Human Reproduction, Vol.25, No.12 pp. 3012–3024, 2010
Advanced Access publication on October 1, 2010 doi:10.1093/humrep/deq266

human reproduction

ORIGINAL ARTICLE *Embryology*

Effect of ICSI on gene expression and development of mouse preimplantation embryos

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Submitted on May 9, 2010; resubmitted on August 16, 2010; accepted on September 15, 2010

BACKGROUND: *In vitro* culture (IVC) and IVF of preimplantation mouse embryos are associated with changes in gene expression. It is however not known whether ICSI has additional effects on the transcriptome of mouse blastocysts.

METHODS: We compared gene expression and development of mouse blastocysts produced by ICSI and cultured in Whitten's medium ($ICSI_{WM}$) or KSOM medium with amino acids ($ICSI_{KSOMaa}$) with control blastocysts flushed out of the uterus on post coital Day 3.5 (*in vivo*). In addition, we compared gene expression in embryos generated by IVF or ICSI using WM. Global pattern of gene expression was assessed using the Affymetrix 430 2.0 chip.

RESULTS: Blastocysts from ICSI fertilization have a reduction in the number of trophoblastic and inner cell mass cells compared with embryos generated *in vivo*. Approximately 1000 genes are differentially expressed between ICSI blastocyst and *in vivo* blastocysts; proliferation, apoptosis and morphogenetic pathways are the most common pathways altered after IVC. Unexpectedly, expression of only 41 genes was significantly different between embryo cultured in suboptimal conditions (WM) or optimal conditions ($KSOM_{aa}$).

CONCLUSIONS: Our results suggest that fertilization by ICSI may play a more important role in shaping the transcriptome of the developing mouse embryo than the culture media used.

Key words: ICSI / embryos / microarray / gene expression / culture medium

Introduction

It is estimated that 1% of children in the western world are born with the help of assisted reproductive techniques (ART). Whereas these techniques are thought to be safe, several studies report an increased risk of unique complications associated with the use of IVF and ICSI (Anthony, 2002; Cox *et al.*, 2002; Hansen *et al.*, 2002; Schieve *et al.*, 2002; DeBaun *et al.*, 2003). The widespread use of ICSI, currently accounting for over 60% of the fertilization procedures in USA (Society for Assisted Reproductive Technology, 2008 www.sart.org) has sparked controversy because ICSI bypasses several physiologic events which would usually occur during fertilization. ICSI involves introduction of the sperm tail into the ooplasm and may be associated with suboptimal sperm nuclear decondensation (Markoulaki *et al.* 2007). In addition, ICSI-generated zygotes cleave at a slower rate, have a reduced hatching rate and a reduced cell number. ICSI zygotes also exhibit shorter calcium oscillations with an altered pattern (Kurokawa and Fissore, 2003). Importantly, Fernandez-Gonzalez *et al.* found that ICSI-generated mice using fresh spermatozoa develop long-term consequences, such as obesity and organomegaly, whereas ICSI-generated mice using frozen–thawed spermatozoa had a more severe phenotype, including aberrant growth, abnormal behavior, early aging and increased incidence of tumors (Fernandez-Gonzalez *et al.*, 2008).

In our earlier studies, we showed that *in vitro* culture (IVC) of zygotes to the blastocyst stage alters their global gene expression patterns (Rinaudo and Schultz, 2004; Rinaudo *et al.*, 2006). In addition, IVF is associated with additional changes in global gene expression and development (Giritharan *et al.*, 2007). In order to investigate the effect that ICSI has on mouse preimplantation embryo

development, and expand upon our prior IVC and IVF studies, we carried out experiments fertilizing embryos by ICSI and culturing embryos in different conditions, both optimal and suboptimal. Specifically, morphologic and gene expression studies were performed on blastocysts fertilized by ICSI and grown *in vitro* either in Whitten's medium (WM) ($ICSI_{WM}$ group) or in KSOM with amino acids ($ICSI_{KSOMaa}$ group) compared with those fertilized and grown *in vivo* (*in vivo* group). In addition, we compared the current data from ICSI embryos cultured in WM to our previous data from IVF embryos cultured in WM. Therefore, any comparisons between the IVF_{WM} and $ICSI_{WM}$ groups would tease out the effects of gamete manipulation using IVF and ICSI.

Materials and Methods

Collection of mature oocytes, preimplantation mouse embryos and ICSI procedure

Oocytes and embryos were isolated from super-ovulated dams as previously described (Rinaudo and Schultz, 2004). Briefly, CF-1 female mice were injected with 5 IU pregnant mare's serum gonadotrophin and 46–48 h later with 5 IU hCG. The following morning, oocytes were obtained from the ampullae. Spermatozoa were collected from B6D2F1/J male mice (10–11 weeks old). After mice were sacrificed, the two caudal epididymides were removed, punctured gently with 30-G needles under a dissecting microscope and transferred with sterile forceps into 1 ml pre-warmed (37°C) EGTA Tris-HCl buffered solution containing 10 mM Tris–HCl, pH 8.2, 50 mM EGTA and 50 mM NaCl in a 1.5 ml microcentrifuge tube and then incubated at 37°C for 10 min. The top 0.8 ml sperm suspension was carefully aspirated and used immediately for ICSI. Only oocytes with first polar body were microinjected using a Piezo drill (PMM Controller, Prime Tech, Ibaraki, Japan), as described (Li *et al.*, 2009). Then fertilized oocytes were washed and cultured in either WM or KSOM with amino acids to the blastocyst stage under 5% CO_2 in humidified air at 37°C. Late-cavitating blastocysts of similar morphology were harvested at ~112 h post-hCG for ICSI groups and 96 h post-hCG for control *in vivo* group. Each treatment was repeated four times. Each time, sperm from one male and oocytes from two to three females were used for fertilization of ICSI groups. For *in vivo* groups, eggs were collected from two to three females after overnight breeding as 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 X-100 (Sigma-Aldrich, USA) in minimum essential medium (MEM; Invitrogen Corporation, USA) for 3–5 seconds, washed 3–5 times in MEM + polyvinylpyrrolidone (PVP; Sigma-Aldrich) and incubated in MEM + PVP containing 100 μg/ml propidium iodide (Sigma-Aldrich) for 30 seconds. The embryos were then washed 3–5 times in MEM + PVP and fixed overnight in 100% ethanol containing 25 μg/ml of bizbenzamide (Sigma-Aldrich) at 4°C. The embryos were mounted on a clean glass slide using glycerol and kept in a dark chamber until observed under fluorescent light using a standard Leica microscope (Model DMRB, Leica Microsystems AG, Germany).

The embryos were observed by fluorescent microscopy, and the numbers of ICM cell (blue) and trophectoderm (TE: red) nuclei were counted and photographed. The staining procedure was repeated at least three times per treatment group with different sets of blastocysts ($n \geq 12$).

