocative and open the possibility that important alterations of fundamental process are taking place. In fact, knock out animals for several of these genes have very specific anomalous phenotypes. For example, *Srf* (Arsenian *et al.* 1998, Li *et al.* 2005a, 2005b, Niu *et al.* 2005) and *Eomes* (Russ *et al.* 2000) knock out mice

exhibit abnormal gastrulation and *Ube2a* mutation has been shown to reduce the embryonic growth (Roest *et al.* 2004). In addition, mutation in the *Eomes* gene, is shown to cause abnormal trophoblast development (Russ *et al.* 2000), a particular important findings in our study, given the reduced number of TE cells in the IVF blastocysts. In addition, *Gata4* is able to induce visceral trophoblast from ES cells (Fujikura *et al.* 2002).

Whether differences in gene expression will persist throughout embryonic development is not known. However, a group of genes involved in cardiac development (*Gata4* (Kuo *et al.* 1997, Tevosian *et al.* 2002), *Adam19* (Kurohara *et al.* 2004), *Fgf4*, and *Fgfr2* (Feldman *et al.* 1995, Wang *et al.* 2005), and *Bmp4* (Liu *et al.* 2004, Zakin & De Robertis 2004) are down-

Table 4 Gene ontology of family of genes statistically different between *in vitro* fertilization (IVF) and *in vitro* culture (IVC) generated blastocysts.

Gene ontology up-regulated genes in IVF when compared with IVC	
1) Protein targeting: $P=0.0063$	Dmgdh (1.72), Ormdl1 (1.72), 1700034H14Rik (1.64), Al317395 (1.85), Kpna4 (1.92)
2) Biosynthesis: $P=0.0079$	Nos1 (1.59), Sc4mol (1.61), Cds2 (1.72)
Protein biosynthesis	Nmt2 (1.52), Eif2c1 (1.72), Impact (1.75), Mrps21 (1.82), Mtrf11 (2.00), Pthr1 (2.00)
Protein amino acid glycosylation	Nipsnap1 (1.52), Ogt (1.52), Sfxn1 (1.52), Timm22 (1.56), Edem1 (1.61), Mrps21 (1.82), St3gal3 (1.82)
Gene ontology: down-regulated in IVF when compared with IVC	
1) Enzyme linked receptor protein signaling pathway: $P=0.0080$	Prkca (1.55), Acvr1 (1.65), Ptpro (1.73), Htra1 (1.82), Nrp1 (1.84), EphA7 (1.88), Ltbp1 (1.89), Ephb2 (1.92)
cAMP-mediated signalling	Ptger3 (1.70), Oprm1 (1.96), Rims2 (2.00), Gnal (2.23)
2) Steroid metabolism: $P=0.0070$	Pbx1 (1.64), Akrlc21 (1.68)
Steroid/lipid transport	Osbp2 (1.62), Abca1 (1.65), Apoa1 (1.75), Vldlr (2.07)
3) Heart development: $P=0.0094$	Acvr1 (1.65), Tgfb2 (1.76), Nrp1 (1.84), Nrp2 (2.08), Myocd (2.20)

regulated, and *Cebpa* knock outs show abnormalities in adipogenesis, hematopoiesis, hepatic, and lung morphology and premature death (Flodby *et al.* 1996, Zhang *et al.* 2004). It is possible that these changes are temporary and disappear, but could persist and affect long term development, extending the Barker hypothesis (Barker 1998) to the preimplantation period.

IVC and IVF embryos were more different from each other than was expected. For example, there was a progressive and significant decrease in TE cell number as the time in culture and degree of manipulation increased (*in vivo* > IVC > IVF). The number of ICM cells was reduced after IVC, but more so in the IVC group. This indicates a specific effect on the TE cells either in terms of proliferation or survival in the IVF embryos. Interestingly, a statistically higher number of IVF embryos developed from the two-cell stage to the blastocyst stage when compared with IVC embryos. In agreement to these findings, more genes were statistically abnormal after IVC (IVC versus *in vivo*: 4660 different genes) than after IVF (IVF versus *in vivo* comparison: 1912 different genes). These latter findings were surprising and contrary to our expectation. In fact, it is well known that embryos develop more slowly in culture when compared with the *in vivo* counterpart. Additional time spent in culture, and in particular the first 24 h of development, would have been expected to slow the development even further. Our findings, however, suggest that a change in environment (like the one present in IVC embryos fertilized *in vivo* and then cultured *in vitro*) appear to be more stressful to the embryos; one could speculate that embryos benefit from being in the same environment from fertilization onward, no matter how artificial the initial environment is. A change in environment represents a stressful shock, requiring time to adapt to the new condition. The timing of this particular stressor is important in determining its effect. It would be interesting to perform complementary experiments (IVF and transfer of zygotes in the fallopian tubes) to test this hypothesis.

Direct comparison of differentially expressed genes in IVF versus IVC reveals that several abnormally regulated IVF genes are involved in brain development. Mutation of *Nos1* has been shown to increase neurogenesis and mutation of *Nrp1*, *Nrp2* (down-regulated 1.8 and 2 times respectively) has been reported to induce abnormal brain development (Gu *et al.* 2003) and abnormal neurogenesis and axon guidance (Chen *et al.* 2000). *Tgfb2*, in addition to causing abnormal brain development, is associated with abnormal cardiovascular, respiratory, and reproductive morphology. This indicates that although the IVF procedure shows fewer alterations in the level of specific gene expression when compared with IVC, some of the important morphogenic genes are altered in the IVF embryos.

