Article

Gene expression in the preimplantation embryo: in-vitro developmental changes

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Abstract

The regulation of early embryo development and the mechanism of implantation remains poorly understood, due to the large number of genes and the complexity of the systems involved. The effect of in-vitro culture on embryos also remains unclear, which raises concerns about the safety of assisted reproductive technology. Changes in the expression of several individual genes in cultured embryos have been reported previously, but a large-scale comparison has not yet been performed to investigate the effects of in-vitro culture systems on embryo development. This study investigated established gene expression profiles of more that 20,000 genes from in-vitro cultured mouse embryos at eight different stages (oocytes, zygote, 2-, 4-, 8-cell embryo, compacting embryo, morula and blastocyst) using microarray technology, and compared these profiles with in-vivo embryos. In most stages of development there was little significant difference in overall expression patterns between in-vitro and in-vivo embryos. In addition, the expression patterns of developmentally important genes from several different categories, such as apoptosis, glycolysis, adhesion and methylation, were examined and compared between in-vitro and in-vivo embryos. Among the genes examined, DNA methyltransferase 1 (DNMT1) shows a significantly higher ($P < 0.05$) expression level in cultured embryos. Cadherin-11 also demonstrates a slightly different pattern, although the difference is not statistically significant. All the other genes have remarkably similar expression patterns between in-vitro and in-vivo embryos throughout preimplantation stages.

Keywords: *gene expression profiles, microarray, preimplantation embryos*

Introduction

It has been known for years that mammalian embryos undergo major changes in their gene expression patterns throughout most stages of preimplantation development. Following fertilization, the transition from oocyte to early embryo starts with degradation of maternal transcripts and the onset of new transcription, known as embryonic genome activation (EGA) or zygotic genome activation (ZGA). Extensive changes in gene expression continue as embryos develop further. A second wave of major gene activation, named mid-preimplantation gene activation (MGA), precedes and accompanies the dramatic morphological changes when embryos go through the process of compaction and develop further into morulae and blastocysts (Latham *et al*., 1991; Wassarman and Kinloch, 1992; Edwards, 2003; Hamatani *et al*., 2004).

Previous studies have explored genome-wide expression and regulation in preimplantation mouse embryos using several techniques, including mRNA differential display (Ma *et al*., 2001), large-scale cDNA library analysis (Ko *et al*., 2000) and suppression subtractive hybridization (SSH) (Zeng and Schultz, 2003). In each study, the roles of several genes were identified in early embryonic development. However, these studies were subject to the limitations of the techniques used. Differential display is known to have a high degree of false positives, and rare transcripts may not be picked up since the technique has a bias toward abundant transcripts. In the case of cDNA libraries, the accuracy or completeness of representation can be a limitation. More recently, two studies were published using microarrays to study gene expression changes during mouse preimplantation development (Hamatani *et al*., 2004; Wang *et al*., 2004). This approach

provides a robust and comprehensive method to analyse a large number of genes involved in early embryo development and reveals overall gene expression changes throughout the preimplantation stages. In addition, Bermudez and colleagues reported gene expression profiles on small numbers of pooled oocytes using microarray technology (Bermudez *et al*., 2004). These technological breakthroughs further enhance understanding on genetic regulations of preimplantation embryos, where limited amounts of materials are available.

The long-term effect of culture on preimplantation embryos has been debated over recent years and has become a safety concern for assisted reproductive technologies. Some retrospective studies have suggested that children conceived by assisted reproduction have a higher incidence of Angelman and Beckwith–Wiedemann syndromes. On the molecular level, several reports have been published on the effects of in-vitro culture on animal behaviour and the imprinting status of genes such as H19 and LIT1 under certain conditions (Cox *et al*., 2002; DeBaun *et al*., 2003; Ecker *et al*., 2004). However, there were some residual confounding factors in the epidemiological studies that could not be completely eliminated due to the nature of the experiments. In addition, the number of cases may still be too small to reach statistically significant conclusions. However, these reports suggest that in-vitro culture may affect embryo development through changes in the regulation of gene expression. This study was designed to address this question by comparing global gene expression profiles from all the stages of preimplantation development *in vitro* and *in vivo*.

This study examined the gene expression profiles of mouse embryos cultured *in vitro*, including eight different stages, oocyte, zygote, 2-, 4-, 8-cell, compacting embryo, morula and blastocyst, using DNA microarray technology. An extensive, thorough database of expression profiles of these embryos was established for future reference. The numbers of genes up- and down-regulated between different embryo stages were also examined. To examine the impact of culture systems on preimplantation embryo development, the gene expression profiles of in-vitro cultured embryos were compared with those of in-vivo derived embryos, from a separate study by Wang and colleagues (Wang *et al*., 2004). Finally, several categories of genes that are known to play critical roles in early embryo development were also tracked. Comparisons of expression patterns of these genes, between in-vitro and in-vivo embryos were made to further investigate the impact of culture systems on embryos. These comparisons, between studies conducted by different groups using slightly different techniques, are subject to some limitations that will be discussed. However, so far as is known, this is the first study to evaluate the effects of in-vitro culture on embryos using global gene expression profiles constructed by microarray technology.