RNA extraction and amplification

Total RNA was extracted from four independent biological replicates (10 blastocysts/replicate) using PicoPure RNA Isoloation Kit (Arcturus, USA) according to the manufacturer's instructions. In column DNase treatment was performed using RNase-free DNase set (Qiagen Inc., USA) as described in the user guide of the PicoPure RNA isolation kit to remove the residual DNA. In each pool, five embryo equivalents of total RNA was used for reverse transcription (RT) followed by linear amplification of antisense cDNA strand, fragmentation and biotin labeling by using NuGEN Ovation Biotin System (NuGEN Technologies Inc., 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 (Agilent, USA). Samples with RNA integrity number (RIN) ≥ 8 were selected for amplification. 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 the University of California San Francisco for GeneChip hybridization. The samples (four biological replicates per experimental group) 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).

cDNA preparation for real-time PCR analysis and RT–PCR

RT was accomplished by utilizing the commercially available first strand cDNA synthesis kit (iScript cDNA Synthesis Kit, Bio-Rad Laboratories, USA). The RT reactions were performed by following the kit manufacturer's protocol. For each treatment group the RT was repeated four times with RNA from 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, 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*), Mm99999915_g1 (*Gapdh*) and Mm00433832_m1 (*Nr3c1*). The real-time PCR was also performed using SyBr green PCR supermix (Bio-Rad Laboratories), and primers for *Bdnf*, *Nr3c1* and *Gapdh* genes with 0.1 embryo equivalents of cDNA from each treatment group. Primers which span at least two exons were designed using Primer3 software. Duplicates were used for each real-time PCR reaction; 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 cell number data were analyzed by one-way analysis of variance. Cell numbers were compared using a mean separation procedure when analysis of variance showed significant *F*-values using Fisher's Least Significant Difference method. Results are reported as the mean values for each set of data ± SEM.

Microarray gene expression data analysis

Statistical analysis was carried out using the R software (http://www.r-project.org/) and the appropriate Bioconductor packages (http://www.bioconductor.org/) run under R. In order to remove all the possible sources of variation of a non-biological origin between arrays, densitometry values between arrays were normalized using the RMA (robust multi-array) normalization function implemented in the Bioconductor affylmGUI. Statistically significant differences between groups were identified using the rank product non-parametric test implemented in the Bioconductor RankProd package. Applying a Student's *t*-test with such a limited number of samples (four in each experimental group) is inappropriate as the obtained statistical significance is not robust; in this situation the mean and the standard deviation could be easily biased by outliers. We therefore carried out a non-parametric statistical test as a rough filter to narrow down the list of most relevant genes (Breitling and Herzyk, 2005). This non-parametric method is highly efficient, robust and widely used for microarray data analysis (Cervello *et al.*, 2010). The Rank Product method has proven to be superior to other statistical methods for microarray data analysis in our and other authors' experience (Hong and Breitling, 2008). Moreover, the rank product approach includes a multiple hypothesis test for raw *P*-value correction to ascertain a false positive rate similar to false discovery rate correction. Data of the microarray are available at the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo).

Genes flagged as 'present' or 'marginal' in at least one hybridization, based on raw perfect match and mismatch as determined by 'mas5calls' function on the Bioconductor affymetrix package, were considered expressed genes.

Functional annotations were carried out using the Ingenuity Pathways Analysis platform (http://www.ingenuity.com/).

Results

Validation of microarray data using real-time PCR

Published evidence confirms that microarray data are robust and reliable (Morey *et al.*, 2006). In order to evaluate the reproducibility of the data, we performed multiple quality control analysis. Initially, we only utilized expanded blastocysts of similar morphology and we performed the experiments using four independent biological replicates. We confirmed that the quality of RNA was optimal, utilizing only RNA samples with RIN $\geq$ 8. We then performed *in silico* quality control analysis of the amplified material: we found that cRNA quality was excellent. Finally, we utilized real-time RT–PCR to confirm the microarray results obtained in this study (Fig. 1). In particular, we chose one gene with increased expression in the ICSI group (*Ccgn1*), one gene with decreased expression (*Ube2a*) and two genes with no change in expression between ICSI and *in vivo* blastocysts (*Bdnf* and *Nr3c1*). Results confirm similar trend between microarray and real-time data. The only difference is represented by the statistically significant ($P < 0.05$) increase after RT–PCR in *Nr3c1* expression in $ICSI_{WM}$ blastocysts, although the increase of *Nr3c1* following microarray analysis in both media or RT–PCR in $ICSI_{KSOMaa}$ is not statistically significant.

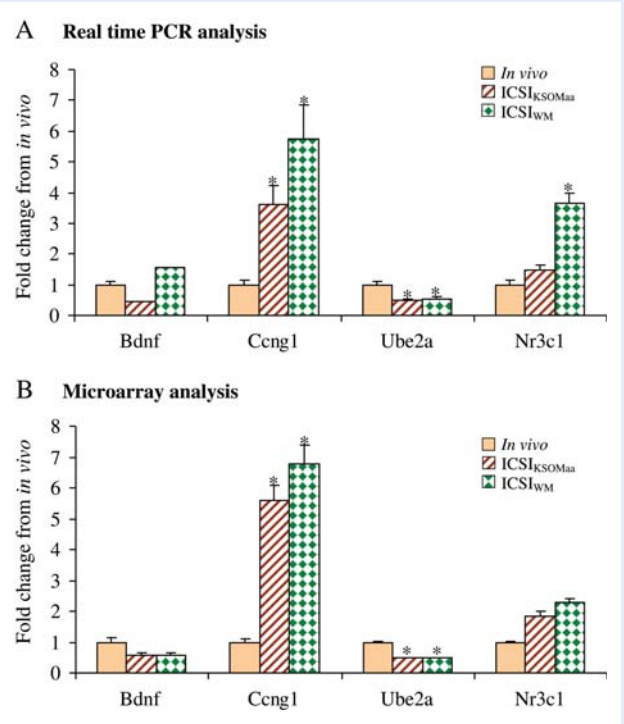

Figure 1 Real-time PCR verification of gene expression data. Expression profile of brain derived neurotrophic factor (*Bdnf*), cyclin G1 (*Ccng1*), ubiquitin-conjugating enzyme E2A (*Ube2a*) and the nuclear receptor subfamily 3, group C, member 1 (*Nr3c1*) genes in murine blastocysts produced by *in vivo* fertilization and culture (*in vivo*), ICSI and IVC in KSOM with amino acids ($ICSI_{KSOMaa}$) and ICSI and IVC in WM ($ICSI_{WM}$) using real-time PCR technique (**A**) and microarray technique (**B**). Bars represent SD. Asterisk (*) indicates significant difference compare with *in vivo* at $P < 0.05$. Of note the genes tested by RT–PCR reflect the changes detected by microarray. The only difference is represented by the statistically significant ($P < 0.05$) increase after RT–PCR in *Nr3c1* expression in $ICSI_{WM}$ blastocysts, while the increase of *Nr3c1* following microarray analysis in both media or after RT–PCR in $ICSI_{KSOMaa}$ is not statistically significant.

Effect of ICSI and the culture medium on blastocyst cell number

Expanded mouse blastocysts are easily recognizable and therefore offer an excellent end-point for morphologic assessment. 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 nuclei in expanded blastocysts. Only expanded blastocysts at the same stage of development were used for analysis (Table I). *In vivo* embryos had on average two additional ICM cells compared with $ICSI_{KSOMaa}$ and three additional ICM cells compared with $ICSI_{WM}$ embryos ($P < 0.05$). The $ICSI_{WM}$ and IVF_{WM} embryos had reduced number of TE cells ($n = 36$ and 33 cells, respectively) compared with the $ICSI_{KSOMaa}$ ($n = 43$ cells) and the *in vivo* control

Table I The method of fertilization affects the distribution of cells in ICM and TE of embryos.

