Focus on ART

Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos

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Abstract

Culture of preimplantation embryos affects gene expression. The magnitude of the effect on the global pattern of gene expression, however, is not known. We compared global patterns of gene expression in blastocysts cultured from the one-cell stage in either Whitten's medium or KSOM + amino acids (KSOM/AA) with that of blastocysts that developed *in vivo*, using the Affymetrix MOE430A chip. The analysis revealed that expression of 114 genes was affected after culture in Whitten's medium, whereas only 29 genes were mis-expressed after culture in KSOM/AA. Expression Analysis Systematic Explorer was used to identify biological and molecular processes that are perturbed after culture and indicated that genes involved in protein synthesis, cell proliferation and transporter function were down-regulated after culture in Whitten's medium. A common set of genes involved in transporter function was also down-regulated after culture in KSOM/AA. These results provide insights as to why embryos develop better in KSOM/AA than in Whitten's medium, and highlight the power of microarray analysis to assess global patterns of gene expression.

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Introduction

Preimplantation embryos can develop in media of composition ranging from simple balanced salt solutions and carbohydrates to very complex constituents (e.g. Ham's F-10) with the further addition of serum or a feeder layer of somatic cells (Lane 2001, Natale *et al.* 2001, Niemann & Wrenzycki 2000). The blastocysts that develop after culture are known to be developmentally competent because, after embryo transfer, live offspring are born (Ecker *et al.* 2004). Nevertheless, embryo culture can perturb gene expression (Ho *et al.* 1995). Of particular note is that expression of imprinted genes appears particularly sensitive to culture conditions. For example, appropriate maternal monoallelic expression of *H19* is observed after culture in K modified simplex optimized medium (KSOM) + amino acids (KSOM/AA), whereas biallelic expression is found after culture in Whitten's medium (WM) (Doherty *et al.* 2000). Moreover, biallelic expression is accompanied by loss of DNA methylation of cytosine residues on the paternal allele in the differentially methylated domain essential for repression of the paternal allele

(Doherty *et al.* 2000). We elected to assess the effect of embryo culture on global patterns of gene expression in embryos cultured in these two media using oligonucleotide microarrays, because of these aforementioned differences in maintaining or not maintaining appropriate monoallelic maternal *H19* expression.

Until recently, quantitative analysis of high-resolution, two-dimensional protein gels (Latham *et al.* 1991), mRNA differential display (Ma *et al.* 2001), analysis of expressed sequence tags derived from libraries of various preimplantation stages (Ko *et al.* 2000, Sharov *et al.* 2003) and analysis of selected genes (Ho *et al.* 1995) have been used. Although these approaches have shed some light on the molecular basis underlying preimplantation development, they offer limited insight because only a small number of genes can be readily analyzed. Microarray techniques provide a powerful approach to study patterns of gene expression on a global scale (Hamatani *et al.* 2004, Wang *et al.* 2004). The ability to amplify the small amounts of mRNA present in preimplantation mouse embryos, which can only be isolated in limited numbers, makes it feasible to generate enough material for microarray analysis. We used Affymetrix oligonucleotide arrays (MOE430A chip)

containing more than 22 000 transcripts and variants, together with a T7-based linear double amplification method, and report here changes in the global patterns of gene expression that occurred during preimplantation development in mouse blastocysts cultured in WM or KSOM/AA from the one-cell stage. We report that, after culture in WM, 114 genes were mis-expressed, whereas only 29 were mis-expressed after culture in KSOM/AA. Of note is that 14 common genes were mis-expressed in either medium and are involved in ion and water transport.

Materials and Methods

Collection of preimplantation mouse embryos and RNA extraction

Mouse CF-1 $\times$ B6D2F1/J embryos were isolated from superovulated mice as previously described (Zeng & Schultz 2003). Briefly, CF-1 female mice were injected with 5IU equine chorionic gonadotrophin and 42–46 h later with 5IU human chorionic gonadotrophin (hCG); the females were then mated to B6D2F1/J males overnight. The next morning, zygotes were obtained from the ampullae and cultured in either WM (Whitten 1971) or KSOM/AA (Ho *et al.* 1995) to the blastocyst stage in 5% CO₂–5% O₂–90% N₂ at 37 °C. Cultures were initiated with more embryos when WM was used, to compensate for the poorer development in WM compared with that in KSOM/AA. After culture in either medium, late-cavitating blastocysts of similar morphology were harvested at 96 h post-hCG. Embryos that developed *in vivo* were harvested 96 h post-hCG and those that looked morphologically similar to embryos that developed *in vitro* were collected and used for analysis. Cell numbers were determined by differential labeling of inner cell mass and trophectoderm cells as previously described (Handyside & Hunter 1984). The numbers of total cells and of inner cell mass cells after culture in WM were 70.6 ± 4.3 (mean $\pm$ S.E.M.) and 16.6 ± 1.2 respectively. Corresponding numbers after culture in KSOM/AA were 81.8 ± 3.8 and 23.9 ± 1.7 respectively. All animal experiments were approved by the Institutional Animal Care and Use Committee and were consistent with NIH guidelines.