Materials and methods

Embryo collection and culture

Oocytes and zygotes were collected from superovulated female B6C3F1 × B6D2F1 male mice at a standard predetermined hour, around 21–23 h post-human chorionic gonadotrophin (HCG; Embryotech, Boston, MA, USA). Zygotes were treated with hyaluronidase and then transferred to M2 where they were graded and pooled. Zygotes were cultured in Quinn's Advantage Cleavage Medium (CooperSurgical Inc., Trumbull, CT, USA) supplemented with 10% Plasmanate (Bayer Healthcare, Elkhart, IN, USA) under oil and incubated in a humidified 5% CO_2 atmosphere at 37°C through the blastocyst stage. Embryos at different stages were graded carefully, pooled and collected. Three replicates were performed at each stage. Depending on the stage, each replicate contained: 300 oocytes, 300 1-cell embryos, 150 2-cell embryos, 75 4-cell embryos, 40 8-cell embryos, 30 compacting embryos, 20 morulae and 15 blastocysts. The embryo collection and culture for in-vivo study were as previously described (Wang *et al*., 2004). Embryos were collected from F1 (C57BL6 × CBA) female mice superovulated with subsequent injection of HCG. The collections were made at 20–24 h after HCG (zygote), 31–32 h (early 2-cell), 46–48 h (late 2-cell), 54–56 h (4-cell), 68–70 h (8-cell), 76–78 h (16-cell), 86–88 h (early blastocyst), 92–94 h (mid-blastocyst) and 100–102 h (late blastocyst). Oocytes and embryos were collected in pools of 30–134, depending on the stages. The detailed numbers can be found in the Supplemental Table 1 in the paper of Wang *et al*. (2004).

RNA extraction, amplification, labelling and hybridization

RNA was isolated from embryos with the PicoPure Isolation Kit (Arcturus, CA, USA) according to the manufacturer's instructions. Two round amplifications were performed for each replicate to generate cRNA probes from total RNA samples similar to the method previously published (Baugh *et al*., 2001). First of all, cDNA was synthesized by poly(dT) primers from total RNA. The cDNA were then amplified using T7 polymerase to generate cRNA. The cRNA from first round amplification was used to generate more cDNA by random priming, followed by a second T7 amplification. The cDNA was labelled and hybridized to Murine Genome 430A GeneChips array (Affymetrix, CA, USA) following manufacturer's instructions.

Results

Data analysis was performed using dChip, a software packing implementing model-based expression analysis of oligonucleotide arrays (Li and Wong, 2001). Data were normalized and calculated by model-based expression analysis where the PM/MM difference model was used. Hierarchical clustering was performed with distance metric (1-Pearsons' correlation) and centroid linkage method was chosen. *P*-value threshold for significant clusters was set at 0.0005 for samples. The criteria for setting filtered gene list were chosen by setting variation across samples at $0.5 <$ standard derivation/mean < 10, with the expression level ≥200 in ≥50% samples. For comparison of two stages on the number of genes up-/down-regulated, the threshold was set to be a 2-fold change. In addition, the absolute value of the difference (experiment–baseline or baseline–experiment) was greater than 100, with *P*-value ≤0.05. Student's *t*-test was performed to test the standardized values of different gene expression levels between in-vitro and in-vivo embryos. The entire dataset has been submitted to ArrayExpress (http://www.ebi.ac.uk/arrayexpress).

Typically, one gene is represented by one probe set on an Affymetrix GeneChip, but sometimes one gene is determined by several probe sets. Although it is more precise to use the term 'probe sets', for simplicity and clarity, 'genes' has been used as a substitute for 'probe sets' in this paper.

Mouse zygotes, and later stages of embryos, including 2-cell, 4-cell, 8-cell, compacting embryos, morulae, and blastocysts, all cultured from zygotes, were carefully staged by morphology and collected separately. For each stage, about 300 cells were collected per tube, i.e. 300 oocytes, 300 zygotes, 150 2-cell embryos, 75 4-cell embryos, 40 8-cell embryos, 30 compacting embryos, 20 morulae, and 15 blastocysts were pooled and collected in separate tubes. Messenger RNAs were extracted, amplified, labelled and hybridized to Affymetrix Murine Genome Array M430A chips.

The gene expression profiles of eight different stages were established and a total of 24 chips were used with three replicates for each stage (**Table 1**). The average Pearson correlation coefficient (γ) at each stage ranged from 0.962 to 0.994. These high γ values demonstrate that the procedures involved in this experiment, including sample collections, RNA extraction, cDNA amplification and microarray hybridization were reproducible.

Hierarchical clustering analysis was performed to identify embryonic stages that share a common pattern of gene expression. A dendrogram was constructed in a sequential manner, using a ranked series of clusters. This analysis clustered samples with similar global gene expressions in nearby branches. **Figure 1** shows the hierarchical clustering analysis of different stages of preimplantation stage embryos with three replicates for each stage. The tight clustering of each replicate on each stage further confirms the highly reproducible procedures for embryo grading, collection and experimental protocols. It is worth noting that the three replicates of 8-cell embryo clusters mixed with those of compacting embryos, indicating similar gene expression profiles in these two embryo stages, despite their distinct morphologies.

