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Dynamics of Global Gene Expression Changes during Mouse Preimplantation Development

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Summary

Understanding preimplantation development is important both for basic reproductive biology and for practical applications including regenerative medicine and livestock breeding. Global expression profiles revealed and characterized the distinctive patterns of maternal RNA degradation and zygotic gene activation, including two major transient waves of de novo transcription. The first wave corresponds to zygotic genome activation (ZGA); the second wave, named mid-preimplantation gene activation (MGA), precedes the dynamic morphological and functional changes from the morula to blastocyst stage. Further expression profiling of embryos treated with inhibitors of transcription, translation, and DNA replication revealed that the translation of maternal RNAs is required for the initiation of ZGA. We propose a cascade of gene activation from maternal RNA/protein sets to ZGA gene sets and thence to MGA gene sets. The large number of genes identified as involved in each phase is a first step toward analysis of the complex gene regulatory networks.

Introduction

Preimplantation development encompasses the period from fertilization to implantation, and is marked by a number of critical events (reviewed in Edwards, 2003). A growing mouse oocyte, arrested at diplotene of its first meiotic prophase, is transcriptionally and translationally active, but a large number of synthesized mRNAs are not used for immediate translation; they are rather stored to support oocyte maturation and early preimplantation embryogenesis (Bachvarova, 1985; Wassarman and Kinloch, 1992). Oocytes arrest in metaphase of their second meiotic division, where transcription stops and translation of mRNA is reduced. Fertilization triggers the completion of meiosis, followed by the formation of a 1-cell embryo containing haploid paternal and maternal pronuclei. Each pronucleus then undergoes DNA replication before entering the first mitosis to produce a 2-cell embryo containing two diploid "zygotic" nuclei.

Meiotic maturation triggers the degradation of maternal transcripts, which is 90% complete by the 2-cell stage. Transcription from the newly formed zygotic genome, known as zygotic genome activation (ZGA) or embryonic genome activation (EGA), occurs in two phases: a minor activation (minor ZGA) before cleavage and a major activation (major ZGA) at the 2-cell stage. Based on BrUTP incorporation studies, the minor ZGA occurs primarily in the male pronucleus (Aoki et al., 1997). This initial zygotic transcription is weak and results in synthesis of a small set of polypeptides that transiently increase at the 2-cell stage (Latham et al., 1991). The major ZGA promotes a dramatic reprogramming of gene expression pattern, coupled with the generation of novel transcripts that are not expressed in oocytes. Thus, the genetic program governed by maternal transcripts/proteins must be switched to that dominated by transcripts/proteins from the newly formed zygotic genome (reviewed in Kanka, 2003; Latham and Schultz, 2001; Nothias et al., 1995; Schultz, 2002; Thompson et al., 1998).

Following two additional cleavages, 8-cell embryos undergo the process of "compaction," in which loosely associated cells adhere to generate the tightly organized cell mass of the morula, resulting in the formation of a communicating polarized epithelium. This is followed by the differentiation of the morula into the blastocyst, composed of pluripotent cells of the inner cell mass (ICM) that will give rise to the embryo proper, and the differentiated cells of the trophectoderm (TE) that will give rise to extraembryonic tissues.

The study of preimplantation development can be facilitated by large-scale genomic approaches that specify the large cohorts of genes active at each stage, and analyze the activity of all of them simultaneously (reviewed in Ko, 2001). Although scarcity of materials has hampered the full exploitation of such methodologies, significant progress has been made in the last few decades. For example, PCR-based differential display and subtractive cDNA library construction techniques have been successfully applied to mouse preimplantation embryos (e.g., Zeng and Schultz, 2003). To identify genes specifically expressed in the preimplantation embryos, expressed sequence tag (EST) projects have been conducted, accumulating a total of 140,111 ESTs in the public sequence database (Ko et al., 2000; Marra et al., 1999; Okazaki et al., 2002; Rothstein et al., 1992; Sasaki et al., 1998; Sharov et al., 2003). This large number of ESTs provided a means to recover genes that function specifically in preimplantation embryos (Hwang et al., 2001; Knowles et al., 2003), and to identify stage-specific genes that are expressed predominantly in each stage of preimplantation embryos (Ko et al., 2000).

Microarray analysis has proved to be the most powerful approach for the global gene expression profiling (Schena et al., 1995). To apply the method to preimplantation embryos, we established the NIA 22K 60-mer oligo microarray, which is enriched for genes expressed in stem cells and preimplantation embryos, and is optimized for use with tiny amounts of RNA (Carter et al., 2003). The microarray accommodates 21,939 gene features derived from the NIA cDNA collection (Sharov et al., 2003). For convenience, we will use the term "gene"

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instead of "gene feature" in descriptions of microarray data.

Here, we report the global gene expression profiles of preimplantation embryos at all stages (unfertilized egg, fertilized egg [1-cell embryo], 2-cell embryo, 4-cell embryo, 8-cell embryo, morula, and blastocyst). Furthermore, to study the details of ZGA and maternal transcript degradation, we also profiled the global gene expression of in vitro cultured embryos treated with α-amanitine (α-AM), cycloheximide (CHX), aphidicolin (AC), and dimethyl sulfoxide (DMSO) from soon after the formation of pronuclei up to the late 2-cell stage.

Results

Gene Expression Profiles of Embryos Retrieved In Vivo

We collected four sets of 500 embryos from each stage of preimplantation development: unfertilized egg, fertilized egg, 2-cell embryo, 4-cell embryo, 8-cell embryo, morula, and blastocyst (Figure 1A). To obtain proper biological and technical replicates, RNAs were extracted separately from each batch of 500 embryos, and an aliquot from each RNA sample, equivalent to 25 embryos, was used for probe labeling and hybridization to the array. This minimizes complications caused by embryo-to-embryo variations in gene expression levels (Peng et al., 2003). A universal mouse reference RNA (Stratagene) was used for all hybridizations so that all stages could be compared (Weil et al., 2002). For biological and technical replicates, we performed four replicates of hybridizations for each stage of embryos, for a total of 614,292 gene expression measurements. All primary microarray data and bioinformatic annotations are available at our website (http://lgsun.grc.nia.nih.gov/cDNA), GEO (http://www.ncbi.nlm.nih.gov/geo/), and ArrayExpress (http://www.ebi.ac.uk/arrayexpress/).