Total RNA was extracted from pools of 80 embryos (120 ng total RNA) using Trizol containing 2 μ l Pellet Paint (Novagen, Madison, WI, USA) according to the manufacturer's instructions (Invitrogen). Total RNA was dissolved in 10 μ l sterile water and stored at –80 °C. RNA mass and size distribution were determined using the Agilent Bioanalyzer with RNA 6000 Nano LabChips (Palo Alto, CA, USA).

cDNA preparation for microarray analysis

Total RNA samples were submitted to the Penn Microarray Facility for target preparation and GeneChip

hybridization. Total RNA yield was 74–133 ng per replicate pool. This total RNA was used for linear, two-round amplification by *in vitro* transcription (Affymetrix Small Sample Target Labeling Assay version II, www.affymetrix.com). cRNA yield after the first amplification was 1.5–5 μ g, and 0.5 μ g of each replicate was used as input template for the second amplification. Final yield of biotinylated cRNA was 74–138 μ g, of which 15 μ g per replicate was fragmented and hybridized to Affymetrix GeneChips. cRNA samples were hybridized to MOE430A GeneChip, then washed and stained on fluidics stations and scanned at 3 μ m resolution according to the manufacturer's instructions (GeneChip Analysis Technical Manual, www.affymetrix.com).

Analysis of the microarrays

Microarray Analysis Suite 5.0 (MAS; Affymetrix, Santa Clara, CA, USA) was used to quantify microarray signals with default analysis parameters and global scaling to target mean = 150. Quality control parameters for all samples were within the following ranges: scale factor 1–2.7, background 42–88, percent genes detected 37–44% on MOE430A, actin 3'/5' signal ratio 1.5–2.8, and GAPDH 3'/5' signal ratio 2.3–7.5. The MAS metrics output was loaded into GeneSpring v5 (Silicon Genetics, www.silicongenetics.com) with per-chip normalization to the 50th percentile and per-gene normalization to the median. A filtered list was created of all genes detected (MAS 'P' call) in at least five of six replicates of the *in vivo* group.

Independent analyses were applied to identify genes with statistically significant differences in any of the two culture conditions (WM or KSOM/AA). The GeneSpring pairwise comparison (Welch *t*-test with ANOVA, $P = 0.05$, Benjamini and Hochberg multiple testing correction) was conducted between all the possible pair combinations (*in vivo*, WM, KSOM/AA). The GeneSpring multi-class analysis was applied to the entire sample set (Welch *t*-test with Welch ANOVA, $P = 0.05$, Benjamini and Hochberg multiple testing correction). A one-way ANOVA for microarrays (lgsun.grc.nia.nih.gov/ANOVA/index.html, default parameters) was also conducted. A non-redundant list was compiled containing candidate genes called significantly different in at least one analysis.

Genes exhibiting altered expression after culture were imported to Expression Analysis Systematic Explorer (EASE) to test for over-representation of annotation classes (Hosack *et al.* 2003). EASE is a program that provides statistical methods (reported as an EASE score) for discovering biological themes within gene lists, using previously published annotation databases. Over-representation does not refer to abundance of gene expression, but rather describes a class of genes that have similar functions – for example, transcription factors – regardless of their expression level, and appear more often in a list of interest

Figure 1 Hierarchical clustering analysis of all samples. Unsupervised clustering in GeneSpring was used to analyze similarities among replicate samples across all stages tested. Replicate sample numbers are indicated at the bottom of the figure. Colors correspond to relative RNA abundance for the ~9000 genes detected (Affymetrix 'Present' call), each of which is represented by one horizontal bar. W, Whitten's medium; K, KSOM + amino acids.

than would normally be predicted by their distribution among all genes assayed. The method incorporates jack-knife iterative re-sampling of Fisher exact probabilities, with Bonferroni multiple testing corrections. An EASE score was calculated for likelihood of over-representation in the annotation categories GO Biological Process, GO Cell Component, GO Molecular Function, KEGG Pathway, and SwisProt keyword. GeneSpring 'genomes' were built for each of these annotation categories so that EASE scores

Figure 2 Differential expression profiles of genes after culture in either Whitten's medium (WM) (A) or KSOM/AA (KAA) (B).

from all subset lists could be visualized in parallel across all treatment and control groups, and pattern identification methods similar to those applied at the gene level were used to find functional themes in the data set. All reported expression differences from gene-based or annotation-based profiling were re-tested for statistical significance by multi-class ANOVA as appropriate.

Real time RT-PCR analysis

Blastocysts developed *in vivo* and *in vitro* (WM and KSOM/AA) were collected as described above and total RNA was isolated. Two embryo equivalents of template RNA were used for each real-time RT-PCR assay according to the manufacturer's procedure using ABI Prism Sequence Detection System 7000 (Applied Biosystems, Foster City,

Table 1 Genes up-regulated after culture in Whitten's medium.

Fold change	Gene symbol	Gene name	Unigene number
2.648	<i>Spnb2</i>	beta-spectrin 2, non-erythrocytic	Mm.123110
2.193	<i>Nsap1l</i> -(pending)	NS1-associated protein 1-like	Mm.196461
1.616	<i>2610016C23Rik</i>	RIKEN cDNA 2610016C23 gene	Mm.34156
1.595	<i>Ptpns1</i>	protein tyrosine phosphatase, receptor type, S	Mm.258771
1.461	<i>Ptpns1</i>	protein tyrosine phosphatase, non-receptor type substrate 1	Mm.4420
1.276	<i>Ptpn21</i>	protein tyrosine phosphatase, non-receptor type 21	Mm.1682

Table 2 Genes down-regulated after culture in Whitten's medium.