The comparison of IVC versus *in vivo* has been the subject of two prior publications (Rinaudo & Schultz

2004, Rinaudo *et al.* 2006). Overall, our new results confirm our past experiments. There are some notable differences in number of genes statistically different (4660 genes in the present comparison IVC versus *in vivo* in 20% oxygen versus 1159 genes in the past comparison), but the classes of genes altered are the same. The differences in gene number are likely due to the differences in laboratory environment, RNA amplification technique (double amplification protocol versus linear amplification using the NuGen protocol used in the present experiments) and the gene chip used in this study. In fact, in the prior experiments, we used the Affymetrix MOE 430A chip, containing only 22 000 transcripts. The present set of experiments used the NuGen small sample amplification protocol because this protocol is robust and requires a reduced amount of starting RNA. In particular, the NuGen linear amplification protocol conserves the correct ratio between mRNAs that are expressed at different levels and does not distort transcript abundance. (Goff *et al.* 2004, Barker *et al.* 2005).

Our study is the first one to offer a complete overview of the changes in the global pattern of gene expression in IVF versus *in vivo* generated mouse embryos and completes our prior findings (Rinaudo & Schultz 2004, Rinaudo *et al.* 2006).

Other authors have analyzed the effect of the fertilization method on mammalian embryos and their findings are complementary to ours. Whitworth *et al.* (2005) analyzed the differences between IVF and *in vivo* embryos in the porcine model by using a tissue-specific 19 968 spot cDNA microarray. Interestingly, cluster analysis revealed that IVF and control embryos were different at the four-cell stage, but they did not separate in independent branches at the blastocyst stage, indicating a less dramatic effect of culture on embryos at this latter stage. One hypothesis advanced by the authors is that poor IVF quality embryos were removed at (or after) the four-cell stage therefore allowing for a more uniform 'healthy' blastocyst population. This could be a species-specific phenomenon. However, the two classes of embryos had significant differences: there were 1696 differentially expressed genes between the two groups and similar gene pathways were modified; for example, RNA metabolism and ribonucleoprotein complex were up-regulated in IVF embryos, a finding consistent with our observation.

Global pattern of gene expression in *in vitro* versus *in vivo* produced embryos was also analyzed by Corcoran *et al.* (2006) in bovine embryos. They found 384 genes differentially regulated in IVF embryos. There was a general down-regulation of genes involved in transcription and translation, as well as genes involved in cellular metabolism.

One potential limitation of our experiments is the choice of WM, a medium that is known to be suboptimal. In fact, we have shown in the past that

WM results in higher number of mis-regulated genes than KSOM medium with amino acids (Rinaudo & Schultz 2004) and that a high oxygen concentration (20% or atmospheric oxygen) has an additional and synergistic negative effect on the gene expression pattern (Rinaudo *et al.* 2006). However, we specifically decided to use WM to stress the system and therefore, potentially magnify the effects of a different method of fertilization. In this fashion, we wanted to glimpse the potential mechanism by which the method of fertilization affects embryo health. Future experiments will be conducted to assess the effect of an improved media and different methods of fertilization on the global pattern of gene expression.

Another important aspect to note is that we compared mRNA abundances between blastocysts that appeared identical at the time of the selection (at 60× using a stereomicroscope), but when later examined for cell numbers, had different quantity of cells. Therefore, the results described could simply be explained by the fact that the blastocysts were at slightly different stage of development. We exclude this possibility, because we were careful to select only late cavitating blastocysts of similar morphology, as we have done in our prior work (Rinaudo & Schultz 2004, Rinaudo *et al.* 2006). In addition, the cell number differences only involve the TE cells, while the number of ICM cells is similar in all three groups. For example, the IVC group and IVF group have a comparable number of ICM cells (12 and 13 respectively), but a statistically different number of TE cells (40 and 34 respectively). If the blastocysts were truly at different stages of development we would expect the ICM number to be lower in the IVF group. Indeed, we think that all the groups are in same stage of differentiation and the difference in cell numbers are secondary to increase incidence of apoptosis or decrease in cell proliferation, as confirmed by our microarray data. Other authors (Devreker & Hardy 1997) have documented a significant change in cell number, mitotic, and apoptotic index in blastocysts cultured in different media.

In conclusion, the *in vitro* produced mouse embryos in the specific culture conditions used are at a disadvantage when compared with *in vivo* embryos. It remains to be investigated if these changes are only temporary or are associated with long-term consequences. For example, it is unclear if the down-regulation of the embryonic development genes is associated with delayed patterning in the cultured embryos. Presence of a long-term effect would be in agreement with the Barker hypothesis that holds that *in utero* events are associated with long lasting predisposition to disease (Barker 1998). Another question to be elucidated is whether changes reported in one species might be present in another species. At present, for example, it appears that bovine embryos are affected by the large offspring syndrome (Niemann & Wrenzycki 2000), while human embryos are not (Olivennes *et al.*

2002). More studies need to be performed to evaluate what is the effect of other forms of artificial fertilization (i.e. ICSI) on embryo development.

Acknowledgements

F D has been supported by a grant from Fondo A Griffini e J Miglierina awarded by Amministrazione Provinciale di Varese, Italy in 2006. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. This work has been partially supported by an ASRM/Serono grant, a Bechtel grant and a WRHR grant to P R. The authors wished to thank Dr Emin Maltepe for his valuable suggestions.

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Received 4 October 2006

First decision 26 October 2006

Revised manuscript received 18 March 2007

Accepted 22 March 2007