Global Outlook by Pairwise Comparison, Hierarchical Clustering, and Principal Component Analysis

To obtain a first perspective on global gene expression changes, we performed a pairwise comparison of gene expression microarray data for all preimplantation stages (Figure 1B). Log-ratio plots identified two major transitions in the gene expression patterns: fertilized eggs to 2-cell embryos and 4-cell embryos to 8-cell embryos. These transitions separated preimplantation embryos into three phases: unfertilized eggs and fertilized eggs (Phase I); 2-cell embryos and 4-cell embryos (Phase II); and 8-cell embryos, morula, and blastocyst (Phase III). Grouping the preimplantation stages into three major phases was also supported by independent, more quantitative analyses: hierarchical clustering and principal component analysis (PCA) (Figures 1C and 1D). In this case, 12,179 genes that showed statistically significant changes with False Discovery Rate (FDR) $\leq$10% by ANOVA-FDR test (details in supplemental data [http://www.developmentalcell.com/cgi/content/full/6/1/117/DC1]) during preimplantation development were used for the analysis.

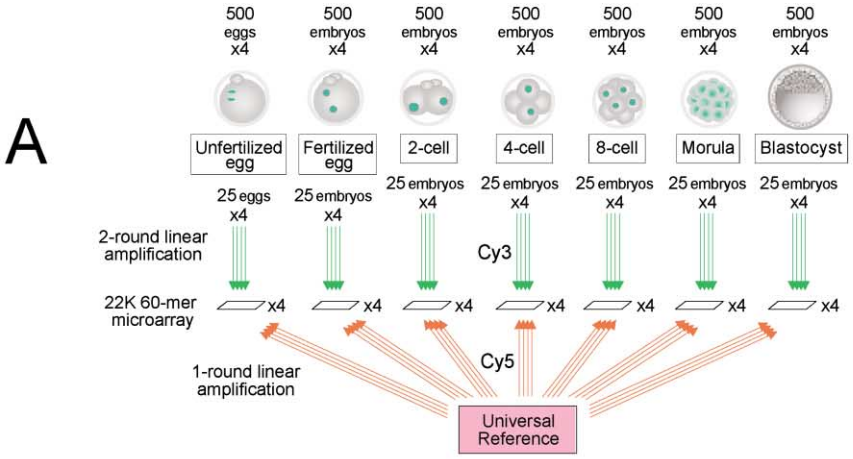
The timing of the first major transition from fertilized eggs to 2-cell embryos corresponded well to the well-known major gene expression changes of ZGA. However, the timing of the second major transition from 4-cell to 8-cell embryos was a surprise, because the most conspicuous morphological changes during preimplantation development occur after the 8-cell stage, followed by compaction and blastulation. This suggests that the dynamic changes of gene expression patterns that occur between the morphologically similar 4-cell and 8-cell stages result in the overt morphological changes in the subsequent stages, compaction and bifurcation into two cell lineages.

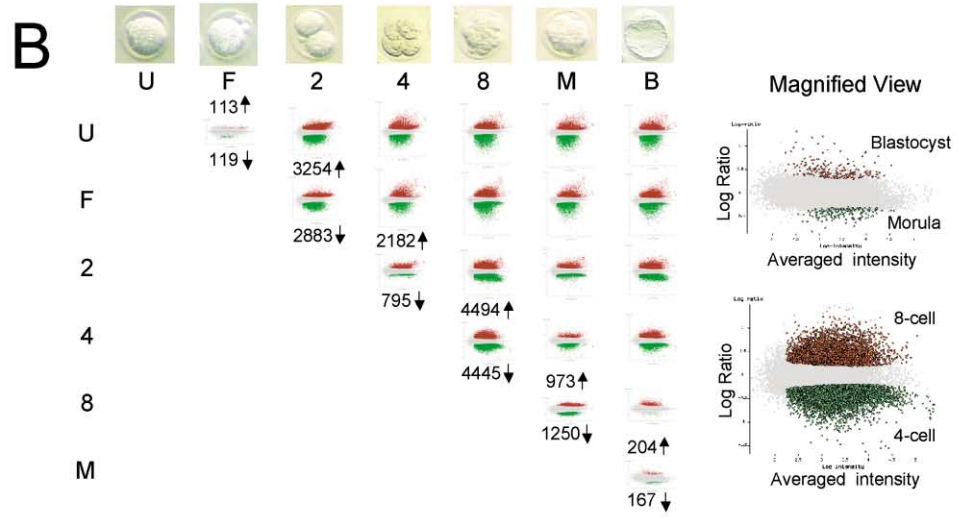
Time-Course Analysis of Individual Genes

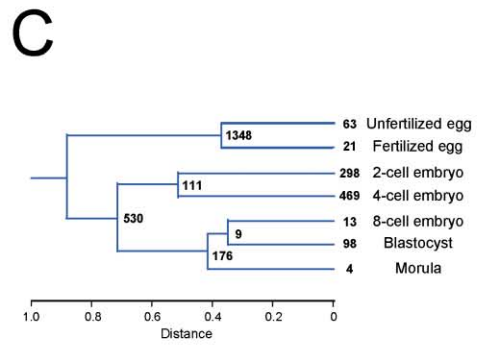
Global analyses do not provide information about the expression changes of individual genes over time. For example, "Are any genes included both in the first major transition and the second major transition?" Such a question has to be addressed by distinguishing individual genes. We analyzed 12,179 statistically significant genes by a k-means nonhierarchical clustering method (Saeed et al., 2003) and identified nine clusters (Figure 2A). Selected genes from each cluster were shown as examples of well-known genes (Figure 2B). The current microarray data were mostly consistent with the previously reported expression patterns of genes (see supplemental data for details).

Gene expression patterns of these clusters can be assigned to three main groups. The first group appears to represent genes that were first activated from the zygotic genome (Clusters 1, 4, 5, and 8). Cluster 1 (381 genes) and Cluster 4 (1570 genes) showed a steady increase throughout preimplantation stages, whereas Cluster 5 (2522 genes) and Cluster 8 (653 genes) peaked at the 2-cell and 4-cell stages, respectively, and then declined. The second group appears to represent genes that are abundant in oocytes, but degraded during preimplantation development (Clusters 7 and 9). Cluster 7 (498 genes) showed a dramatic decrease throughout preimplantation stages, whereas Cluster 9 (1091 genes) showed the most rapid decrease in earlier stages. The third group appears to represent genes that follow a combination of these two patterns (Clusters 2 and 3). Cluster 2 (2990 genes) started with maternally stored RNAs, peaked at the 8-cell stage, and then declined. Cluster 3 (1185 genes) showed degradation of maternally stored transcripts, followed by compensation by zygotic activation. Cluster 6 (1289 genes) appears to contain genes from both the second and third groups. In fact, Cluster 6 contains *Mater* (the second group; the expression of this well-known maternal transcript decreased throughout preimplantation stages [Tong et al., 2000]; Cluster 6b in Figure 2A), and *Dtr*/*Hb-egf* (the third group; the expression of this well-known gene that can mimic blastocysts by triggering the implantation reaction in uterine lumen [Paria et al., 2001] increased from the 8-cell to morula stage; Cluster 6a in Figure 2A).