Fold change	Gene symbol	Gene name	Unigene number
0.14	<i>5730469M10Rik</i>	RIKEN cDNA 5730469M10 gene	Mm.27227
0.20	<i>Snap91</i>	synaptosomal-associated protein, 91 kDa	Mm.4870
0.21	<i>Ndr1</i>	N-myc downstream regulated 1	Mm.4063
0.21	<i>9030418M05Rik</i>	RIKEN cDNA 9030418M05 gene	Mm.45994
0.26	<i>Ptk6</i>	PTK6 protein tyrosine kinase 6	Mm.4497
0.27	<i>Ndr1</i>	N-myc downstream regulated 1	Mm.30837
0.27	<i>Ccnd1</i>	cyclin D1	Mm.22288
0.28	<i>Msx1</i>	homeo box, msh-like 1	Mm.259122
0.30	<i>Slc7a3</i>	solute carrier family 7 (cationic amino acid transporter, y + system), member 3	Mm.5140
0.31	<i>S100a10</i>	S100 calcium binding protein A10 (calpactin)	Mm.1
0.31	<i>Aqp8</i>	aquaporin 8	Mm.9970
0.33		EST, weakly similar to CAMP [<i>Mus musculus</i>] [<i>M.musculus</i>]	Mm.270928
0.34	<i>Lcp1</i>	lymphocyte cytosolic protein 1	Mm.153911
0.34	<i>Il6</i>	interleukin 6	Mm.1019
0.35	<i>Pip5k2a</i>	phosphatidylinositol-4-phosphate 5-kinase, type II, alpha	Mm.8771
0.36	<i>1810018M05Rik</i>	RIKEN cDNA 1810018M05 gene	Mm.29379
0.38	<i>Tdgf1</i>	teratocarcinoma-derived growth factor	Mm.5090
0.40		hypothetical protein C030011B15	Mm.202715
0.40	<i>Arhu</i>	ras homolog gene family, member U	Mm.28954
0.40	<i>Efnb1</i>	ephrin B1	Mm.3374
0.41	<i>Upp</i>	uridine phosphorylase	Mm.4610
0.42	<i>Car4</i>	carbonic anhydrase 4	Mm.1641
0.43	<i>Slc15a2</i>	solute carrier family 15 (H ⁺ /peptide transporter), member 2	Mm.63479
0.44	<i>C77258</i>	eomesodermin homolog (<i>Xenopus laevis</i>)	Mm.200692
0.45	<i>1810017F10Rik</i>	RIKEN cDNA 1810017F10 gene	Mm.25295
0.46	<i>Lgals3</i>	lectin, galactose binding, soluble 3	Mm.2970
0.46	<i>Nid1</i>	nidogen 1	Mm.4691
0.47	<i>Ccni</i>	cyclin I	Mm.22711
0.47	<i>Cldn6</i>	claudin 6	Mm.86421
0.47	<i>Tex20</i>	testis expressed gene 20	Mm.28388
0.47	<i>Btg1</i>	B-cell translocation gene 1, anti-proliferative	Mm.16596
0.47	<i>D6Ertd263e</i>	DNA segment, Chr 6, ERATO Doi 263, expressed	Mm.195955
0.48	<i>Fem1b</i>	feminization 1 homolog b (<i>C. elegans</i>)	Mm.24069
0.48	<i>Np95</i>	nuclear protein 95	Mm.42196
0.48	<i>Niban</i>	niban protein	Mm.157700
0.48	<i>Ube2a</i>	ubiquitin-conjugating enzyme E2A, RAD6 homolog (<i>S. cerevisiae</i>)	Mm.27541
0.49	<i>Grhl1</i> -(pending)	grainyhead like 1 (<i>Drosophila</i>)	Mm.25552
0.49	<i>Gpx1</i>	glutathione peroxidase 1	Mm.1090
0.49	<i>Dkk1</i>	dickkopf homolog 1 (<i>Xenopus laevis</i>)	Mm.7960
0.49	<i>Tgut</i> -(pending)	tRNA-guanine transglycosylase	Mm.24178
0.50	<i>Enpp1</i>	ectonucleotide pyrophosphatase/phosphodiesterase 1	Mm.27254
0.50	<i>Atp6v0c</i>	ATPase, H ⁺ transporting, V0 subunit C	Mm.30155
0.50	<i>1110013H04Rik</i>	RIKEN cDNA 1110013H04 gene	Mm.7863
0.51	<i>Rpl13a</i>	ribosomal protein L13a	Mm.180458
0.51	<i>Ftl1</i>	ferritin light chain 1	Mm.7500
0.52	<i>Spp1</i>	secreted phosphoprotein 1	Mm.260317
0.52	<i>D10Ucla1</i>	DNA segment, Chr 10, University of California at Los Angeles 1	Mm.41272
0.52	<i>Lamr1</i>	Laminin receptor 1	Mm.4071
0.53	<i>Oaz1</i>	ornithine decarboxylase antizyme	Mm.683
0.53	<i>Tex19</i>	testis expressed gene 19	Mm.12669
0.53	<i>Ftl2</i>	ferritin light chain 2	Mm.30357
0.54	<i>Ldh1</i>	lactate dehydrogenase 1, A chain	Mm.29324
0.54	<i>D16Bwg1494e</i>	hypothetical protein MNCb-4137	Mm.246388
0.54	<i>Ephx2</i>	epoxide hydrolase 2, cytoplasmic	Mm.15295
0.54	<i>Tcl1</i>	T-cell lymphoma breakpoint 1	Mm.18154
0.54	<i>Slc15a2</i>	solute carrier family 15 (H ⁺ /peptide transporter), member 2	Mm.63479
0.55	<i>Rps8</i>	ribosomal protein S8	Mm.3381
0.56	<i>Plp2</i>	proteolipid protein 2	Mm.18565
0.56	<i>Pla2g10</i>	phospholipase A2, group X	Mm.4214
0.56	<i>Ppap2a</i>	phosphatidic acid phosphatase 2a	Mm.2598
0.56	<i>MGC36305</i>	hypothetical protein MGC36305	Mm.203937
0.56	<i>Ppp1ca</i>	protein phosphatase 1, catalytic subunit, alpha isoform	Mm.1970