Treatment	# ICM cells (mean ± SEM)	# TE cells (mean ± SEM)	# Total cells (mean ± SEM)
$ICSI_{KSOMaa}$ ($n = 31$)	11.51 ± 0.46^{b}	43.32 ± 1.22^{b}	54.83 ± 1.76^{b}
$ICSI_{WM}$ ($n = 12$)	10.79 ± 0.79^{b}	36.92 ± 1.22^{c}	46.92 ± 2.87^{c}
IVF_{WM} ($n = 63$)	12.84 ± 0.42^{ab}	33.51 ± 1.28^{c}	46.35 ± 1.45^{c}
In vivo ($n = 46$)	13.80 ± 0.50^{a}	49.67 ± 1.50^{a}	63.48 ± 1.70^{a}

Numbers are expressed as means ± SEM. Values with different superscripts in each column differ significantly ($P < 0.05$).

embryos ($n = 49$, $P < 0.05$). Of note, the development of embryos after ICSI, was excellent. More than 70% of the injected eggs survived and more than 90% of the surviving eggs reached the 2-cell stage in both culture media. The blastocyst development rates for 2-cell embryos cultured in $ICSI_{KSOM}$ and $ICSI_{WM}$ were 80.7 and 76.4%, respectively.

Effect of ICSI and *in vitro* embryo culture on blastocyst global gene expression

To analyze the global pattern of gene expression we used the mouse Affymetrix 430 2.0 microarray chip, which is believed to represent the complete mouse genome. Out of 12 035 total non-redundant genes with known gene symbols, 8847 (73.5%) were present in $ICSI_{WM}$, 8408 (69.8%) in $ICSI_{KSOMaa}$ and 7758 (64.4%) in *in vivo* blastocysts. Pair-wise comparison revealed that a significant number of genes are differentially regulated following $ICSI_{WM}$ (947) or $ICSI_{KSOMaa}$ (1016) compared with *in vivo* control embryos (Fig. 2, $P < 0.05$). Most of the differentially regulated genes between the ICSI groups and *in vivo* are common ($ICSI_{KSOMaa}$: 78.9%—802/1016; $ICSI_{WM}$: 84.6%—802/947), with only 215 genes being uniquely different between $ICSI_{KSOMaa}$ and *in vivo* and 145 between $ICSI_{WM}$ and *in vivo*. This finding is important because it provides additional evidence of the robustness of the data. Indeed, unsupervised hierarchical clustering analysis confirms these findings (Fig. 3). The samples segregated into two major clustering branches (ICSI and *in vivo*). However, all the replicate samples of the ICSI embryos cultured in WM and $KSOM_{aa}$ did not show a clear separation, indicating that the ICSI procedure itself affects the transcriptome of the blastocyst and that the culture media plays only a minor role.

The entity of fold changes was higher than expected: 20.6% (210/1016) of the genes were changed more than 5-folds between $ICSI_{KSOMaa}$ and *in vivo*, and 20.4% (194/947) were different between $ICSI_{WM}$ and *in vivo* blastocysts.

Remarkably, a reduced number of genes were differentially expressed in $ICSI_{KSOMaa}$ embryos compared with $ICSI_{WM}$ (41 genes, $P < 0.05$); in addition only three genes were changed more than 3-fold between the two groups, indicating a minor effect of culture on gene expression following ICSI procedure.

The list of significantly different genes after each pair-wise comparison with the relative expression profiles is summarized in Supplementary data, Table S1.

Pathways analysis (Table II) reveals that similar classes are altered in embryos generated by ICSI and *in vivo*, independently of the culture media used. In particular there is an increase in alteration of pathways indicating mitochondrial dysfunction (ubiquinone biosynthesis, oxidative phosphorilation and mitochondrial dysfunction pathways) and alteration of metabolic pathways (N glycan degradation, branch chained amino acids and butanoate metabolism). Only the hepatocytes growth factor (HGF) signaling pathway and glycolysis/gluconeogenesis pathway are uniquely different between $ICSI_{KSOMaa}$ and *in vivo*, while several classes are uniquely changed between $ICSI_{WM}$ and *in vivo* [Oct4-dependent pathway; production of reactive oxygen species (ROS) in macrophages; CDK5 signaling; TR/RXR Activation].

Functional categories analysis (Fig. 4 and Supplementary data, Table S2) shows that cell function, development and metabolic genes are affected by the method of fertilization and culture media used. Whereas all conditions used affect these functions, it is clear that the method of fertilization plays a more important role in altering the gene expression pattern than the media used to culture embryos.

In fact, only 41 genes and two pathways (one-carbon pool by folate and RhoA signaling pathways) were different between $ICSI_{KSOMaa}$ and $ICSI_{WM}$, while ~1000 genes were different between ICSI embryos and *in vivo* embryos.

Effect of the method of fertilization (IVF or ICSI) on gene expression

Comparison of $ICSI_{WM}$ data with our previously generated gene expression data-IVF_{WM} (Giritharan *et al.*, 2007) showed differential expression of 981 genes. Eighteen percent (177/981) of these genes were changed more than 5-fold.

Overall multiple functional categories involved in cell function, development and metabolism (Supplementary data, Table S2) and pathways (Table II) are statistically different between $IVF_{WM}$and $ICSI_{WM}$.

Analysis of transporter genes (Table III) and imprinted and methylation genes (Table IV) reveals that several genes are affected by the method of fertilization.

Discussion

This study, for the first time, offers a complete overview of the changes in the global pattern of gene expression in ICSI or *in vivo* generated mouse embryos. This provides an overview of the transcriptional consequences of fertilizing eggs by different techniques and complements our previous work on the effect of different media and oxygen concentration on the transcriptome of mouse blastocysts (Rinaudo and Schultz, 2004; Rinaudo *et al.*, 2006; Giritharan *et al.*, 2007). The most notable finding is that the ICSI procedure plays a more important role in determining the blastocyst gene expression pattern than the culture media used (WM or $KSOM_{aa}$).

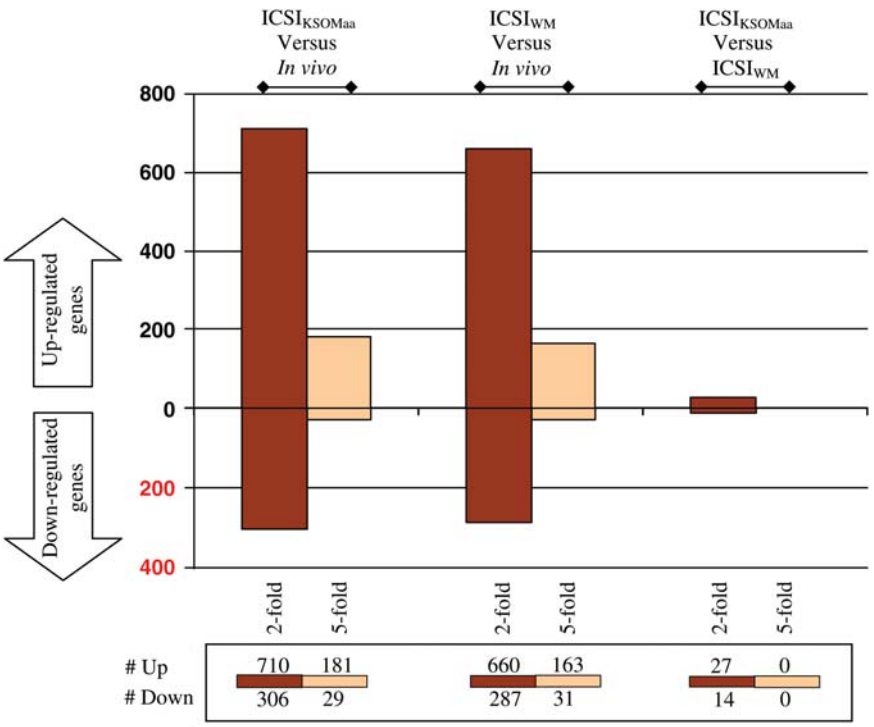

Figure 2 Summary of up-regulated and down-regulated genes for the following comparisons: ICSI$_{KSOMaa}$ versus *in vivo*, ICSI$_{WM}$ versus *in vivo*, ICSI$_{KSOMaa}$ versus ICSI$_{WM}$. Each comparison in the graph shows the number of genes statistically different when comparing the four replicates of one group with the four replicates of the other group. The numbers below each comparison indicate how many genes are statistically up-regulated or down-regulated ($P < 0.05$) more than 2-fold or 5-fold.