Although the dynamics of actual gene expression changes of individual genes was very complex, as shown in the 3D representation of all gene expression patterns (Figure 2C), the k-means clustering provided a good overview of expression trends. One of the most intriguing general trends is the transient de novo transcription of many genes, forming a wave-like expression







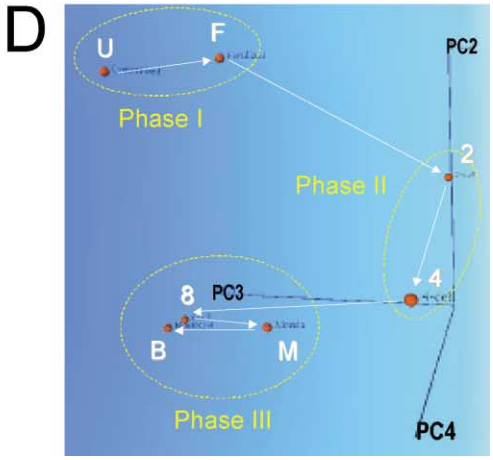


Figure 1. Expression Profiling of Preimplantation Embryos

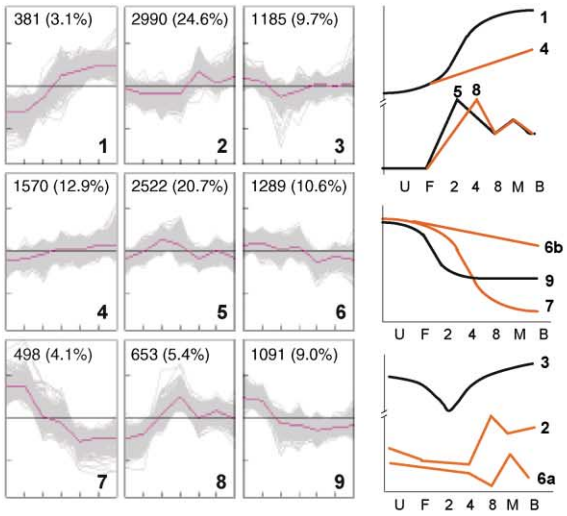
(A) Experimental design. See text and supplemental data for details.

(B) A matrix of scatter plots. U, F, 2, 4, 8, M, and B denote unfertilized egg, fertilized egg, 2-cell embryo, 4-cell embryo, 8-cell embryo, morula, and blastocyst, respectively. Each scatter plot shows the comparison of gene expression between embryo stages. A horizontal axis represents the averaged log (intensity) of genes, whereas a vertical axis represents the log (ratio) of signal intensity for each gene between one stage and another stage. Colored spots (red and green) represent genes that passed the FDR = 10% statistical test. Red spots represent array features with higher expression at the stage described as a column head, whereas green spots represent array features with lower expression. Magnified views of two representative scatter plots are also shown (far right column).

(C) Hierarchical clustering analysis. Numerical values represent the number of genes specific to each cluster or stage. See supplemental data for a list of these stage-specific genes.

(D) Principal component analysis.

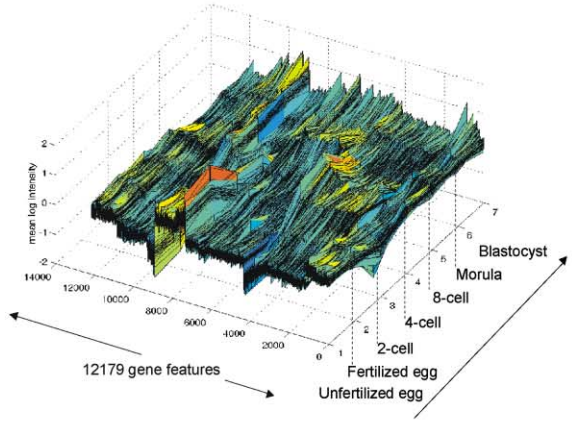
A



B

Cluster 1	Cluster 2	Cluster 3
Rex1/Zfp42	Nanog/Enk	Pou5f1/Oct4
Krt2-8/EndoA	Lefty1/Ebaf	E-cadherin
Cd9	Gja4/Cx45	Dnmt1
Gja1/Cx43	Gata3	Dnmt2
Hmgb2/Hmg2	Sox2	Dnmt3b
Cbx3	Errb3	Lefty2
Hdac1	Smarca1/Snf2l	Spp1/Osteopontin
Set	Tcfap2c/AP-2γ	Mecp2
Dnmap1	Shh	Cbx1
Gata6	Mybl2/Bmyb	Morf4l1/Mrg15
Nme1/NM23A	Pcaf	Tex20/Sall4
Rab1	Dnmt3a	Fragilis/Ifitm3
Pabpc1	Glut2	Pelo
Esrra	Tsix	Sfrs3/SRp20
Cluster 4	**Cluster 5**	**Cluster 6**
Zfp57	Dppa2	Mater
Dppa5/Esg1	Dppa3/Stella	Ddx4/Vasa
Sirt1/Sir2	Aatf/Trb	Zp3
Mbd3	Gdf3	Gata4
Tead2	Brachyury/T	Frap1/mTOR
N-myc/Nmyc1	L-myc/Lmyc1	Fsrg1/Brd2
Sp1	Zfp52	Baz1a
Gata1	Zfp393	Cdc45l
Gata2	Suv39h1	Ilk
Fgfr1	Hdac3	Dtr/HB-EGF
Glut3	Cbx8	Smarca5
Pem	Fgfr4	Brd4
Racgap1	Kpna2/Importinα2	Son
Idb2	U2af1-rs1	Nfkbia/IκBα
Ndr1	Tbp	Nfkb1/NF-κB
Cluster 7	**Cluster 8**	**Cluster 9**
Gdf9	Smarca4/Brg1	Mos/c-mos
Obox1	Igf2r	Kit/c-kit
Ezh2	Mbd2	Stat3
Mad2l2	Piwil2	Cry1
Tcl1	Hmg17/Hmgn2	Oosp1
Bmi1	Yy1	Nobox/Og2x
Bmal1/Arntl	Mcl1	Cpeb1
Clock	Vhlh	Magoh
Zar1	Lyar	Catna1
Rfpl4	Top2a	N-cadherin
Mek1/Map2k1	Dppa4	Rac1
Eps8	Rbbp2	Hells/Lysh
Stk13/AurkC	Amhr2	Morf4l2/MrgX
Zfp68	Papola	Baz1b
Rnf35/2cz61	Apex1/Ref-1	Zp1
Sfmbt1	Fbxo15	Zp2
Spindlin	Fxr2h	Scml2