Next, the numbers of genes up- and down-regulated between different stages were investigated. The result is shown in **Figure 2**. With a 2-fold change as a threshold, 382 genes were up-regulated between egg and zygote, and 1783 new transcripts were added when embryos developed from zygote to 2-cell stage. Between the 2- to 4-cell and 4- to 8-cell stage, 717 and 831 genes were up-regulated respectively. Surprisingly, very few newly transcribed genes were observed between 8-cell and compacting embryos, where embryos undergo dramatic morphological changes. When embryos developed from compacting embryos to morulae, 839 genes were up-regulated, while 229 new transcripts were observed from morulae to blastocyst. As for genes that were down-regulated, 4, 1946, 352, and 1079 genes were down regulated from egg to zygote, zygote to 2-cell, 2- to 4-cell, and 4- to 8-cell respectively. Similarly, very few transcripts were degraded between 8-cell and compacting embryos. For later embryonic stages, 331 genes were down-regulated between compacting embryos and morulae, while 191 genes were down-regulated when embryos developed from morulae to blastocysts.

A previous genome-wide study has described gene activity in the preimplantation mouse embryo with microarrays (Wang *et al*., 2004). In contrast to the present study, where embryos were cultured *in vitro* from zygotes, the embryos used by Wang *et al*. were all collected *in vivo* at corresponding developmental stages. Their results were compared with those of the present study to investigate the difference between in-vivo and in-vitro culture in terms of gene expression. The collection of zygotes for in-vivo study was performed 20–24 h after HCG treatment, compared with 21–23 h for in-vitro study. Different platforms of chips were used (Affymetrix U74Av2 GeneChip in Wang's study; Affymetrix 430A 2.0 GeneChip in the present study). The sequence clusters of 430A 2.0 GeneChip were created from the more current UniGene database compared with those on U74Av2. Since the probe sets on these two platforms are quite different, direct comparison is nearly impossible. To solve this problem, about 8000 common probes on these two different chips were used to achieve a meaningful comparison of genes up-/down-regulated at different time points, both *in vitro* and *in vivo*. A list of common probe sets for the two different types of chips was obtained using the 'best match' comparison obtained from the Affymetrix website. In general, the matching probe sets have very high sequence identity. A full explanation of the criteria used can be found on www.affymextrix.com.

The 2-cell mouse embryos in the present study were collected 5–6 h after the first cleavage, about halfway before they went on to the next stage. These embryos were compared with the 'mid 2-cell stage' of the in-vivo embryos. The result of the comparison is shown in **Figure 3**. For up-regulated genes, except when embryos developed from 2- to 4-cell, overall, both cultured embryos and in-vivo embryos have similar trends. Significant numbers of genes were up-regulated when embryos developed from zygote to 2-cell, and almost no newly transcribed genes were seen between 8-cell and compacting embryos. The number of up-regulated genes increased again between compacting embryos/morulae, and then decreased at morulae/blastocyst stage. For the in-vitro embryos, a significant number of genes were down-regulated between 1- and 2-cell stages, which probably reflects the degradation of maternal transcripts. Transcript degradation in embryos obtained *in vivo* seemed to shift to a later stage, as the same amount of degradation was seen between 2- to 4- cell stages. After the 4-cell stage, the number of down-regulated genes for both culture system and in-vivo embryos were comparable.

As shown in **Figure 3**, there was a significant difference between in-vitro and in-vivo embryos in terms of up-regulated gene numbers when they developed from 2- to 4-cell. Gene ontology analysis on these 693 genes (1027 up-regulated genes for *in vivo* and 334 up-regulated genes for *in vitro*) was performed and shown in **Figure 4**. Among these 693 genes, metabolism-related genes accounted for roughly 66% of the total number: 33% of these genes were categorized as protein metabolism, 3% for carbohydrate metabolism, 6% for lipid metabolism, and 24% for

Table 1. Stage points of the gene expression profiles in preimplantation embryos.

Stage	*No. of replicates*	*Range of γ*	*Average γ*
Oocyte	3	0.985–0.992	0.989
Zygote	3	0.993–0.994	0.994
2-cell	3	0.943–0.973	0.962
4-cell	3	0.967–0.986	0.978
8-cell	3	0.980–0.992	0.985
Compacting embryos	3	0.969–0.991	0.978
Morulae	3	0.985–0.993	0.989
Blastocyst	3	0.992–0.994	0.993