Overall, there is a net reduction of ICM and TE cells in the ICSI group compared with the *in vivo* control. The reduction of cells is larger in TE cells (~26% less cells in ICSI$_{WM}$ and 14% in ICSI$_{KSOMaa}$) than ICM (~10% less in ICSI embryos) of ICSI embryos. Since TE cells will give rise to the placenta, this suggests that the method of fertilization could affect more placenta formation than the embryo itself. The cell number data confirm findings of other investigators, who found that, in various species, ICSI-generated zygotes cleave at a slower rate and have reduced hatching rate and reduced cell number (Dumoulin *et al.*, 2000; Bedford *et al.*, 2003; Malcuit *et al.*, 2006).

Although it is not possible to compare the present mouse findings with human data, it is interesting to note that lower hCG levels were found in human IVF gestations than in *in vivo* conception, implying reduced placental mass (Zegers-Hochschild *et al.*, 1994). Assuming that an embryo with lower cell numbers will grow less, these findings could explain the fact that ART children have lower birthweight at term (McDonald *et al.*, 2009).

The gene expression results confirm the morphologic studies. The ICSI embryos have a very distinct transcriptome compared with embryos fertilized *in vivo*, as shown by the unsupervised hierarchical clustering analysis (Fig. 3). ICSI embryos have an increased dysregulation (both up- and down-regulation) in the expression of genes related to cellular function, development and metabolism. The down-regulation of several genes involved in cellular development and differentiation (Fig. 4 and Supplementary data, Table S2) implies that an alteration of developmental strategy could follow the preimplantation stress and explains the reduced number of cells in ICSI embryos. Overall, the increased number of dysregulated genes indicates that these embryos have an increased metabolism; this would suggest decreased fitness according to the 'quiet embryo' hypothesis (Leese *et al.*, 2008).

Pathway analysis offers additional insights (Table II): mitochondrial dysfunction and a disproportionately higher number of metabolic pathways occurring in mitochondria (inositol, butanoate, urea cycle and branched amino acid metabolism) are altered following ICSI. Individual gene analysis confirms these findings. For example, *Cox6b2* (cytochrome *c* oxidase subunit VIb polypeptide 2) gene is increased more than 20-fold in ICSI embryos. This gene produces an isoform of cytochrome *c* oxidase, the terminal enzyme in the mitochondrial respiratory chain, which catalyzes the electron transfer from reduced cytochrome *c* to molecular oxygen (Huttemann *et al.*, 2003). Up-regulation of mitochondrial oxidative phosphorylation genes indicates the increased metabolic need of the embryo.

Of note, mitochondrial dysfunction can result in reduced post-implantation development (Thouas *et al.*, 2006) and can have long-lasting effects. For example, it has been hypothesized as the mechanism inducing the insulin-resistant phenotype observed in offspring of patients with type 2 diabetes (Petersen *et al.*, 2004).

Among the signaling pathways modified after ICSI, the γ amino butyric (GABA) receptor signaling and invasiveness signaling are prominent. Autocrine and paracrine GABA signaling via GABA$_A$ receptors slow preimplantation embryonic growth by decreasing proliferation

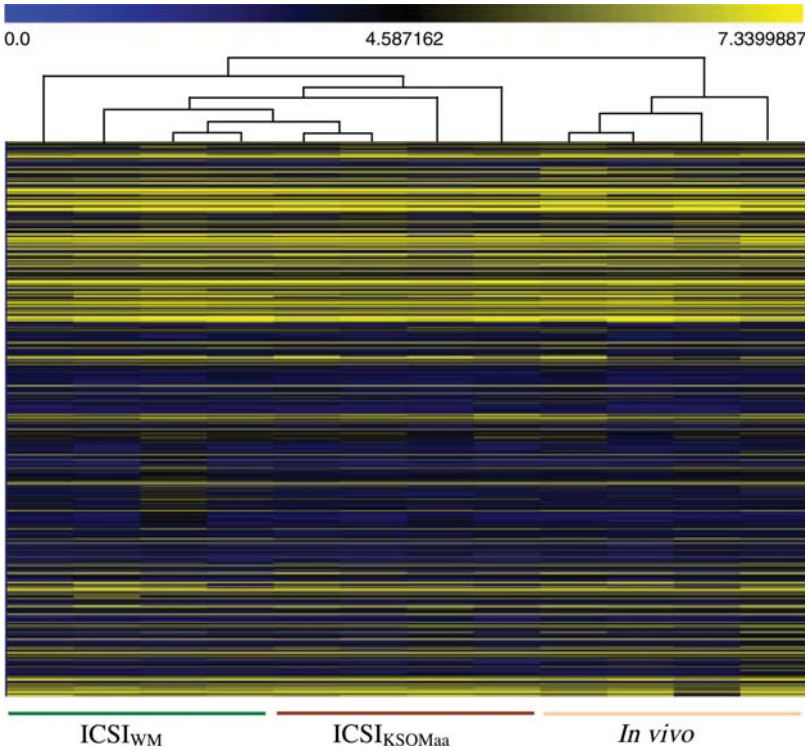

Figure 3 Hierarchical clustering with heat-map of $ICSI_{KSOMaa}$, $ICSI_{WM}$ and *in vivo* embryos based on their gene expression profile. Unsupervised clustering was performed to analyze similarities among replicate samples across all treatment groups tested. Colors correspond to relative RNA abundance for the transcripts detected; each is represented by a horizontal bar in the heat-map. Yellow indicates high expression and blue denotes low expression.

because of increased cellular arrest in the S phase (Andang *et al.*, 2008). Alteration of invasiveness signaling could indicate suboptimal placentation. For example, *Timp1*, an inhibitor of cellular invasion (Paiva *et al.*, 2009) and *Itgb5* (integrin beta 5), an integrin involved in implantation (Massuto *et al.*) are up-regulated in ICSI placentas.

Alteration of multiple additional genes involved in placentation is present. This finding provides a molecular justification of the significant reduction in TE cells found in ICSI embryos. *Prl2c2* (prolactin family 2, subfamily c, member 2) is expressed in large trophoblastic giant cells during the invasion (Adamson *et al.*, 2002) and it is down-regulated 25-fold in ICSI embryos. *Psg28* (pregnancy-specific glycoprotein 28) is expressed in giant cells and spongiotrophoblast of the placenta (Kromer *et al.*, 1996), is implicated in immunomodulatory function to prevent rejection of embryo/fetus (Wynne *et al.*, 2006) and is down-regulated more than 11-fold in ICSI blastocysts. *Bex1* (brain-expressed X-linked 1) located close to *Xist* on the X chromosome, is down-regulated more than 16 times in ICSI embryos; while it does not appear to be epigenetically regulated, it is highly expressed in trophectodermal cells (Williams *et al.*, 2002). Down-regulation of these genes could be associated with reduced placental development.