C



D

Pair-wise comparison between stages	Fertilized egg to 2-cell	2-cell to 4-cell	4-cell to 8-cell	8-cell to morula	morula to blastocyst
Number of genes siginificantly increased for the first time during preimplantation stages	**3254**	**1177**	**4216**	**511**	**97**

	Total number of genes	MGA genes	
Cluster 1	**381**	**25** 0.8%	6.6%
Cluster 2	**2990**	**2752** 82.7%	92.0%
Cluster 3	**1185**	**409** 12.3%	34.5%
Cluster 4	**1570**	**65** 2.0%	4.1%
Cluster 5	**2522**	**0** 0.0%	0.0%
Cluster 6	**1289**	**0** 0.0%	0.0%
Cluster 7	**498**	**5** 0.2%	1.0%
Cluster 8	**653**	**0** 0.0%	0.0%
Cluster 9	**1091**	**73** 2.2%	6.7%
Others		887	
Total (1-9)	12179	4216	

E

	Total number of genes	Egg-specific genes		EST(+) in U/F cDNA libraries	
Cluster 1	**381**	**0** 0.0%	0.0%	**214** 3.5%	56.2%
Cluster 2	**2990**	**5** 3.7%	0.2%	**826** 13.6%	27.6%
Cluster 3	**1185**	**9** 6.7%	0.8%	**669** 11.0%	56.5%
Cluster 4	**1570**	**1** 0.7%	0.1%	**694** 11.4%	44.2%
Cluster 5	**2522**	**13** 9.7%	0.5%	**949** 15.6%	37.6%
Cluster 6	**1289**	**19** 14.2%	1.5%	**932** 15.3%	72.3%
Cluster 7	**498**	**49** 36.6%	9.8%	**452** 7.4%	90.8%
Cluster 8	**653**	**0** 0.0%	0.0%	**452** 7.4%	69.2%
Cluster 9	**1091**	**38** 28.4%	3.5%	**892** 14.7%	81.8%
Total (1-9)	12179	134		6080	

Figure 2. Time-Course Analysis of Individual Genes

(A) General trends of expression changes analyzed by k-means clustering method. The nine clusters were further classified into three super groups by visual inspection as shown in the three schemes in the far right column. Black lines show known patterns of gene expression, whereas red lines indicate novel patterns of gene expression.

pattern. For example, among the 4445 genes identified with decreasing expressions from the 4-cell to 8-cell stage (Figure 1B), 37.8% fell into Cluster 5, which constituted 66.6% of the total genes in this cluster. Similarly, 19.9%, 7.9%, 12.9%, and 8.0% fell into Cluster 6, Cluster 7, Cluster 8, and Cluster 9, which accounted for 68.7%, 70.5%, 87.9%, and 32.5%, respectively. The results indicate that the majority of genes downregulated during the 4-cell to 8-cell stage were also upregulated during ZGA (from the fertilized egg to 2-cell/4-cell stage: Cluster 5 and Cluster 8). The remaining genes were downregulated as part of the degradation of maternal transcripts (Clusters 6, 7, and 9: see the maternal degradation section below). A large number of genes showing a transient wave of expression were unexpected.

To distinguish the transcripts downregulated from the maternal RNA pools from those downregulated from the products of activated zygotic genomes, we reanalyzed all the genes on the array in a pairwise manner with statistical significance of 10% FDR, and identified genes with expression levels that increased for the first time at a specific stage of preimplantation development (Figure 2D). This approach is more discriminating to analyze the transcripts produced from the zygotic genomes, and still correlates well with the k-means clustering results as shown in the case of genes from the 4-cell to 8-cell stage (Figure 2D).

We discuss characteristics of genes below, starting with genes activated at each preimplantation stage, and then genes in the maternal RNA degradation group.

Genes Activated from Fertilized Eggs to 4-Cell Embryos

Genes that increased their expression levels between fertilized eggs and 2-cell stage and were grouped in Clusters 1, 5, and 8 correspond to ZGA. Cluster 1 (381 genes) showed a continuous increase throughout preimplantation development (Figures 2A and 2B), though slight changes were also observed during the Phase III stage (8-cell, morula, and blastocyst). The functional assignments of these genes by Gene Ontology (GO) terms (Ashburner et al., 2000) characterized this cluster as related to basic cellular machineries represented by GO terms such as "ribosome," "ribonucleoprotein complex," "proton transport," "ion transport," "ribonucleotide triphosphate metabolism," "proteasome complex," and "RNA binding" (Supplemental Table S1A).

The transcripts in both Cluster 5 and Cluster 8 transiently increased during the 2-cell and 4-cell stages, decreased from the 4-cell to 8-cell stage, and then slightly increased again at the morula stage (Figures 2A and 2C). Cluster 5 genes peaked at the 2-cell stage, whereas those in Cluster 8 peaked at the 4-cell stage. However, because both clusters began to increase their expression between the fertilized egg and 2-cell stages, they were treated in the same manner here. These genes seemed to be the main contributors to the global gene expression profile in Phase II (2-cell and 4-cell embryos), but not in Phase III. According to the major GO terms associated with these genes by MAPPFinder (Doniger et al., 2003), Cluster 5 was characterized by "triacylglycerol metabolism," "inositol/phosphatidylinositol phosphatase," "receptor signaling protein serine/threonine kinase," and "protein-nucleus import," whereas Cluster 8 was represented by "lysosome," "sphingolipid metabolism," "DNA-directed RNA polymerase," "translation initiation factor," "helicase," "RNA binding," and "RNA processing" (Supplemental Tables S1B and S1C).