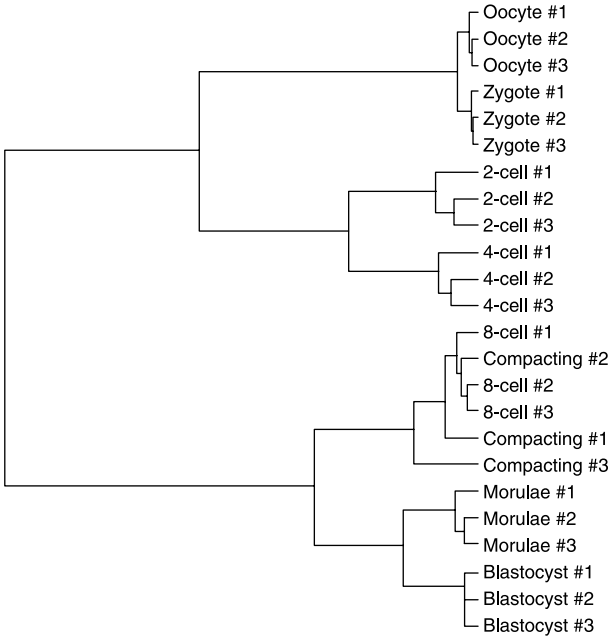

Figure 1. Hierarchical clustering of gene expressions across the preimplantation development time course. Note the closeness and some intermixing of the 8-cell and compacting embryos, which indicates similar expression profiles.

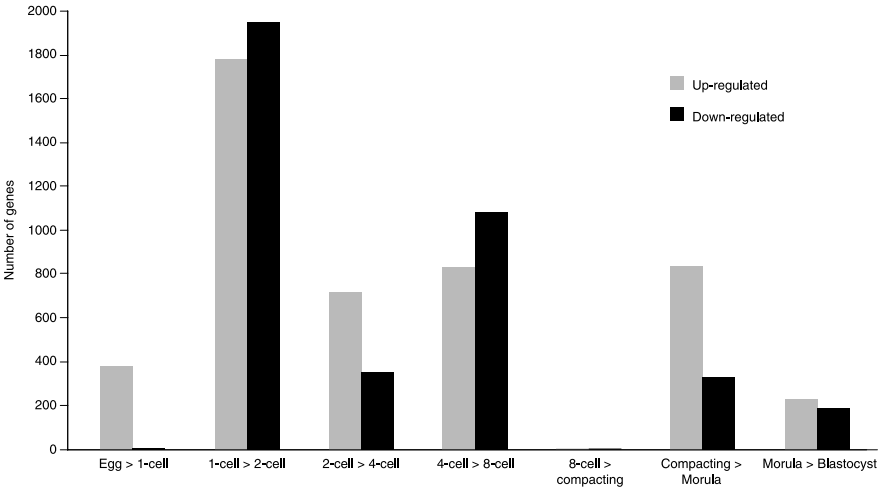

Figure 2. Number of genes up- and down-regulated between different developmental stages. The threshold was set as 2-fold change. Note significant numbers of genes were up- and down-regulated when embryos developed from 1- to 2-cell stage, while almost no change was observed between 8-cell and compacting embryos.

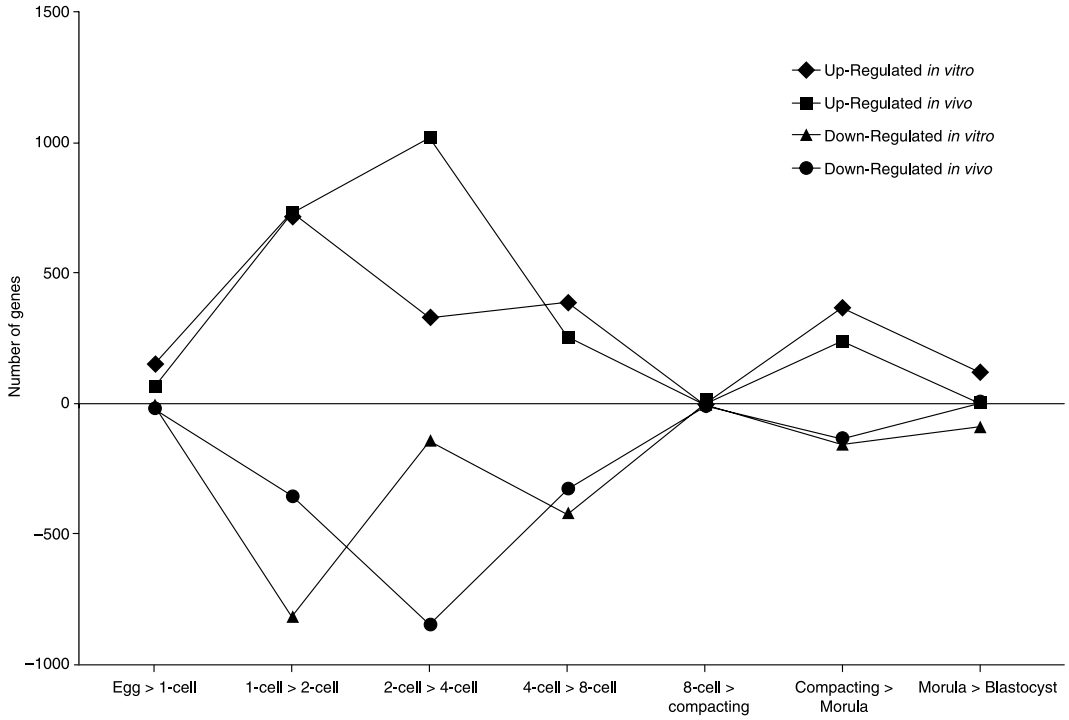

Figure 3. Developmental profile of gene regulation for both *in vitro* and *in vivo*. Y-axis represents the number of genes up- or down-regulated, using 2-fold change as threshold. Note the similar trends of up-regulated genes for both *in vivo* and *in vitro*, except the stage between 2- and 4-cell stage.