Table 2 Continued

Fold change	Gene symbol	Gene name	Unigene number
0.57	<i>MGC27616</i>	hypothetical protein MGC27616	Mm.50610
0.57	<i>Osgep</i>	O-sialoglycoprotein endopeptidase	Mm.29642
0.57	<i>S100a10</i>	S100 calcium binding protein A10 (calpactin)	Mm.1
0.57	<i>Gpx4</i>	glutathione peroxidase 4	Mm.2400
0.57	<i>1500032D16Rik</i>	RIKEN cDNA 1500032D16 gene	Mm.28349
0.57	<i>Hmg20b</i>	high mobility group 20 B	Mm.38474
0.57	<i>Slc16a1</i>	solute carrier family 16 (monocarboxylic acid transporters), member 1	Mm.9086
0.58	<i>Prss8</i>	protease, serine, 8 (prostasin)	Mm.5875
0.58	<i>4930471M23Rik</i>	RIKEN cDNA 4930471M23 gene	Mm.247985
0.58	<i>Rps17</i>	ribosomal protein S17	Mm.42767
0.58	<i>2410015N17Rik</i>	RIKEN cDNA 2410015N17 gene	Mm.29898
0.58	<i>MGC28180</i>	Mus musculus, clone IMAGE:3995663, mRNA	Mm.267131
0.59	<i>Rpl13a</i>	ribosomal protein L13a	Mm.180458
0.59	<i>Prkcz</i>	protein kinase C, zeta	Mm.28561
0.60	<i>Nap111</i>	nucleosome assembly protein 1-like 1	Mm.3797
0.61	<i>Rpl10a</i>	ribosomal protein L10A	Mm.2424
0.61	<i>1110020C13Rik</i>	RIKEN cDNA 1110020C13 gene	Mm.29865
0.62	<i>Igf2bp1</i>	insulin-like growth factor 2, binding protein 1	Mm.12919
0.63	<i>Renbp</i>	renin binding protein	Mm.28280
0.63	<i>Lsm4</i> -(pending)	U6 snRNA-associated SM-like protein 4	Mm.14585
0.63	<i>Prdx2</i>	peroxiredoxin 2	Mm.42948
0.63	<i>5730578N08Rik</i>	RIKEN cDNA 5730578N08 gene	Mm.46480
0.64	<i>Tmsb10</i>	thymosin, beta 10	Mm.3532
0.65	<i>Dlg5</i>	discs, large homolog 5 (Drosophila)	Mm.68971
0.65	<i>Taldo1</i>	transaldolase 1	Mm.29182
0.65	<i>Scotin</i> -(pending)	scotin gene	Mm.196533
0.66	<i>Klf9</i>	Kruppel-like factor 9	Mm.19788
0.66	<i>Ssfa1</i>	sperm specific antigen 1	Mm.29504
0.67	<i>Ifi30</i>	interferon gamma inducible protein 30	Mm.30241
0.67	<i>2410002K23Rik</i>	RIKEN cDNA 2410002K23 gene	Mm.182051
0.67	<i>Gnb2-rs1</i>	guanine nucleotide binding protein, beta 2, related sequence 1	Mm.5305
0.67	<i>Rps17</i>	ribosomal protein S17	Mm.42767
0.67	<i>2310009N05Rik</i>	RIKEN cDNA 2310009N05 gene	Mm.29247
0.68	<i>Ran</i>	RAN, member RAS oncogene family	Mm.7521
0.68	<i>0610011C19Rik</i>	RIKEN cDNA 0610011C19 gene	Mm.27792
0.68	<i>Rps14</i>	ribosomal protein S14	Mm.43778
0.68	<i>Rpl13</i>	ribosomal protein L13	Mm.42578
0.68	<i>D11Ert672e</i>	DNA segment, Chr 11, ERATO Doi 672, expressed	Mm.27670
0.68	<i>Actb</i>	actin, beta, cytoplasmic	Mm.297
0.69	<i>Txn1</i>	thioredoxin 1	Mm.1275
0.69	<i>1300011C24Rik</i>	RIKEN cDNA 1300011C24 gene	Mm.186
0.69	<i>Dnajb1</i>	DnaJ (Hsp40) homolog, subfamily B, member 1	Mm.2982
0.70	<i>LOC224023</i>	similar to RIKEN cDNA 1200009K10	Mm.38788
0.72	<i>Ptdsr</i>	Phosphatidylserine receptor	Mm.24997
0.73	<i>DXImx38e</i>	DNA segment, Chr X, Immunex 38, expressed	Mm.28850
0.73	<i>Rps5</i>	ribosomal protein S5	Mm.5291

CA, USA). To confirm the ability of this microarray analysis to resolve the differences in expression level, three genes that showed a statistically significant decrease were selected. The corresponding ABI TaqMan Assay-on-Demand probe/primer sets used were Mm00431846-m1 (*Aqp8*), Mm00500526-m1 (*Slc7a3*) and Mm00451610 (*Slc15a2*). Three replicates were used for each real-time PCR reaction; a minus template served as control. Quantification was normalized to a constant amount of pEGFPN2 RNA spiked into the RNA cocktail before the RNA extraction. Data were analyzed within the log-linear phase of the amplification curve obtained for each probe/

primer using the comparative CT method (ABI PRISM 7700 Sequence Detection System, User Bulletin no. 2).