Taken together, these data suggest that ZGA contributes mainly to the preparation of basic cellular machinery during Phase II stages (2-cell and 4-cell) rather than to dramatic biological and morphological events in Phase III (8-cell, morula, and blastocyst).

Genes Activated from 4-Cell to 8-Cell Embryos

We identified 4216 genes whose expression first significantly increased from the 4-cell to 8-cell stage (Figure 2D). This particular fraction represents genes that initiate transcription between the 4-cell and 8-cell stages. Among those, 3329 genes were included in the 12,179 significant genes used for k-means clustering analysis, where 82.7% and 12.3% of genes fell into Cluster 2 and Cluster 3, respectively (Figure 2D). Genes in Cluster 2 showed a transient increase from the 4-cell to 8-cell stage, followed by an immediate decrease from the 8-cell to the morula stage. This group of genes is responsible for the second major transition and the separation of Phase II embryos (2-cell and 4-cell) from Phase III embryos (8-cell, morula, and blastocyst). This pattern of gene expression has not been reported previously and has thus been of particular interest. We call this phenomenon "mid-preimplantation gene activation" (MGA).

Interestingly, the MGA gene list includes many known genes with critical functions in preimplantation embryos (Figure 3). The microarray comparison between ES and trophoblast stem (TS) cells (Tanaka et al., 2002) further subgrouped these genes into those with higher expression in ES cells, such as *Pou5f1*, *Nanog*, *Lefty1*, *Fragilis*, *Gja7*, *Ctbp2*, and *Mxi1*, and those with higher expression in TS cells, such as *Gata3*, *Irx3*, *EndoA*, *Bmp8b*, *Sin3b*, and *Gata6*. These genes may be key regulators in the differentiation into the ICM and TE, as has already been demonstrated for some of them.

(B) Representative genes in each cluster.

(C) 3D-view of gene expression patterns. The expression changes of 12,179 genes were plotted in 3D-graphs by MatLab: *x* axis represents individual genes; *y* axis represents the developmental stages from unfertilized eggs to blastocysts; and *z* axis represents log intensities of individual genes.

(D) Genes upregulated for the first time during preimplantation development. The 4216 genes that significantly increased their expression between the 4-cell and 8-cell stages in the FDR = 10% statistical test in pairwise comparison were designated as "mid-preimplantation gene activation" (MGA) and were further subdivided in the lower table. 82.7% and 12.3% of MGA genes fell into Cluster 2 and Cluster 3, respectively. They correspond to 92.0% of total genes in Cluster 2 and 34.5% of genes in Cluster 3, respectively.

(E) The correlation of the genes in each cluster with egg-specific genes identified through the analysis of EST frequency (middle column) and with genes which were represented as ESTs in cDNA libraries made from unfertilized eggs or fertilized eggs (right column) (Sharov et al., 2003).