Alteration of multiple cellular transporters could indicate the placenta function is compromised. Among the transporter genes (Table III), *Slc7a12* (solute carrier family 7, member 12) stands out for being down-regulated more than 18-fold in ICSI embryos; this gene is involved in the cationic amino acid transport (Closs *et al.*, 2006). Dysregulation of other transporter genes has been associated with different metabolic or storage diseases. Although so far none of the following diseases have been linked to ART, alterations of these genes implies metabolic stress. *Slc35c1* (solute carrier family 35, member C1) encodes a GDP-fucose transporter located in the Golgi apparatus. Mutations in this gene result in congenital disorder of glycosylation type IIc (Lubke *et al.*, 2001). *Slc39a4* (solute carrier family 39, member 4) encodes for a transmembrane protein required for zinc uptake in the intestine. Mutations in this gene result in acrodermatitis enteropathica, a rare inherited defect in the absorption of dietary zinc (Wang *et al.*, 2002). *Slc46a1* (solute carrier family 46 member 1) is a transmembrane proton-coupled folate transporter that facilitates the movement of folate and heme across membranes. Mutations in this gene cause a recessive form of folate malabsorption (Qiu *et al.*, 2006).

Because abnormal DNA methylation can follow culture *in vitro*, we paid particular attention to imprinting and methylation genes (Doherty *et al.*, 2000; Rolaki *et al.*, 2007). Several imprinted genes are different between ICSI embryos and *in vivo* embryos (Table IV). Among them *Cd81* and *Peg10* are changed more than 10-fold. *Cd81* encodes a tetraspanin that functions as an organizer of multi-molecular membrane complexes and as a result, regulates cell migration, fusion and signaling (Hemler, 2005). *Cd81* is located close to the cluster of imprinted genes on murine chromosome 7 which is syntenic with the Beckwith–Wiedemann syndrome associated cluster of imprinted gene on human chromosome 11p15.5 region (Paulsen *et al.*, 1998). Lim *et al.* (2009) reported that children born after ICSI with

Table II Overrepresented canonical pathways in comparisons among $ICSI_{KSOM}$, $ICSI_{WM}$ and *in vivo* embryos ($P < 0.05$).

Ingenuity canonical pathways	Ratio	Gene symbol
Altered pathways with fold change in ICSI embryos (both $ICSI_{WM}$ and $ICSIK_{SOM}$) compared with *in vivo* embryos		
Signaling		
GABA receptor signaling	0.109	*Abat* (4.6, 3.6), *Gabra5* (−3.1, −3.3), *Gabrb2* (−2.7, −2.5), *Gabrg1* (−2.2, −2.3), *Slc6a13* (6.5, 5.7)
Invasiveness signaling (Glioma)	0.107	*F2r* (3.6, 5.3), *Itgb5* (4.2, 4.5), *Rhod* (5.3, 4.4), *Rhoj* (−2.7, −2.7), *Rras* (3.7, 3.9), *Timp1* (4.8, 5.9)
Metabolism		
Inositol metabolism	0.143	*Adi1* (−2.5, −2.5), *Aldh16a1* (3.3, 3.7), *Blvrb* (9.5, 11.1), *Cyba* (6.5, 8.5), *Dhrs1* (5.4, 4.3), *Dhrs13* (3.7, 3.7), *Lamb2* (4.7, 4.5), *Ndor1* (4.1, 4.1), *Ndufa6* (3.6, 3.4), *Recql4* (3.7, 3.5), *Retsat* (3.3, 3.5), *Tm7sf2* (3.9, 3.6)
Butanoate metabolism	0.133	*Abat* (4.6, 3.6), *Aldh2* (3.5, 3.5), *Dcxr* (14.3, 15.1), *Ech1* (4.4, 5.7), *Hadha* (4.0, 4.1), *Nlgn1* (−2.6, −2.8), *Oxct1* (3.9, 4.1), *Sdhb* (3.8, 4.0)
N-glycan degradation	0.133	*Man1b1* (4.1, 3.4), *Man1c1* (3.1, 4.2), *Man2c1* (3.8, 4.2), *Manea* (3.2, 3.5)
Urea cycle and metabolism of amino groups	0.118	*Acy3* (6.1, 4.9), *Ass1* (4.1, 3.7), *Oat* (3.4, 4.0), *Pycrl* (4.0, 3.4)
Valine, leucine and isoleucine degradation	0.101	*Abat* (4.6, 3.6), *Aldh2* (3.5, 3.5), *Bcat2* (4.7, 5.4), *Bckdhb* (4.4, 4.5), *Ech1* (4.4, 5.7), *Hadha* (4.0, 4.1), *Oxct1* (3.9, 4.1)
Glutathione metabolism	0.094	*Gpx3* (8.0, 7.7), *Gpx7* (5.3, 6.2), *Gstk1* (5.8, 4.7), *Gstm2* (10.6, 13.4), *Mgst3* (4.3, 3.9), *Oplah* (3.9, 4.0)
Mitochondrial function		
Ubiquinone biosynthesis	0.127	*Bckdhb* (4.4, 4.5), *Ndufa1* (4.0, 3.9), *Ndufa13* (3.8, 3.6), *Ndufa3* (4.1, 4.2), *Ndufa6* (3.6, 3.4), *Ndufb3* (3.8, 4.0), *Ndufb7* (4.1, 4.0), *Ndufs7* (4.4, 4.2), *Ndufs8* (3.3, 3.3)
Oxidative phosphorylation	0.105	*Atp5e* (3.4, 3.7), *Atp5g2* (3.2, 3.9), *Cox6b2* (22.9, 24.9), *Cox7a1* (7.0, 5.8), *Ndufa1* (4.0, 3.9), *Ndufa13* (3.8, 3.6), *Ndufa3* (4.1, 4.2), *Ndufa6* (3.6, 3.4), *Ndufb3* (3.8, 4.0), *Ndufb7* (4.1, 4.0), *Ndufs7* (4.4, 4.2), *Ndufs8* (3.3, 3.3), *Ppa2* (6.6, 7.7), *Sdhb* (3.8, 4.0), *Uqcr* (4.1, 4.2)
Mitochondrial dysfunction	0.092	*Cox6b2* (22.9, 24.9), *Cox7a1* (7.0, 5.8), *Gpx7* (5.3, 6.2), *Ndufa13* (3.8, 3.6), *Ndufa3* (4.1, 4.2), *Ndufa6* (3.6, 3.4), *Ndufb3* (3.8, 4.0), *Ndufb7* (4.1, 4.0), *Ndufs7* (4.4, 4.2), *Ndufs8* (3.3, 3.3), *Sdhb* (3.8, 4.0), *Snca* (−2.6, −2.6)
Pathways uniquely changed between $ICSI_{WM}$ and *in vivo* embryos		
Signaling		
Role of Oct4 in mammalian embryonic stem cell pluripotency	0.044	*Fgf4* (−2.3), *Rxrb* (3.5)
CDK5 signaling	0.034	*Egr1* (−2.6), *Ppp1r11* (3.3), *Ppp1r3a* (−2.5)
TR/RXR activation	0.033	*Ppargc1a* (−2.2), *Rxrb* (3.5), *Ucp2* (3.2)
Production of nitric oxide and reactive oxygen species in macrophages	0.031	*Map3k4* (3.7), *Mapk13* (3.9), *Ppp1r11* (3.3), *Ppp1r3a* (−2.5), *Rnd2* (3.8)
Pathways uniquely changed between $ICSIK_{SOM}$ and *in vivo* embryos		
Signaling		
HGF signaling	0.04	*Ccnd1* (−2.2), *Elf3* (3.4), *Elf5* (3.7), *Map3k6* (3.5)
Metabolism		
Glycolysis/gluconeogenesis	0.042	*Aldh3b2* (3.4), *Galm* (3.2), *Ldha* (4.0), *Pfkm* (3.7)
Pathways uniquely changed between $ICSIK_{SOM}$ and $ICSI_{WM}$ embryos		
Signaling		
RhoA signaling pathways	0.019	*Myl9* (2.05), *Sept4* (2.04)
Metabolism		
One-carbon pool by folate	0.048	*Mthfd2* (2.33)
Pathways changed between IVF_{WM} and $ICSI_{WM}$		
Signaling		
Invasiveness signaling (Glioma)	0.125	*F2r* (−7.87), *Itgb5* (−3.41), *Rhod* (−3.28), *Rhoj* (2.35), *Rnd2* (−2.86), *Rras* (−4.83), *Timp1* (−6.65)