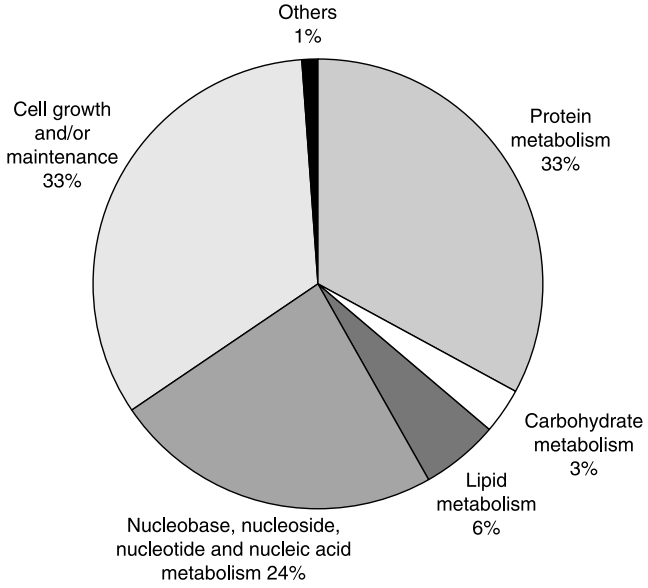

Figure 4. A significant number of genes (693 genes) were observed between in-vitro and in-vivo embryos in terms of up-regulated gene numbers when they developed from 2- to 4-cell. This pie chart shows the gene ontology analysis of these 693 genes. Note that metabolism-related genes account for 66% of the total genes.

nucleobase, nucleoside, nucleotide and nucleic acid metabolism. The remaining 33% of the genes belonged to the category of cell growth/maintenance and others.

There have been many reports on the expression of individual genes at specific stages of the preimplantation embryo. The expression patterns of several genes known to be of importance in the control of development were examined. Since the activity of these genes has been previously described only at specific stages, it was intended to clarify their role by determining the expression profile. The impact of long-term culture was also examined, by comparing the expression patterns between the cultured embryos and embryos derived *in vivo*. **Figure 5a**, **b**, and **c** show three genes that have been demonstrated to be involved in apoptosis. E2F-1, a member of a transcription factor family consisting six E2F species, is tightly regulated through cell cycles. It is postulated that E2F-1 plays an important role in the apoptotic pathways in early embryos (de Bruin *et al*., 2003). Palena and colleagues reported that E2F-1 mRNA was abundant in eggs, gradually decreased during the first rounds of cleavage and was newly detected at high concentrations in blastocysts (Palena *et al*., 2000). However, in in-vitro microarray data, E2F-1 was present at the egg and zygote stage. The expression level increased at the 2-cell stage, then decreased dramatically at the 4-cell stage, and became almost undetectable after the 8-cell stage. The in-vivo pattern of E2F-1 was quite similar; the expression level began to decrease sharply after the 2-cell stage, and remained at a low level after the 8-cell stage (**Figure 5a**).

Caspases are a family of intracellular proteases that are involved in initiating the cellular events of apoptosis in preimplantation stage embryos. However, very little is known about where and when these genes are expressed in these embryos. The expression of caspase-6, which is an effector caspase, has been demonstrated in rat blastocysts (Hinck *et al*., 2003). The data show that the expression level of caspase-6 remains low before the 2-cell stage, and begins to increase and reach the highest level in compacting embryos, then starts to decrease again. In-vivo data show a remarkably similar expression pattern for this gene compared with embryos in the present culture system (**Figure 5b**). Janus kinase 2 (Jak2), which is a member of the Janus protein tyrosine kinase family, is involved in cytokine signal transduction and in the regulation of cell growth and gene expression. It has been previously reported that Jak2 mRNA level is highest in unfertilized oocytes, gradually decreasing until the 4-cell stage, and remains at low levels until the blastocyst stage (Ito *et al*., 2004). The present data show that the expression level of this gene slightly increases at the 1- and 2-cell stage; gradually decreases at the 4-cell stage, then sharply decreases at the 8-cell stage and remains low until blastocyst. For this gene, in-vivo data once again show an expression pattern comparable with in-vitro cultured embryos (**Figure 5c**).