Gene	U-B	U-B Mean Log-Ratio (/ Universal reference)							FDR in pairwise comparison						K-mean	ES vs. TS	
	Av. Intensity	U	F	2	4	8	M	B	F / U	2 / F	4 / 2	8 / 4	M / 8	B / M	Cluster	Av. Intensity	ES/TS Fold Change
Ebaf/Lefty-1	2.73	-0.09	-0.33	-0.36	-0.15	0.14	0.00	0.01			▲				2	3.48	41.66
Pou5f1/Oct4	3.25	0.81	0.71	0.40	0.37	0.74	0.95	0.68	▼		▲				3	3.11	11.08
Gja7/Cnx45	3.42	-0.23	-0.34	-0.25	-0.41	0.10	-0.23	-0.07			▲	▼			2	3.32	4.87
Ddah2	3.64	-0.82	-0.87	-0.81	-0.73	-0.17	-0.38	-0.31			▲				2	3.92	4.76
Nanog/Enk	3.41	0.23	0.16	0.11	0.12	0.41	0.42	0.43			▲				2	3.57	4.64
Otx2	2.97	0.47	0.45	0.47	0.44	1.07	0.73	0.91			▲				2	2.69	4.59
Anp32a/pp32	2.68	-0.58	-0.66	-1.09	-0.93	-0.67	-0.65	-0.52	▼		▲				3	2.95	3.76
Pdgfc	2.84	-0.47	-0.48	-0.53	-0.57	-0.17	-0.52	-0.31			▲	▼			2	2.74	3.46
Egln3	3.39	-0.69	-0.58	-0.58	-0.76	0.22	-0.24	0.20			▲				2	3.01	3.32
Fxyd6	3.56	0.25	0.14	-0.01	0.04	0.49	0.28	0.52			▲				2	4.20	3.25
Enah/Mena	3.67	0.05	0.09	0.02	-0.12	0.24	0.35	0.22			▲				2	3.73	3.18
Ifitm3l/Fragilis	3.56	-0.79	-0.91	-1.02	-1.06	-0.73	-1.02	-0.84			▲	▼			3	3.75	3.06
Itm2a	3.26	-0.12	-0.45	-0.58	-0.40	0.02	-0.17	-0.08	▼		▲				2	3.11	2.80
Epha4	3.07	0.82	0.43	-0.14	0.00	0.65	0.37	0.55	▼	▼	▲	▼			3	2.84	2.76
Prcad	2.50	0.00	-0.23	-0.26	-0.15	0.19	-0.13	0.16			▲				2	2.41	2.66
Stat4	2.67	0.03	-0.08	-0.24	-0.23	0.03	-0.16	-0.07			▲				-	2.83	2.28
Gas5	3.05	-0.42	-0.45	-0.26	-0.19	0.10	0.07	-0.01			▲				4	3.30	2.13
Ctbp2	3.50	0.96	0.58	-0.46	-0.22	0.22	0.27	0.60	▼	▼	▲			▲	3	3.61	2.12
Mxi1/Mad2	3.13	-0.68	-0.71	-0.64	-0.64	-0.34	-0.59	-0.43			▲				2	3.09	1.92
Jmj	3.40	0.29	0.13	-0.01	-0.03	0.38	0.35	0.36			▲				2	3.83	1.91
Ccnd1	3.38	-0.45	-0.40	-0.60	-0.63	-0.41	-0.54	-0.45			▲				-	3.38	1.84
Smarca1	2.68	0.03	0.15	0.11	0.09	0.53	0.26	0.43			▲				2	2.30	1.77
Fshprh1	3.14	-0.15	-0.16	-0.07	-0.07	0.33	-0.01	0.18			▲	▼			2	2.91	1.73
Dner	2.76	0.29	0.03	0.14	0.11	0.38	0.19	0.23			▲				-	2.86	1.71
Atp1b1	3.64	-0.48	-0.51	-0.75	-0.82	-0.28	-0.21	0.41		▼	▲			▲	2	3.90	1.69
Mkrn3/Zfp127	2.95	0.17	0.01	0.05	-0.06	0.74	0.30	0.57			▲	▼			2	2.63	1.67
Smc1l2	3.48	0.55	0.43	0.35	0.29	0.67	0.48	0.61			▲				-	2.91	1.55
Rbbp4	4.08	-0.56	-0.61	-0.75	-0.59	-0.16	-0.21	-0.15			▲				2	4.35	1.49
Dtx1	3.58	0.28	0.23	0.39	0.17	0.46	0.34	0.42			▲				-	3.26	1.44
Gli3	3.44	0.60	0.78	0.15	-0.02	0.21	-0.09	0.06		▼	▲	▼			9	2.92	1.43
Mybl2/Bmyb	3.72	0.20	0.01	0.04	0.00	0.40	0.18	0.32			▲				2	3.57	1.38
Melk	2.97	-0.06	-0.04	-0.39	-0.31	0.00	-0.14	0.02		▼	▲				3	3.12	1.36
Ptprf	3.39	0.28	-0.31	-0.77	-0.72	-0.18	-0.17	0.35	▼	▼	▲			▲	3	3.97	1.35
Pecam	3.26	0.42	0.27	0.26	0.24	0.86	0.50	0.71			▲	▼			2	2.77	1.33
Crtr1	2.97	0.25	0.12	-0.06	0.04	0.46	0.25	0.37			▲				2	2.90	1.31
Fancc	3.12	0.32	0.31	0.15	0.11	0.43	0.20	0.33			▲				3	2.97	1.29
Txn1	3.46	-1.58	-1.62	-1.73	-1.74	-1.11	-1.02	-0.15			▲			▲	2	4.51	1.27
Utf1	4.19	0.40	0.30	0.57	0.40	1.03	0.78	0.91			▲				2	4.18	1.24
Pdgfrb	2.92	-0.86	-0.70	-0.74	-0.96	-0.59	-0.83	-0.62			▲				-	2.93	1.24
Cir	3.98	0.25	0.13	-0.18	-0.04	0.53	0.35	0.44		▼	▲				2	3.48	1.24
Notch3	3.85	-0.09	-0.19	-0.11	-0.18	0.18	-0.05	0.09			▲				2	3.93	1.22
Fancl/Pog	3.35	-0.24	-0.30	-0.23	-0.24	0.13	-0.01	0.06			▲				2	3.31	1.21
Arx	2.70	0.50	0.24	0.27	0.20	0.79	0.53	0.57			▲				2	2.29	1.20
Dnmt3a	3.12	0.28	0.13	0.17	0.19	0.50	0.30	0.38			▲				2	2.91	1.19
Bmp15	3.41	0.43	0.58	0.17	0.12	0.35	0.18	0.27		▼	▲				3	2.99	1.19
Diap2	2.51	-0.58	-0.50	-0.73	-1.08	-0.30	-0.01	0.16			▲				2	3.14	1.16
Emd	3.56	-0.14	-0.19	-0.21	-0.18	0.16	0.05	0.17			▲				2	3.55	1.16
Ncoa2	3.74	0.89	0.64	0.57	0.41	1.41	0.89	1.31			▲	▼			2	2.66	1.15
Fxyd4	3.16	0.37	0.13	0.06	0.20	0.98	0.62	0.96			▲	▼			2	2.68	1.14
Slc2a2/Glut2	3.42	0.55	0.38	0.39	0.41	1.37	0.88	1.26			▲				2	2.61	1.14
Dhx9/Ddx9	2.79	0.37	0.27	0.41	0.29	0.67	0.47	0.56			▲				2	2.42	1.14
Pcaf	3.39	0.25	0.21	0.39	0.31	0.56	0.34	0.46			▲				2	3.05	1.13
Ddx21	3.92	0.20	0.06	0.23	0.10	0.85	0.43	0.73			▲	▼			2	3.66	1.11
Il6st	2.90	0.13	0.02	-0.03	-0.07	0.24	-0.05	0.10			▲	▼			-	2.70	1.11
Shh	3.10	0.28	0.18	0.14	0.08	0.76	0.39	0.63			▲	▼			2	2.74	1.08
Gja4/Cnx37	3.18	0.38	0.27	0.23	0.20	0.86	0.43	0.73			▲	▼			2	2.82	1.07
Mllt3	3.30	0.24	0.16	0.01	-0.03	0.41	0.12	0.29			▲	▼			2	2.95	1.07
Snai2	3.41	-0.41	-0.53	-0.34	-0.57	0.59	-0.07	0.51			▲	▼			2	2.56	1.07
Terf2	3.08	-0.09	-0.31	-0.09	-0.21	0.00	-0.19	-0.17			▲				-	3.13	1.06
Bmp6	3.64	0.43	0.28	0.18	0.16	0.51	0.26	0.43			▲	▼			3	3.14	1.04
Selpl/P-selectin	3.53	0.29	0.13	0.08	0.07	0.85	0.38	0.69			▲	▼			2	2.98	1.04
Inha	3.41	0.19	0.06	0.17	0.08	0.32	0.18	0.21			▲				-	3.14	1.04
Dnmt2	2.57	0.25	0.10	-0.53	-0.65	-0.12	-0.29	-0.17		▼	▲				3	2.49	1.04
Gjb1/Cnx32	3.46	0.37	0.18	0.33	0.26	0.85	0.53	0.73			▲	▼			2	2.79	1.03
Phf1/Tctex3	3.78	0.11	0.04	0.10	-0.01	0.21	0.11	0.17			▲				-	3.58	1.03
Tcl1b5	4.00	0.41	0.44	0.16	-0.01	0.38	0.13	0.31		▼	▲	▼			3	3.57	1.02
Star	3.43	0.50	0.22	0.31	0.29	1.28	0.73	1.16			▲	▼			2	2.58	1.02
Ncor2	3.44	0.13	0.06	0.08	0.07	0.29	0.10	0.22			▲				-	3.34	1.02