Continued

Table II *Continued*

Ingenuity canonical pathways	Ratio	Gene symbol
Metabolism		
Urea cycle and metabolism of amino groups	0.176	*Acy3* (−3.30), *Aldh18a1* (−5.21), *Cps1* (3.72), *Oat* (−3.77), *Pycrl* (−3.31), *Srm* (−4.52)
Glutathione metabolism	0.125	*Ggt1* (−3.80), *Gpx4* (−3.72), *Gpx7* (−4.64), *Gstk1* (−3.40), *Gstm2* (−5.77), *Mgst1* (2.19), *Mgst3* (−3.05), *Oplah* (−5.19)
Phospholipid degradation	0.103	*Dgka* (−3.23), *Hmox1* (−3.99), *Lamb2* (−3.89), *Napepld* (−3.84), *Pla1a* (−3.25), *Pla2g10* (−2.96), *Plcd1* (−5.23), *Pld3* (−3.05), *Sphk2* (−3.39)
Selenoamino acid metabolism	0.132	*Cbs* (−5.85), *Cth* (−5.93), *Edf1* (−3.16), *Ggt1* (−3.80), *Mars2* (−3.00)
Glycerophospholipid metabolism	0.092	*Agpat2* (−3.34), *Dgka* (−3.23), *Hmox1* (−3.99), *Lamb2* (−3.89), *Napepld* (−3.84), *Pcyt2* (−4.84), *Pemt* (−2.59), *Pla1a* (−3.25), *Pla2g10* (−2.96), *Plcd1* (−5.23), *Pld3* (−3.05), *Sphk2* (−3.39)
Glutamate metabolism	0.132	*Abat* (−5.49), *Cad* (−4.01), *Cps1* (3.72), *Nadsyn1* (−2.47), *Tgm4* (−3.55)
Inositol metabolism	0.131	*Adi1* (2.20), *Aldh16a1* (−3.80), *Blvrb* (−6.23), *Cyba* (−7.99), *Dhrs1* (−4.05), *Dhrs13* (−3.73), *Hmox1* (−3.99), *Lamb2* (−3.89), *Ndor1* (−3.62), *Recql4* (−4.66), *Tm7sf2* (−4.01)
Pyrimidine metabolism	0.111	*Apobec1* (2.38), *Cad* (−4.01), *Dhodh* (−4.03), *Mad2l2* (−3.24), *Nme3* (−5.20), *Nme4* (−3.70), *Nt5m* (−3.21), *Pold1* (−4.68), *Pold2* (−9.11), *Pold4* (−3.58), *Poli* (−3.79), *Polr2i* (−5.06), *Polr2l* (−5.06), *Polrmt* (−3.35), *Trub2* (−3.57), *Uckl1* (−3.20), *Upp1* (−3.39)
Mitochondrial function		
Mitochondrial dysfunction	0.115	*Cox6b2* (−14.50), *Cox7a1* (−5.05), *Cox7b2* (−2.33), *Dhodh* (−4.03), *Gpx4* (−3.72), *Gpx7* (−4.64), *Hsd17b10* (−3.24), *Ndufa3* (−4.04), *Ndufb3* (−3.61), *Ndufb7* (−3.25), *Ndufs2* (−3.71), *Ndufs7* (−3.88), *Ndufs8* (−3.32), *Park7* (−3.28), *Ucp2* (−3.22)
Oxidative phosphorylation	0.091	*Atp5e* (−3.69), *Cox6b2* (−14.50), *Cox7a1* (−5.05), *Cox7b2* (−2.33), *Ndufa1* (−3.10), *Ndufa3* (−4.04), *Ndufb3* (−3.61), *Ndufb7* (−3.25), *Ndufs2* (−3.71), *Ndufs7* (−3.88), *Ndufs8* (−3.32), *Ppa2* (−5.10), *Uqcr* (−4.16)
Ubiquinone biosynthesis	0.127	*Bckdhb* (−4.41), *Edf1* (−3.16), *Ndufa1* (−3.10), *Ndufa3* (−4.04), *Ndufb3* (−3.61), *Ndufb7* (−3.25), *Ndufs2* (−3.71), *Ndufs7* (−3.88), *Ndufs8* (−3.32)

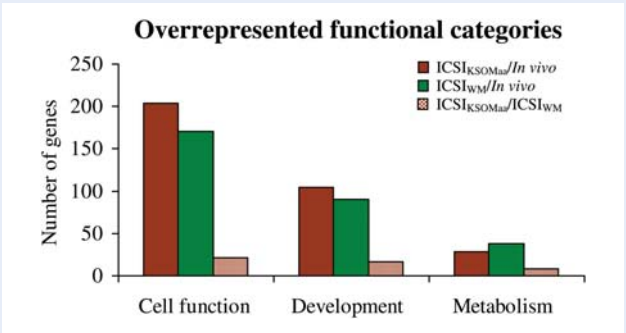

Figure 4 A total number of differentially expressed genes in cellular, developmental and metabolic functional catergories overrepresented in ICSI$_{KSOMaa}$, ICSI$_{WM}$, and *in vivo* embryos ($P < 0.05$). Detail information of cellular, developmental and metabolic functions with differentially regulated genes in each comparison is listed in Supplementary data, Table S2.

Beckwith–Wiedemann syndrome have an altered methylation in this region of chromosome 11. It is, however, important to remember that this gene is imprinted in mice but not humans and therefore conclusions valid in one model system may not be valid in another species. *Peg10* (Paternally expressed 10) is an imprinted gene down-regulated more than 18-fold in ICSI embryos; it plays an important role in placental development and its deletion is associated with early embryonic lethality (Rawn and Cross, 2008).

Interestingly, additional genes that are not imprinted but are located on the X chromosome and therefore potentially subjected to selective epigenetic controls were altered. Among these, *Ott* (ovary testis transcribed) is a mouse X-linked multigene family gene specially expressed during meiosis in testis and ovary (Kerr *et al.*, 1996), while *Slc10a3* (solute carrier family 10 member 3) belongs to the sodium/bile acid cotransporter family and was originally cloned from placental tissue; it maps to a GC-rich region of the X chromosome and was identified by its proximity to a CpG island (Geyer *et al.*, 2006).