Results and Discussion

Hierarchical cluster analysis

Blastocysts that developed after culture from the one-cell stage in either WM or KSOM/AA (four replicates of each) or blastocysts that developed *in vivo* (six replicates) were used for expression profiling. We elected to initiate culture at the one-cell stage, rather than the two-cell stage, as in the previous study that assessed the

Figure 3 Real-time PCR verification of microarray data. Three genes exhibiting decreased expression after culture in either WM or KSOM/AA were selected for analysis. The level of expression relative to embryos that developed *in vivo* by either microarray analysis or real-time PCR was set as 1. IV MA/RT, relative level of expression by embryos that developed *in vivo* (IV), assessed by either microarray (MA) or real-time (RT) PCR; KSOM/AA MA and KSOM/AA RT, relative levels of expression after culture in KSOM/AA assessed by MA or RT respectively; WM MA and WM RT, relative levels of expression after culture in WM assessed by MA or RT respectively.

effect of culture on expression of *H19* (Doherty *et al.* 2000), because the experimental design of many studies incorporates culture initiating at the one-cell stage. Moreover, culture initiated at the one-cell stage more closely mimics the experimental procedures performed in human IVF clinics.

An unsupervised hierarchical clustering was performed using genes that were present at least in five of six *in vivo* control groups. Of the 22 690 genes present on the MOE430A chip, we found that, on average, 39.2% were present in at least five of six *in vivo* replicates, and three of four WM or KSOM/AA replicates respectively. Although, the clustering dendrogram revealed that the embryos that developed *in vivo* clustered together, embryos cultured in either WM or KSOM/AA did not (Fig. 1). Analysis of the branch tree distances revealed that only minimal differences existed between the WM and KSOM/AA group and overall between the *in vivo* group and the cultured groups: the minimal branch distance between the *in vivo* replicates was 0.354 and the final clustering branch was close to one (0.999). Instead, the cultured groups had a minimal branch distance of 0.544 and the more distant replicates branched at a distance of 1.027. This suggests that, although embryo culture leads to perturbations in global patterns of gene expression, the differences between the expression profiles for embryos cultured under the two different conditions are actually quite small.

Effect of culture in WM on gene expression

Expression of 114 genes ($114/8868 = 1.28\%$) was statistically different between the two groups and satisfied the *post hoc* correction (Fig. 2) when GeneSpring pairwise comparison (Welch *t*-test with ANOVA, $P = 0.05$, Benjamini and Hochberg multiple testing correction) was conducted between the control group and the WM group. It is possible that some of the differences observed after culture in WM (or KSOM/AA) are sex-specific. Of these genes, only six were up-regulated (Table 1), whereas 108 were down-regulated (Table 2). It is interesting to note that only 2/6 (33%) and 41/108 (38%) were up-regulated or down-regulated more than twofold – that is, many of the changes were small. It is becoming more widely appreciated that modest changes in the expression of a gene can result in profound differences. An example is that changing the expression of the transcription factor OCT4 by 50% can result in changes in cell differentiation (Niwa *et al.* 2000). Thus small changes in expression of a critical transcription factor or a gene that acts in the early steps of a signal transduction pathway could function as a molecular switch that would lead to widespread and dramatic changes in gene expression.

To confirm that the differences detected from the microarray analyses were real, three genes that exhibit decreased expression after culture in either WM or KSOM/AA (see below) were selected for real-time PCR analysis. Results of this experiment confirmed that a decrease in expression was observed (Fig. 3), strongly suggesting that the changes in gene expression observed from the microarray analyses are valid.

EASE analysis using GO Biological Process and other annotation categories was implemented to ascertain if any biological theme(s) could be discovered from the list of genes with expression altered by culture in WM. EASE analysis identified five such biological processes: ribosome biogenesis and protein synthesis, cell organization and biogenesis, apoptosis, regulation of cell cycle and proliferation, and selenium binding (Table 3). Note that genes can be common to more than one process (e.g. *Rp13*).

Formation of a functional ribosome requires exquisite coordinate expression of rRNA and ribosomal proteins. Decreased expression of several ribosomal proteins for both the large and small subunits would adversely affect ribosome biogenesis, which would in turn have a negative effect on the rate of protein synthesis and development. For example, cyclin synthesis is required for cell proliferation and decreasing the rate of protein synthesis would inevitably slow down the rate of cell proliferation. In fact, culture in KSOM/AA results in blastocysts that have greater cell numbers when compared with blastocysts that develop in media that do not contain amino acids (Erbach *et al.* 1994, Ho *et al.* 1995, Biggers *et al.* 2000, Summers *et al.* 2000). The reduced level of expression of cyclin D1, which regulates cell cycle progression (Stacey 2003), and of Ran, which is involved in spindle formation (Kalab *et al.*

Table 3 EASE analysis of differentially expressed genes after culture in Whitten's medium.