The energy sources and metabolism of embryos at different stages have been intensively investigated for many years, with the main focus on enzyme activities and concentrations. This study examined mRNA transcripts of several enzymes throughout preimplantation development. The result is shown in **Figure 5d** and **e**. Lactate dehydrogenase, an enzyme that converts lactate to pyruvate, has been reported to be highest at the zygote stage, decreasing at the blastocyst stage (Lane and Gardner, 2000). In in-vitro data, the concentration of mRNA for this enzyme started high in oocytes, increased slightly at zygote, then decreased afterwards and became almost undetectable after the 8-cell stage. The in-vivo data did not show the slight increase at the 2-cell stage, but overall the expression pattern throughout preimplantation stage was similar (**Figure 5d**). Hexokinase, which catalyses the first reaction in glycolysis, increases during later stages of human and mouse embryos. The enzymatic activity reached the highest level in blastocysts (Brinster, 1968). A previous study observed an increased level of hexokinase gene expression in blastocysts in comparison with morulae (Johnson *et al*., 1997). In contrast, the present data show that the expression level of this gene peaks at the 8-cell stage and begins to decrease after compaction. The morulae have slightly higher expression levels than the blastocyst, although the difference may not be significant. The expression pattern of this gene for in-vivo embryos is slightly different from the present data as the level remained consistent after the 8-cell stage. However, the difference was not statistically significant (**Figure 5e**).

Adhesion molecules play an important role in preimplantation embryonic development. Integrins, which are heterodimers composed of an α and β subunit, have been intensively studied (Humphries, 2000). In the present study, alpha 6 showed a strong presence before the zygote stage, decreased to a low level and began to increase again in morulae and blastocysts. On the whole, the expression pattern of this gene for in-vivo embryos was comparable (**Figure 5f**). Cadherin-11, which belongs to a gene superfamily of integral membrane glycoproteins that mediate cell adhesion (Getsios and MacCalman, 2003), showed a steadily increased level after the zygote and peaked at the blastocyst in the present data. It is worth noting that in-vivo data showed quite a different pattern for this gene, with the expression level dropping to the lowest point at the 4-cell stage, and steadily increasing again until morulae, although the difference was not significant (**Figure 5g**). Both the in-vitro and in-vivo observations differ from a previous publication in which alpha 6 integrin was first detected in late blastocysts (Bloor *et al*., 2002).

DNA methylation, which is an epigenetic modification of the genome, is essential for embryonic development (Robertson, 2002). DNA methyltransferase 1 (DNMT1), a DNA cytosine methyltransferase, which has been identified in human and mouse previously, is not expressed in the embryo after implantation, but is ubiquitously expressed afterwards in somatic proliferating cells (Chen *et al*., 2003; Liu *et al*., 2003). In this study, this gene had a unique expression pattern *in vitro*: it showed a sharp surge at 1-cell, and then decreased significantly to base level after the 2-cell stage (**Figure 5h**). Interestingly, the in-vivo data showed a pattern distinct from the current data, especially at the zygote stage. The expression of this gene was low before 2-cell stage and remained low afterwards. Unlike in-vitro data, the peak at the zygote stage was not observed.