Gene	U-B	U-B Mean Log-Ratio (/ Universal reference)							FDR in pairwise comparison						K-mean	ES vs. TS	
	Av. Intensity	U	F	2	4	8	M	B	F / U	2 / F	4 / 2	8 / 4	M / 8	B / M	Cluster	Av. Intensity	ES/TS Fold Change
Tgfb3	3.38	0.24	0.03	0.03	-0.07	0.44	0.16	0.24			▲	▼			2	2.97	1.01
Nkx2-5	4.15	0.01	-0.18	-0.16	-0.12	0.33	0.06	0.22			▲				2	4.10	1.01
Cpeb3	3.55	0.18	0.05	-0.07	-0.13	0.33	0.10	0.32			▲				2	3.04	1.01
Znfn1a4	3.24	0.22	0.07	0.05	0.04	0.27	0.19	0.23			▲				-	2.95	1.01
Cenpb	4.17	0.26	0.16	0.23	0.08	0.55	0.36	0.51			▲				2	3.59	1.00
Sirt2	3.15	0.07	0.05	0.10	0.00	0.24	0.10	0.13			▲				-	3.14	-1.01
Egf	3.39	0.94	0.80	0.15	0.05	0.40	0.12	0.32	▼		▲	▼			9	2.86	-1.01
Nr5a1/SF-1	3.42	0.35	0.13	0.32	0.16	0.98	0.54	0.82			▲	▼			2	2.70	-1.02
Ccnd2	2.80	-0.57	-0.66	-0.55	-0.71	-0.33	-0.68	-0.64			▲	▼			-	2.77	-1.04
Rnf33/2czf45	3.17	0.24	0.18	0.30	0.08	0.35	0.23	0.26			▲				-	3.21	-1.07
Dnmt3b	3.31	0.61	0.64	0.07	0.26	0.59	0.81	1.00	▼		▲				3	3.97	-1.07
Fkbp1a	3.75	-0.60	-0.80	-0.93	-0.84	-0.58	-0.69	-0.56			▲				3	4.45	-1.08
Srebf2	3.11	-1.17	-1.26	-1.35	-1.60	-1.06	-1.23	-0.87			▲			▲	2	4.17	-1.08
Scmh1	4.43	-0.40	-0.61	-0.52	-0.37	0.21	-0.12	0.14			▲	▼			2	4.75	-1.08
Cul3	2.55	-0.32	-0.18	0.09	-0.43	0.47	0.10	0.41			▲				2	2.59	-1.08
Yap	4.13	0.77	0.92	-0.05	-0.14	0.09	-0.11	0.05	▼		▲				9	3.73	-1.09
Ptger2	2.59	-0.05	-0.26	-0.18	-0.23	0.22	0.00	0.06			▲				2	2.65	-1.09
Creb3	2.99	0.46	0.42	0.47	0.38	0.92	0.57	0.74			▲	▼			2	2.44	-1.10
Ly6e/Sca-2	3.72	0.15	-0.09	-0.06	-0.04	1.19	0.65	1.14			▲				2	3.27	-1.11
Phtf	3.48	0.08	-0.19	-0.34	-0.42	0.33	-0.12	0.25			▲	▼			2	3.09	-1.15
Aqp8	3.94	0.19	0.04	-0.04	-0.02	0.62	0.27	0.63			▲	▼			2	3.36	-1.15
Camk2d	3.28	-0.91	-0.93	-0.79	-1.03	-0.42	-0.61	-0.43			▲				2	3.19	-1.16
Calm1	4.24	0.32	0.27	0.45	0.19	0.86	0.48	0.73			▲	▼			2	3.62	-1.17
Nfkb2	3.75	-0.51	-0.48	-0.43	-0.54	-0.25	-0.44	-0.22			▲				2	3.66	-1.18
Itga4	2.45	0.12	0.04	0.26	-0.03	0.34	0.07	0.17			▲				-	2.22	-1.20
Dab2	2.95	-0.12	-0.34	-0.58	-0.85	-0.45	-0.16	-0.25			▲				3	2.64	-1.21
Top3b	3.54	-0.18	-0.33	-0.31	-0.46	-0.22	-0.15	-0.11			▲				3	4.00	-1.23
Fgfr1	2.61	0.02	0.03	0.06	-0.13	0.28	0.01	0.15			▲				-	2.53	-1.25
Vdac3	3.65	-0.54	-0.37	-0.50	-0.49	-0.28	-0.39	-0.26			▲				4	3.77	-1.30
Plac1	3.77	0.34	0.26	0.44	0.31	0.79	0.52	0.74			▲	▼			2	3.22	-1.32
Pfn1	3.80	-0.76	-0.87	-0.75	-0.82	-0.22	-0.61	-0.29			▲	▼			2	4.21	-1.34
Ocln	2.27	0.01	-0.06	0.08	-0.03	0.28	0.28	0.37			▲				2	2.61	-1.35
Ercc1	3.56	-0.09	-0.23	-0.20	-0.32	0.46	-0.02	0.37			▲	▼			2	3.35	-1.38
Itga5	2.68	0.13	0.03	0.03	-0.11	0.80	0.20	0.59			▲	▼			2	2.36	-1.40
Nfix	3.81	-0.30	-0.58	-0.81	-0.83	0.09	-0.52	0.03			▲	▼			2	3.34	-1.46
Mitc1/Smif	3.10	0.60	0.52	0.14	0.18	0.58	0.26	0.48	▼		▲	▼			3	2.58	-1.50
Cdh3	3.67	0.60	0.58	0.52	0.36	0.64	0.46	0.59			▲				-	3.83	-1.52
Hint	4.01	-1.23	-1.04	-0.83	-0.77	-0.53	-0.51	-0.09			▲			▲	1	4.57	-1.56
Wt1	3.69	0.33	0.19	0.43	0.27	0.92	0.62	0.88			▲				2	2.85	-1.57
Mll	3.78	-0.19	-0.25	-0.13	-0.25	0.35	0.05	0.30			▲				2	3.69	-1.57
Eif2c4	2.80	0.33	0.30	0.49	0.39	0.80	0.68	0.71			▲				2	2.35	-1.59
Meis1/Evi8	2.94	0.01	-0.27	-0.04	-0.15	0.23	0.08	0.11			▲				2	2.84	-1.61
Gata6	2.96	-1.10	-0.83	-0.57	-0.22	0.17	0.32	-0.09			▲				1	2.33	-1.62
Cldn6	3.11	0.21	0.17	0.15	0.12	0.46	0.18	0.32			▲	▼			2	3.49	-1.73
Itga6	3.62	0.38	0.17	-0.37	-0.36	-0.15	-0.23	-0.12	▼		▲				9	3.89	-1.75
Npc1	2.64	0.51	0.00	-0.39	-0.61	-0.27	-0.10	-0.19	▼	▼	▲				9	2.89	-1.79
Bcl2l	4.26	0.20	0.16	-0.48	-0.44	0.43	0.03	0.43	▼		▲	▼		▲	2	3.95	-1.81
Timp2	3.30	-0.67	-0.48	-0.46	-0.61	-0.22	-0.49	-0.29			▲				2	3.07	-1.91
Stat1	3.27	0.15	-0.08	0.03	-0.06	0.77	0.27	0.60			▲	▼			2	2.88	-1.96
Drctnnb1a	3.06	-0.10	-0.47	-0.43	-0.61	-0.35	-0.42	-0.31	▼		▲				3	3.10	-1.97
Vav3	3.21	0.14	-0.14	-0.12	-0.27	0.80	0.22	0.66			▲	▼			2	2.68	-2.02
Cldn10	2.46	0.38	0.20	0.37	0.14	0.47	0.24	0.48			▲				-	1.91	-2.17
Xist	2.70	-0.03	-0.20	0.04	-0.15	0.20	-0.01	0.06			▲				2	3.10	-2.23
Serpinh1	3.38	-0.65	-0.78	-0.84	-0.88	-0.24	-0.65	-0.41			▲	▼			2	3.40	-2.27
Gjb5/Cnx31.1	3.02	0.06	0.04	0.00	0.01	0.27	0.09	0.26			▲				-	3.27	-2.49
Satb1	3.29	0.97	0.44	0.28	0.32	0.92	0.78	0.83	▼		▲				2	3.13	-2.62
Itm2c	3.35	-0.73	-0.68	-0.72	-0.76	-0.49	-0.72	-0.50			▲				2	3.81	-2.72
Ggta1	3.36	0.23	-0.06	-0.13	-0.36	0.01	-0.29	-0.19			▲	▼			3	3.33	-2.85
Erbb3	3.42	0.31	0.12	0.30	0.38	0.84	0.63	0.87			▲				2	3.53	-2.91
Il16	2.62	-0.48	-0.37	-0.39	-0.69	-0.23	-0.53	-0.39			▲				-	2.16	-3.13
S100a6	3.42	-2.17	-2.27	-2.24	-2.17	-1.92	-2.11	-1.92			▲				2	4.66	-3.21
Bmp8b	3.21	0.11	0.05	-0.03	-0.09	0.22	0.06	0.18			▲				-	3.56	-3.80
Lgals1	3.75	-1.87	-1.94	-1.95	-1.79	-1.56	-1.79	-1.12			▲			▲	2	4.61	-3.92
Sin3b	4.26	-0.16	-0.09	-0.57	-0.46	-0.03	-0.09	0.23	▼		▲			▲	2	4.67	-5.07
Cbx4	3.37	-0.56	-0.52	-0.38	-0.55	0.02	-0.32	-0.03			▲	▼			2	3.04	-6.36
Krt2-8/EndoA	3.62	-1.36	-1.53	-1.46	-1.48	-1.04	-0.39	0.33			▲	▲		▲	1	4.72	-10.68
Irx3	2.96	0.43	0.34	0.26	0.17	1.20	0.66	0.99			▲				2	2.91	-20.06
Gata3	3.45	-0.07	-0.06	-0.45	-0.65	0.09	0.61	0.94	▼		▲	▲		▲	2	3.28	-27.34