EASE gene category	Gene symbol	Gene name
Ribosome biogenesis and protein synthesis	<i>Rps8</i>	ribosomal protein S8
	<i>Rpl13</i>	ribosomal protein L13
	<i>Rpl13a</i>	ribosomal protein L13a
	<i>Rps17</i>	ribosomal protein S17
	<i>Rps14</i>	ribosomal protein S14
	<i>Rps5</i>	ribosomal protein S5
	<i>Rpl10a</i>	ribosomal protein L10a
	<i>Ssfa1</i>	sperm specific antigen 1
	<i>Lsm4</i>	LSM4 homolog, U6 small nuclear RNA associated
	<i>2610016C23Rik</i>	RIKEN cDNA 2610016C23 gene
	<i>Syncrip</i>	synaptotagmin binding, cytoplasmic RNA interacting protein
	<i>Qtrt1</i>	queuine tRNA-ribosyltransferase 1
	<i>P5cr2</i>	pyrroline-5-carboxylate reductase
Cell organization and biogenesis	<i>Gpx4</i>	glutathione peroxidase 4
	<i>Actb</i>	actin, beta, cytoplasmic
	<i>Atp6v0c</i>	ATPase, H ⁺ transporting, V0 subunit C
	<i>Ptpns1</i>	Protein tyrosine phosphatase, non receptor type substrate 1
	<i>Rps8</i>	ribosomal protein S8
	<i>Tmsb10</i>	thymosin, beta 10
	<i>Nap1l1</i>	nucleosome assembly protein 1-like 1
	<i>Rpl13</i>	ribosomal protein L13
	<i>Rps17</i>	ribosomal protein S17
	<i>Rps14</i>	ribosomal protein S14
	<i>Rps5</i>	ribosomal protein S5
	<i>Ran</i>	RAN, member RAS oncogene family
	<i>Arhu</i>	Ras homolog gene family, member U
Apoptosis	<i>Ppap2a</i>	phosphatidic acid phosphatase 2a
	<i>Gpx1</i>	glutathione peroxidase 1
	<i>Fem1b</i>	feminization 1 homolog b (<i>C. elegans</i>)
	<i>Prkcz</i>	protein kinase C, zeta
	<i>Prdx2</i>	peroxiredoxin 2
	<i>Spp1</i>	secreted phosphoprotein 1
	<i>6430628I05Rik</i>	RIKEN cDNA 6430628I05 gene
	<i>Ptdsr</i>	phosphatidylserine receptor
	<i>Hip1</i>	huntingtin interacting protein 1
	<i>Spp1</i>	secreted phosphoprotein 1
	<i>Tdgf1</i>	teratocarcinoma-derived growth factor
	<i>Nap1l1</i>	nucleosome assembly protein 1-like 1
	<i>0610011C19Rik</i>	RIKEN cDNA 0610011C19 gene
Regulation of cell proliferation	<i>Btg1</i>	B-cell translocation gene 1, anti-proliferative
	<i>Ccnd1</i>	cyclin D1
	<i>Ccni</i>	cyclin I
	<i>Np95</i>	nuclear protein 95
	<i>Ppp1ca</i>	protein phosphatase 1, catalytic subunit, alpha isoform
	<i>Ran</i>	RAN, member RAS oncogene family
	<i>9030418M05Rik</i>	RIKEN cDNA 9030418M05 gene
	<i>Arhu</i>	Ras homolog gene family, member U
Selenium binding	<i>Gpx1</i>	glutathione peroxidase 1
	<i>Gpx4</i>	glutathione peroxidase 4
	<i>Prdx2</i>	peroxiredoxin 2

1999) and essential for the nuclear import and export cycle (Quimby & Dasso 2003), could also contribute to retarded development *in vitro*. In a similar vein, reduced expression of claudin (Table 2) and protein kinase C (PKC) ζ , which is an atypical PKC (Tables 2 and 3), may perturb tight junction assembly and function. Tight junction formation, which initiates after compaction, establishes a permeability seal in the trophectoderm that is essential for vectorial fluid transport and blastocoel formation (Fleming *et al.* 2001, Watson & Barcroft 2001). Claudin is a component of tight junctions (Tepass 2003) and PKC ζ , in association with PAR3 and PAR6, localizes with the tight junction protein, ZO-1 (Suzuki *et al.* 2001);

the role of this complex in tight junction assembly or function is unknown.

Decreased expression of secreted phosphoprotein 1 (osteopontin) (Tables 2 and 3), could, in principle, contribute to reduced implantation. Osteopontin contains a GRGDS amino acid sequence that can mediate adhesion to specific integrins implicated in implantation (Lessey 2002). It is unlikely that modest reductions in expression of these genes results in compromised implantation, because a recent study noted no decrease in the incidence of implantation and development to term after embryo transfer of WM-cultured embryos (Ecker *et al.* 2004).

Table 4 Genes down-regulated after culture in KSOM/AA.