Several genes involved in epigenetic regulation appear to be misregulated. Importantly *Wbp7*, *Smarca1* and *Hdac6* are altered in ICSI embryos.

Hdac6 belongs to the histone deacetylases (HDACs) family of enzymes that catalyze the removal of acetyl groups from lysine residues in histones and non-histone proteins, resulting in transcriptional repression. HDACs play a role in cell growth arrest, differentiation and death, and *Hdac6* over expression has been associated with premature chromatin compaction in mouse oocytes and embryos (Verdel *et al.*, 2003).

Smarca1 (SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily a, member 1) encoded protein has helicase and ATPase activity and regulates transcription by altering chromatin structure (Magnani and Cabot, 2009).

Wbp7 (WW domain binding protein 7) is a histone methyltransferase that methylates Lysine 4 of histone H3 resulting in an epigenetic mark favoring transcriptional activation. Mice lacking Wbp7 fail to develop beyond E9.5 (Lubitz *et al.*, 2007).

Table III Differentially expressed transporter genes in ICSI and IVF embryos ($P < 0.05$).

Gene symbol	Gene function	$ICSI_{KSOM}$/ *In vivo*	$ICSI_{WM}$/ *In vivo*	$ICSI_{WM}$/ $ICSI_{KSOM}$	IVF_{WM}/ $ICSI_{WM}$
Slc10a3	Sodium/bile acid cotransporter	−5.36	−4.31	NS	−4.00
Slc12a4	Potassium/chloride transporters	−4.98	−4.07	NS	−4.42
Slc13a2	Sodium-dependent dicarboxylate transporter	−5.19	−4.51	NS	−5.82
Slc16a6	Monocarboxylic acid transporter	−4.33	−3.87	NS	NS
Slc22a9	Organic anion transporter	4.40	4.55	NS	NS
Slc25a28	Mitochondrial iron transporter	−5.30	−5.25	NS	−4.98
Slc27a3	Fatty acid transporter	−3.32	−3.28	NS	−3.78
Slc35c1	GDP-fucose transporter	−3.79	−3.63	NS	−4.02
Slc35d2	UDP-N-acetylglucosamine/UDP-glucose/GDP-mannose transporter	2.64	2.60	NS	2.19
Slc39a11	Metal ion transporter	−3.24	NS	NS	NS
Slc39a4	Zinc transporter	−3.34	−3.57	NS	NS
Slc3a2	Activators of dibasic and neutral amino acid transport	−3.27	−4.25	NS	NS
Slc44a4	Choline transporter	−3.54	−3.40	NS	NS
Slc46a1	Folate/heme transporter	−5.46	−5.44	NS	−7.58
Slc5a12	Sodium/glucose cotransporter	2.64	2.82	NS	NS
Slc5a6	Sodium-dependent vitamin transporter	−3.08	−3.79	NS	−3.95
Slc6a13	Neurotransmitter (GABA) transporter	−5.66	−6.49	NS	−3.90
Slc7a12	Cationic amino acid transporter	16.66	15.09	NS	10.24
Slc7a13	Cationic amino acid transporter	2.38	2.40	NS	2.37
Slco6b1	Organic anion transporter	2.87	2.73	NS	2.61
Slco6c1	Organic anion transporter	3.35	3.34	NS	3.11
Slc44a3	Choline transporter	NS	NS	−2.10	NS
Slc27a2	Fatty acid transporter	NS	NS	−2.42	NS
Slc27a4	Fatty acid transporter	NS	NS	NS	−2.57
Slc30a2	Zinc transporter	NS	NS	NS	−3.74
Slc37a4	Glucose-6-phosphate transporter	NS	NS	NS	−4.56
Slc38a7	Sodium-coupled neutral amino acid transporter	NS	NS	NS	−3.88
Slc39a12	Zinc transporter	NS	NS	NS	2.00
Slc4a2	Anion exchanger	NS	NS	NS	−2.48
Slc5a6	Sodium-dependent vitamin transporter	NS	NS	NS	−3.95
Slc7a3	Cationic amino acid transporter	NS	NS	2.07	−7.65
Slc9a1	Sodium/hydrogen exchanger	NS	NS	NS	−4.18
Slco6d1	Organic anion transporter family	NS	NS	NS	2.40

NS, not significant.

The transcriptome comparison of ICSI embryos cultured in $KSOM_{aa}$ or WM and the comparison of $ICSI_{WM}$ and IVF_{WM} blastocysts provided some of the most unanticipated findings of the study: the method of fertilization plays a more important role than the culture media to determine the transcriptome of the blastocysts.

Regarding the comparison of $ICSI_{KSOMaa}$ and $ICSI_{WM}$, it appears that while $ICSI_{WM}$ embryos have a statistically reduced number of TE cells (18% less), only 41 genes (4%—versus ~1000 genes different between *in vivo* and ICSI blastocysts) were differentially expressed; in addition the fold change differences were small and overall <4-fold. Interestingly, *H19* is the only imprinted gene differentially expressed being up-regulated more than 3-fold in WM. This is not surprising, since culture in WM, as opposite to culture in KSOM, is associated with biallelic expression of the gene (Doherty *et al.*, 2000).

Only three transporter genes are differentially regulated (*Slc44a3*; *Slc27a2*; *Slc7a3*).

Finally only two pathways were statistically different between $ICSI_{KSOMaa}$ and $ICSI_{WM}$ (RhoA signaling and one carbon folate pathways). Members of the Rho family of small guanosine triphosphatases have emerged as key regulators of the actin cytoskeleton, and furthermore, through their interaction with multiple target proteins, they ensure coordinated control of other cellular activities such as gene transcription and adhesion. Down-regulation of *Mthfd2* (methylenetetrahydrofolate dehydrogenase—NADP+-dependent—2) is, however,

Table IV Imprinted and epigenetic genes differentially expressed in ICSI and IVF embryos compared with *in vivo* embryos with their fold changes ($P < 0.05$).

Gene symbol	Gene function	*In vivo*/ $ICSI_{WM}$	*In vivo*/ $ICSI_{KSOMaa}$	$ICSI_{WM}$/ $ICSI_{KSOMaa}$	IVF_{WM}/ $ICSI_{WM}$
Imprinted genes					
Asb4	Ankyrin repeat and SOCS box-containing 4	2.17	2.86	NS	NS
Cd81	CD81 antigen	−13.92	−13.91	NS	NS
H19	H19 fetal liver mRNA	2.08	NS	3.68	NS
Igf2as	Insulin-like growth factor 2, antisense	−3.32	−4.40	NS	−5.41
Kcnq1	Potassium voltage-gated channel, subfamily Q, member 1	NS	NS	NS	2.07
Osbpl5	Oxysterol binding protein-like 5	NS	−3.65	NS	−4.15
Peg10	Paternally expressed 10	−18.85	−21.75	NS	−12.27
Epigenetic regulating genes					
Hdac6	Histone deacetylase 6	−4.37	−4.24	NS	NS
Smarca1	SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily a, member 1	2.82	2.50	NS	NS
Wbp7	WW domain binding protein 7	−4.86	−4.56	NS	−4.59
Mbd2	Methyl-CpG binding domain protein 2	NS	−3.53	NS	NS
Smarca4	SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily a, member 4	NS	NS	NS	−3.36

NS, not significant.

notable as this gene is involved in folate metabolism and therefore could result in abnormal methyl donor availability for methyltransferases.