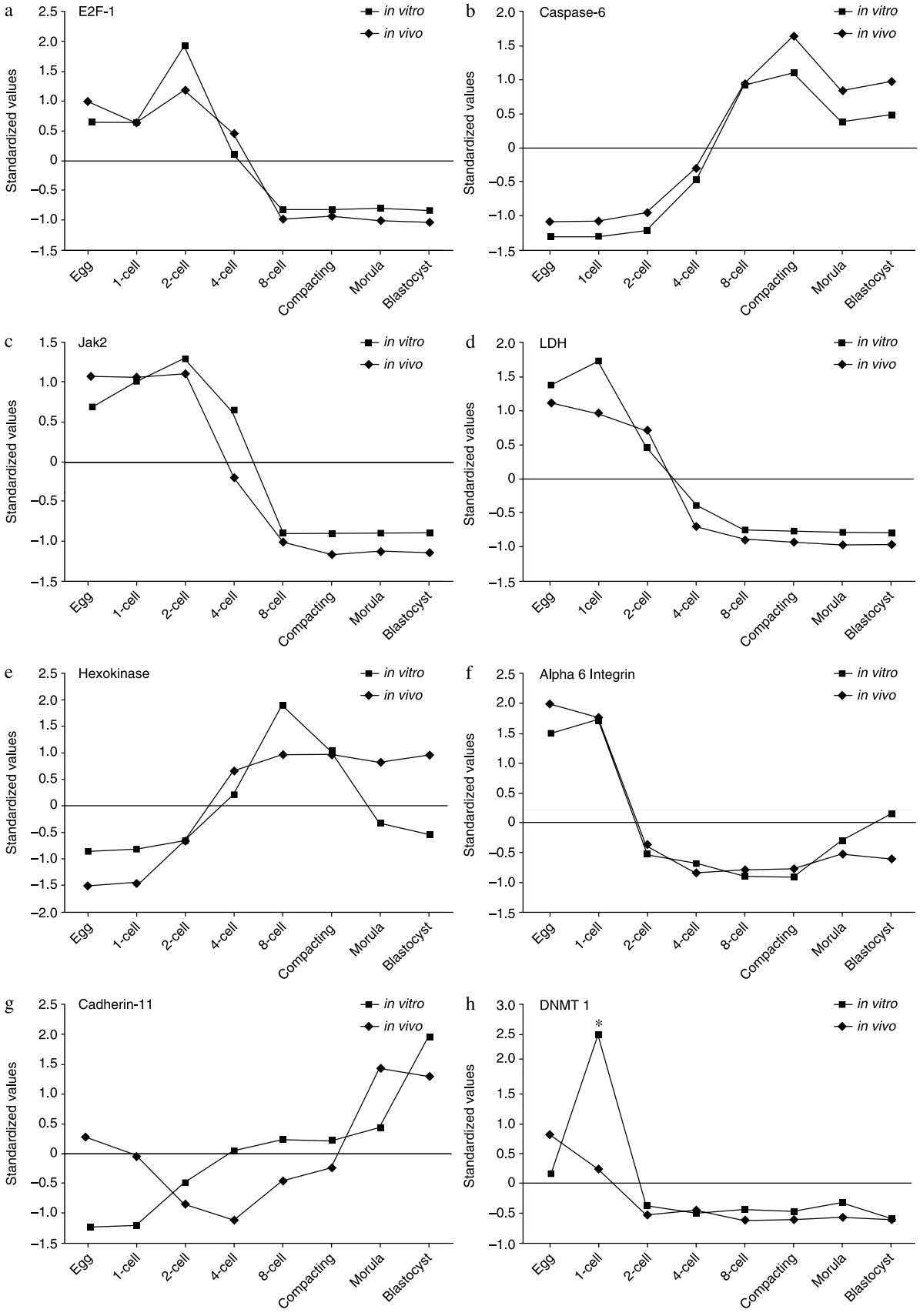

Figure 5. Comparisons of gene expression patterns between cultured embryos and in-vivo embryos. (**a**) e2f-1, (**b**) caspase-6, (**c**) jak2, (**d**) lactate dehydrogenase, (**e**) hexokinase, (**f**) alpha 6 integrin, (**g**) cadherin-11, (**h**) dnmt1. *$P < 0.05$.

Discussion

This study has demonstrated that RNA can be extracted, amplified linearly and hybridized onto DNA chips in a highly reproducible manner from relatively few embryos.

Consistency and reproducibility have been major concerns with microarray studies, especially with small amounts of materials such as preimplantation stage embryos. The high correlation coefficients in the present study showed that reproducible results could be obtained by the methods employed. The slightly lower γ (0.962) observed at the 2-cell stage may be due to the increased variance of gene expression profiles. Typical 2-cell embryos take approximately 10 h to develop into 4-cell embryos. Although morphologically staged as 2-cell embryos, gene expression profiles in later 2-cell may be significantly different from those of early 2-cell.

Hierarchical clustering analysis categorizes the results based on the similarity of the datum points to one another. The outcome of the analysis is shown as cluster diagrams displaying 'terminal branches' where samples or experiments with similar overall gene expressions cluster together. The software clusters samples with the highest similarity of gene patterns. In the present study, all the replicates at the same stage were clustered under same branch, which further indicated the consistency of the experiments. The only exceptions are the 8-cell/compacting embryo stages. Instead of two separate branches, the two stages (six replicates) mingled together in the clustering tree. This indicates that 8-cell/compacting embryos have indistinguishable gene expression patterns, despite their obvious morphological difference.

A significant number of genes were up-regulated when embryos developed from the zygote to 2-cell stage, which corresponded to zygote genome activation. It is well documented that the first burst of transcription, zygote genome activation (ZGA), begins following fertilization and is nearly completed by the 2-cell stage, which corresponds to the present data. In addition, a large number of genes were down-regulated at the same stage, indicating the degradation of maternal transcripts. Transcription remained active afterwards, although the change was not as dynamic as what happened between zygote and 2-cell stage. Surprisingly, very few genes were up-regulated when embryos began to compact, where they underwent significantly morphological change. Thus, the genes involved in compaction are likely transcribed at or before the 8-cell stage.

The relative changes in transcription patterns at different stages in development were striking. At each stage shown in **Figure 3**, there was an inverse relationship between up and down gene regulation. The increase in the number of genes actively transcribed was paralleled by a decrease in transcripts from a remarkably similar number of genes. This observation is repeated *in vitro* and *in vivo*. This strongly suggests that the total number of actively transcribed genes is maintained at a constant level during mammalian preimplantation development. Investigations are continuing into the connection between mRNA synthesis and degradation in the embryo.

Concerns have been raised recently about the safety of assisted reproductive technology, especially on the long-term consequences of culture. Some studies suggest that embryo culture could possibly perturb embryo metabolism and demonstrated specific aberrant animal behaviours (Ecker *et al*., 2004). A recent paper also reported the effects of different culture media on global patterns of gene expression in preimplantation mouse embryos (Rinaudo and Schultz, 2004). Embryos were cultured in different media (Whitten's and KSOM/AA) and the gene expression patterns were compared by microarray. However, only blastocyst stages were used as end-points, and culture media compared are not widely used in clinical IVF settings. In addition, no in-vivo embryos were used for comparison. This study examined gene expression patterns of embryos cultured for an extended period of time in an IVF laboratory setting with careful pH measurements, CO_2 calibration, minimal light exposure, and intensively modified medium used in a typical IVF setting. The results were compared with an independent study (Wang *et al*., 2004) where embryos were obtained *in vivo* at corresponding time points.