Fold change	Common symbol	Gene name	Unigene
0.16	<i>5730469M10Rik</i>	RIKEN cDNA 5730469M10 gene	Mm.27227
0.17	<i>2210417O06Rik</i>	RIKEN cDNA 2210417O06 gene	Mm.25353
0.21	<i>9030418M05Rik</i>	RIKEN cDNA 9030418M05 gene	Mm.45994
0.26	<i>Morc</i>	microorchidia	Mm.26875
0.26	<i>Slc7a3</i>	solute carrier family 7 (cationic amino acid transporter, y+system), member 3	Mm.5140
0.29	<i>Ndr1</i>	N-myc downstream regulated 1	Mm.30837
0.29	<i>Rpl13a</i>	ribosomal protein L13a	Mm.180458
0.32	<i>Il6</i>	interleukin 6	Mm.1019
0.32	<i>Slc15a2</i>	solute carrier family 15 (H+/peptide transporter), member 2	Mm.63479
0.33	<i>Ndr1</i>	N-myc downstream regulated 1	Mm.4063
0.36	<i>Aqp8</i>	aquaporin 8	Mm.9970
0.40	<i>Arhu</i>	ras homolog gene family, member U	Mm.28954
0.40	<i>Asns</i>	asparagine synthetase	Mm.2942
0.41	<i>Fem1b</i>	feminization 1 homolog b (<i>C. elegans</i>)	Mm.24069
0.49	<i>Blnk</i>	B-cell linker	Mm.9749
0.49	<i>Tex20</i>	testis expressed gene 20	Mm.28388
0.50	<i>MGC38363</i>	transmembrane 4 superfamily member 3	Mm.22270
0.52	<i>Ugp2</i>	UDP-glucose pyrophosphorylase 2	Mm.28877
0.52	<i>B4galt6</i>	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6	Mm.26364
0.55	<i>Tpm1</i>	tropomyosin 1, alpha	Mm.121878
0.57	<i>Nupr1</i>	nuclear protein 1	Mm.18742
0.59	<i>Oaz1</i>	ornithine decarboxylase antizyme	Mm.683
0.59	<i>Map17</i> -(pending)	membrane-associated protein 17	Mm.30181
0.60	<i>Prdx2</i>	peroxiredoxin 2	Mm.42948
0.61	<i>Lamr1</i>	Laminin receptor 1	Mm.4071
0.61	<i>Rw1</i> -(pending)	RW1 protein	Mm.10808
0.64	<i>Rab11b</i>	RAB11B, member RAS oncogene family	Mm.35727
0.65	<i>Slc2a3</i>	solute carrier family 2 (facilitated glucose transporter), member 3	Mm.3726

Expression of teratocarcinoma-derived growth factor (*Tdgf1*) (Tables 2 and 3) and *Msx1* (*Hox7*) (Table 2) was also reduced after culture in WM. *Tdgf1* may be an important regulatory gene in gastrulation and early specification of tissues and organs (Xu *et al.* 1999). *Tdgf1* deficiency leads to abnormal failure of cardiomyocytes to differentiate and embryo lethality. *Msx1* is a homeobox gene the expression of which is regulated by BMP signaling and is expressed in several developing organs in vertebrates, including the facial primordia, particularly at the sites where epithelial–mesenchymal interactions occur during organogenesis (Liu *et al.* 2004). The function of either *Tdgf1* or *Msx1* in preimplantation is not known.

Effect of culture in KSOM/AA on gene expression

After culture in KSOM/AA, expression of only 29 genes was affected when compared with embryos that developed *in vivo* (Fig. 2). One gene (CD81) was up-regulated, whereas the others were down-regulated (Table 4); the significance of CD81 up-regulation, which is involved in signal transduction and cell adhesion in the immune system (Levy *et al.* 1998) and possibly sperm–egg fusion (Takahashi *et al.* 2001), is not apparent. The lower number of genes mis-expressed in KSOM/AA than in WM is consistent with KSOM/AA supporting better development *in vitro*. EASE analysis revealed that *Slc7a3*, *Slc15a2*, *Aqp8*, *Slc2a3*, *Cd81*, *Arhu* and *Asns* were over-represented (Table 5). The common feature of these genes is that they

Table 5 EASE analysis of genes differentially expressed after culture in KSOM/AA.

EASE gene category	Gene symbol	Gene name
Carrier activity	<i>Slc7a3</i>	solute carrier family 7 (cationic amino acid transporter, y+system), member 3
	<i>Slc15a2</i>	solute carrier family 15 (H+/peptide transporter), member 2
	<i>9030418M05Rik</i>	RIKEN cDNA 9030418M05 gene
Localized to plasma membrane	<i>Slc2a3</i>	solute carrier family 2 (facilitated glucose transporter), member 3
	<i>Cd81</i>	Cd81 antigen
	<i>Slc7a3</i>	solute carrier family 7 (cationic amino acid transporter, y+system), member 3
	<i>Slc15a2</i>	solute carrier family 15 (H+/peptide transporter), member 2
	<i>Aqp8</i>	aquaporin 8
	<i>Slc2a3</i>	solute carrier family 2 (facilitated glucose transporter), member 3
	<i>Arhu</i>	ras homolog gene family, member U
	<i>Asns</i>	asparagine synthetase

Table 6 Genes differentially expressed after culture in either Whitten's medium or KSOM/AA.