Complementary to the findings of ICSI embryos cultured in different media, the different gene expression pattern between IVF_{WM} and $ICSI_{WM}$ confirms that the method of fertilization plays a fundamental role in determining the transcriptome of the preimplantation embryos. In fact, as many genes are different between IVF_{WM} and $ICSI_{WM}$ (984 gene) as between $ICSI_{KSOMaa}$ and *in vivo* (1016) or $ICSI_{WM}$ and *in vivo* (947).

Pathways analysis confirms that similar pathways (Mitochondrial function pathways and metabolic pathways) are changed between IVF_{WM} blastocysts and $ICSI_{WM}$ embryos as between ICSI embryos (both WM and KSOMaa) and *in vivo* embryos.

Interestingly, among the transporters differentially expressed, a subset is uniquely mis-expressed between IVF_{WM} and $ICSI_{WM}$ blastocysts (Table III). *Slc27a4* is involved in translocation of long-chain fatty acids across the plasma membrane and is required for fat absorption in early embryogenesis (Gimeno *et al.*, 2003). *Slc37a4* (solute carrier family 37 member 4) regulates glucose-6-phosphate transport from the cytoplasm to the endoplasmic reticulum and is involved in ATP-mediated calcium sequestration in the lumen of the endoplasmic reticulum. Mutations in this gene have been associated with various forms of glycogen storage disease (Schaub and Heyne, 1983).

Three epigenetic genes (*Wbp7*, *Kcnq1* and *Smarca4*) were differentially regulated in IVF_{WM} and $ICSI_{WM}$ embryos. *Kcnq1* was down-regulated in ICSI embryos while *Wbp7* and *Smarca4* were up-regulated in ICSI embryos compared with IVF embryos. *Smarca4* protein plays a fundamental roles controlling gene expression during early mammalian embryogenesis (Magnani and Cabot, 2009). *Wbp7* also plays a key role in embryo development, and regulates the apoptosis and differentiation of cells into all three germ layers (Lubitz *et al.*, 2007). Most recent evidence revealed an association of the *Kcnq1* gene with the susceptibility to type 2 diabetes. *Kcnq1* participates in the regulation of cell volume, which is, in turn, critically important for the regulation of metabolism by insulin. *Kcnq1* counteracts the stimulation of cellular K+ uptake by insulin and thereby influences K+-dependent insulin signaling on glucose metabolism (Boini *et al.*, 2009).

Importantly, this study does not address unequivocally whether the differences in gene expression described in ICSI-produced blastocysts are secondary to the ICSI procedure alone or the combination of ICSI and IVC. An ideal experiment would include the transcriptome analysis of blastocysts that were generated by ICSI, with the resulting two pronuclei embryos immediately transferred to recipients to eliminate the effects of *in vivo* culture. Whereas this experiment is feasible, it is technically difficult and requires multiple manipulations. The next best experiment is to observe the gene expression changes of zygote generated *in vivo* and cultured to the blastocyst stage (IVC embryos). We (Rinaudo and Schultz, 2004; Giritharan *et al.*, 2007) and others (Wang *et al.*, 2005; Zeng and Schultz, 2005; Fernandez-Gonzalez *et al.* 2009) have shown that culture conditions play an important role in shaping gene expression in developing embryos and blastocysts. In particular, in one study we showed that culture media and oxygen concentration play an independent role in affecting gene expression, with the oxygen concentration determining a synergistic increase in gene misregulation. IVC of zygotes in atmospheric oxygen (20% O_2) was associated with markedly increasing misregulated genes (20% O_2:WM: 354 genes changed more than 2-fold compared with *in vivo* flushed blastocysts; $KSOM_{aa}$: 102 differentially expressed gene), compared to IVC of zygotes in physiologic oxygen concentration (5% O_2: WM: 45 genes and KSOM 18 genes changed more than 2-fold, respectively; Rinaudo *et al.*, 2006). In a separate study, we showed that a high number of transcripts was statistically

different in blastocysts generated by IVF and cultured in WM 20% O_2 when compared with *in vivo* control blastocysts, but the magnitude of the changes in gene expression was low and only a minority of transcripts (357) was changed more than 2-fold. Surprisingly, IVF embryos were different from IVC blastocysts cultured in WM 20% O_2 (3058 transcripts were statistically different but only 98 transcripts were changed more than 2-fold; Giritharan *et al.*, 2007). Fernandez-Gonzalez *et al.* (2009) compared the gene expressions of IVC embryos cultured in KSOM versus KSOM + fetal calf serum (FCS), and observed that the presence of FCS during IVC affected several genes involved in regulating epigenetic mechanisms. Their results are particularly relevant because the same authors found that adult animals resulting from IVC embryos cultured in KSOM + FCS displayed alteration of post-natal development and behavior (Fernandez-Gonzalez *et al.*, 2004).

The above-mentioned studies and the present findings support the conclusion that each manipulation and culture conditions have an independent effect on the transcriptome of the developing embryo. Interestingly, the ICSI manipulation results in a higher number of genes being changed compared with all the other manipulations analyzed.

It is possible that the different pattern of calcium oscillation following ICSI (Kurokawa and Fissore, 2003) triggers a different wave of gene activation and therefore the effect of different culture media is less prominent. Consistent with this possibility is the finding that the *S100a6* (S100 calcium binding protein A6), a calcium binding protein, is down-regulated more than 25-folds in the ICSI groups. *S100a6* induces conformational changes and post-translational modifications of multiple cytoplasmic proteins (Santamaria-Kisiel *et al.*, 2006). In particular, it interacts with p53 and modulates apoptosis (Grigorian *et al.*, 2001).

Implication for future health

The most important clinical question relates to the long-term significance of the observed gene changes. These changes could be self-limited and isolated, like T-wave changes found on a healthy person EKG following intense physical exercise. In fact, while one rodent study found differences in selected gene expression in blastocysts generated by ICSI with mature spermatozoa or round spermatid injection (ROSI; Hayashi *et al.*, 2003), other studies did not find any obvious long-term health consequences in ROSI mice (Tamashiro *et al.* 1999; Meng *et al.*, 2002). On the other hand, it is possible that some of the differentially regulated genes might alter the development of additional genes, and as a result, potentially affect cellular and tissue development, as asserted by the developmental origin of health and disease hypothesis (Barker, 1998). Indeed, a cohort of ART pubertal children has been found to have slightly worse metabolic parameters than spontaneously conceived children of infertile couples (Ceelen *et al.*, 2008). We believe that our database provides fundamental resources for understanding how the method of fertilization and culture conditions affect mouse preimplantation embryo development. Therefore the provided gene list could be useful to uncover the cellular mechanisms that can lead to long-term health consequences.

Overall, it appears that ICSI, IVF and *in vivo* produced mouse blastocysts have a very different transcriptome.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

G.G.: Conception and design, collection and assembly of data and manuscript writing. M.W.L.: Collection of data. F.S.: Collection of data. F.J.E.: Data analysis and interpretation. J.A.H., A.D., E.M.: Data analysis and interpretation. K.C.K.L.: Collection of data. P.R.: Conception and design, manuscript writing and final approval of manuscript.

Funding

This research was supported by NICHD/NIH through cooperative agreement 1U54HD055764 as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research.

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