Interestingly, the trends for up-regulated genes throughout the entire preimplantation stage between *in vivo* and *in vitro* were strikingly similar, with the exception of the 2- to 4-cell stage. The numbers of genes up-regulated between 1- and 2-cell stage, where embryonic genome activation (EGA) or zygote genome activation (ZGA) occurs, were almost identical (715 *in vitro* versus 724 *in vivo*). The difference accounts for only 0.1% among all the 8000 genes compared. Other stages (with the exception of 2- to 4- cell stage) also showed very similar numbers of up-regulated genes, with 1.6% as the largest difference (4- to 8-cell stage). The similarity was remarkable, considering not only that the embryos were cultured *in vitro* and derived *in vivo* separately, but also that all the experimental procedures, including embryo collection, amplification, and hybridization, were all conducted with different settings in two different laboratories. The concentrations of mRNA observed in the experiments depend on the interaction of synthesis and degradation. Given the possible in-vitro perturbations to either of these very complex processes, it is remarkable that the differences in gene expression are almost non-existent at most of the stages examined.

A noticeable difference in terms of the number of up-regulated genes was observed between 2- to 4-cell stages. One thousand and twenty-seven genes were up-regulated at this stage for in-vivo embryos, while only 334 genes were seen up-regulated on embryos cultured *in vitro*. Gene ontology analysis showed that the difference of more than 600 genes was mainly metabolism-related. The remaining one-third of the genes fell into the category of cell growth and/or maintenance. Among the metabolism category, a major portion of the genes were involved with protein and nucleic acid metabolism. A possible explanation is that the nutrients and the micro-environments are very different for embryos *in vivo* and *in vitro*. Embryos in culture systems are confined in drops where metabolites would be concentrated over extended period of culture time. In addition, signalling pathways such as paracrine and autocrine effects are also crucial for embryo development, especially for stage where embryos are in active metabolism. The causes for the decrease of the up-regulated genes at this stage need to be further investigated.

A significant difference was also observed between in-vivo and in-vitro embryos with respect to down-regulated genes before 2- to 4-cell stages. For in-vitro embryos, the degradation of maternal transcripts seemed to occur at an earlier stage (1- to 2-cell), compared with in-vivo embryos (2- to 4-cell), although the total numbers of down-regulated genes at these two stages for both in-vivo and in-vitro embryos were very close. The early degradation of maternal transcript of in-vitro embryos may also explain the decrease of up-regulated genes at 2- to 4-cell stage, where maternal mRNAs may be indispensable for the transcription of new genes at later stages.

The great similarity of in-vitro and in-vivo gene expression patterns provides evidence that modern IVF culture media cause very little perturbation in gene regulation at most stages of in-vitro development. In addition, observations on those genes that do show a difference provide a basis for further research, particularly in the area of media development.

When the expression pattern of several genes that are known to play important roles in early embryo development was examined, it was found that, in general, the gene expression patterns shown in the present data confirm previous studies. However, the careful application of DNA microarray analysis to multiple stages of development provides a much more complete picture of the molecular biology of the pre-implantation mammalian embryo. With this analysis, a dynamic picture is now available of gene regulation from oocyte to blastocyst. The data, and the in-vivo data, confirm the safety of in-vitro culture by demonstrating both the temporal and quantitative correlation of gene regulation in both systems.

The disparity of the expression levels of certain genes, such as DNMT1, may suggest an important difference in imprinting status and the biological significance cannot be ignored. Further studies are underway to elucidate the role of imprinting effects on early embryo development.

It is significant that the in-vitro and in-vivo studies were performed in different laboratories by different personnel using different strains of mice. This makes the very close similarity of the gene expression profiles very striking. As mentioned above, the DNA chips used for the studies were different, so that only about 8000 common probes could be used for comparison. This may compromise the validity of the overall comparisons since the common probes only represent a portion of the total genes. Whether the gene expression profiles of those non-common probes differ between in-vitro and in-vivo embryo or not still remains unknown. It is not clear whether the strain difference may contribute to divergence of gene expression profiles. The different strains of mice may amplify the difference. In addition, the two-step amplification from the original materials may also introduce a significant amount of noise in both studies. This experimental variation may create an apparent, but unreal difference between the two systems.

In this study, gene expression profiles have been established in more than 20,000 genes of mouse embryos cultured *in vitro* throughout the entire preimplantation stages. The great similarity between embryos *in vivo* and cultured *in vitro* with respect to gene patterns has also been demonstrated. Future studies will focus on the causes of the small but potentially important differences in gene expression and their impact on cultured mouse and human embryos.

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Declaration

The microarray dataset has been submitted to the public database ArrayExpress.

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