Common name	Description	Unigene
<i>Rpl13a</i>	ribosomal protein L13a	Mm.180458
<i>Prdx2</i>	peroxiredoxin 2	Mm.42948
<i>Lamr1</i>	Laminin receptor 1	Mm.4071
<i>Oaz1</i>	ornithine decarboxylase antizyme	Mm.683
<i>Tex20</i>	testis expressed gene 20	Mm.28388
<i>Arhu</i>	ras homolog gene family, member U	Mm.28954
<i>Fem1b</i>	feminization 1 homolog b (<i>C. elegans</i>)	Mm.24069
<i>Aqp8</i>	Aquaporin 8	Mm.9970
<i>Ndr1</i>	N-myc downstream regulated 1	Mm.4063
<i>Slc15a2</i>	solute carrier family 15 (H+/peptide transporter), member 2	Mm.63479
<i>Il6</i>	interleukin 6	Mm.1019
<i>Slc7a3</i>	solute carrier family 7 (cationic amino acid transporter, y+ system), member 3	Mm.5140
<i>Ndr1</i>	N-myc downstream regulated 1	Mm.30837
<i>9030418M05Rik</i>	RIKEN cDNA 9030418M05 gene	Mm.45994

are involved in membrane transport function. Interestingly, expression of *Slc2a3* was altered by culture in KSOM/AA but not in WM. *Slc2a3* is also known as *Glut3*, which is involved in facilitating glucose transport.

Inclusion of amino acids in KSOM has several positive effects on preimplantation development *in vitro*. For example, blastocyst cell number is increased, cavitation occurs earlier, and a greater fraction of blastocysts hatch (Ho *et al.* 1995, Biggers *et al.* 2000). Consistent with these beneficial effects is that there are only minor changes in the global pattern of gene expression after culture in KSOM/AA, although it is formally possible that the differences in salt and energy source concentrations may underlie the changes in gene expression. Recent studies have suggested, however, that ammonia generated by hydrolysis of glutamine in the culture medium may promote deleterious effects on embryo development, and experimentally increasing the ammonia concentrations compromises embryo development (Lane & Gardner 2003). Asparagine synthase (Asns), which catalyzes the ATP-dependent formation of asparagine from aspartate and ammonia, is down-regulated and could impair the

ability of the embryo to convert ammonia to a non-toxic form. Nevertheless, results presented here suggest that inclusion of amino acids in KSOM is unlikely to compromise developmental competence – that is, the ability of transferred blastocysts to implant and develop to term – because the global pattern of gene expression is very similar to that of embryos that develop *in vivo*. Current experiments examining the effect on global patterns of gene expression of including amino acids in WM and omitting them from KSOM should provide information about the role of amino acids in governing gene expression in cultured embryos.

Common genes mis-expressed after culture in either WM or KSOM/AA

Analysis of the data in Tables 2 and 4 revealed 14 genes that were mis-expressed after culture in either WM or KSOM (Table 6). EASE analysis revealed that five of these 14 down-regulated genes (*Slc7a3*, *Slc15a2*, *9030418M0Rik*, *Prdx2*, *Aqp8*) belong to the GO transporter activity family, with the first three belonging to the child ontological carrier activity family (Table 7). Solute carrier family 7 is important in the transport of lysine and arginine, whereas solute carrier family 15 is devoted to the transport of oligopeptides. Aquaporin 8 belongs to a class of membrane channel proteins that facilitate bulk water transport (Agre & Kozono 2003). It is present on the basolateral membranes of the trophectoderm and is probably involved in trans-trophectodermal water movement required for cavitation (Barcroft *et al.* 2003). It will be of interest to determine if over-expressing *Aqp8* stimulates development under either of these culture conditions.

Also of note is the reduced expression of the genes for interleukin-6 (*Il6*) and for peroxiredoxin 2 (*Prdx2*). *Il6* function *in vivo* may stimulate preimplantation embryo development, because the addition of interleukin-6 to culture media results in an increased cell number in blastocysts, prolonged gestation and increased weight of the generated pups (Cheung *et al.* 2003). Peroxiredoxin 2 belongs to a newly defined family of antioxidant proteins that have been implicated in cell proliferation and differentiation, intracellular signaling, protecting proteins from

Table 7 EASE analysis of genes differentially expressed after culture in either Whitten's medium or KSOM/AA.

Gene category	Gene name	Gene description
Plasma membrane	<i>Arhu</i>	ras homolog gene family, member U
	<i>Lamr1</i>	RIKEN cDNA 2700094K13 gene
	<i>Slc7a3</i>	solute carrier family 7 (cationic amino acid transporter, y+ system), member 3
	<i>Slc15a2</i>	solute carrier family 15 (H+/peptide transporter), member 2
	<i>Aqp8</i>	aquaporin 8
Transporter activity	<i>Prdx2</i>	peroxiredoxin 2
	<i>9030418M05Rik</i>	RIKEN cDNA 9030418M05 gene
	<i>Slc7a3</i>	solute carrier family 7 (cationic amino acid transporter, y+ system), member 3
	<i>Slc15a2</i>	solute carrier family 15 (H+/peptide transporter), member 2
	<i>Aqp8</i>	aquaporin 8

oxidative damage, and apoptosis (Fujii & Ikeda 2002). *Prdx2* down-regulation could contribute to the increase incidence of apoptosis that is observed in cultured embryos, when compared with those that develop *in vivo* (Brison & Schultz 1997).

In summary, results presented here highlight the ability of microarray analysis to provide insights into biological processes, in this case preimplantation development. Genes with expression that is affected by culture provide candidates for a standard hypothesis-driven approach to study their function.

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