Figure 3. A List of Genes in Mid-Preimplantation Gene Activation Category

"Av. Intensity" represents averaged log intensity of both Cy3 and Cy5 signals for a gene in all the samples. "Mean Log-Ratio" indicates the log of the ratio of signal intensities for each gene between each embryo stage and a universal reference, averaged among biological replicates for that embryo stage. Filled arrowheads stand for the significant increase or decrease of gene expression in pairwise comparisons between stages.

The functional assignment of these genes by MAPPFinder characterized the function of the MGA genes by the following three representative GO terms: "endopeptidase inhibitor," "intercellular junction," and "DNA (cytosine-5-)-methyltransferase" (Supplemental Table S1D).

The "endopeptidase inhibitor" category included genes such as *Fetub*, *Ambp*, *Aplp2*, *Itih1*, *Timp2*, *Timp3*, *Timp4*, *Spi11*, *Spi12*, *Spint2*, *Serpina3n*, *Serpinb1a*, *Serpinf2*, and *Serpinh1*. These proteins may play a role in extracellular matrix homeostasis during implantation.

The "intercellular junction" category was divided into two subcategories: "tight junction" that included *Cldn6*, *Cldn8*, *Cldn10*, *Cldn12*, *Cldn15*, and *Ocln*; and "gap junction" that included *Gja3*, *Gja4*, *Gja7*, *Gjb1*, and *Gjb5*. The increased expression of connexins (*Gja3*, *Gja4*, *Gja7*, *Gjb1*, and *Gjb5*) and the assembly of multiple types of

gap junction channels at this stage of preimplantation embryos (the 8-cell stage) are consistent with the biological events immediately after this stage, i.e., embryo compaction and cavitation. For example, it has been reported that the trophectoderm epithelium exhibits functional intercellular tight junctions to maintain epithelial integrity during blastocoel expansion (Sheth et al., 2000). Another connexin gene *Gja1* (*connexin43*), though it was not a part of the MGA gene list, was included in Cluster 1 with a dramatic increase in expression through preimplantation stages. Because *Gja1* null mice show normal compaction and blastocyst formation, the loss of this protein's function is compensated by other gap junction molecules (Houghton et al., 2002), and the identification of many connexins showing similar expression patterns supports this hypothesis. In addition, *Atp1b1*, a Na-K-ATPase, and *Aqp8*, a water channel, were also upregulated from the 4-cell to 8-cell stage. These channels are also thought to contribute to the trans-trophectoderm ion gradient and to the formation of a fluid-filled blastocoel (MacPhee et al., 2000; Watson and Barcroft, 2001). These transcripts have to be translated into proteins to be functional, and thus the transcription of these genes must occur earlier than the actual compaction and cavitation. Therefore, the timing of MGA seems consistent with the proposed role of these proteins in compaction and cavitation.

The "DNA (cytosine-5-)-methyltransferase" category included three of the four known DNA methyltransferases (*Dnmt2*, *Dnmt3a*, and *Dnmt3b*). *Dnmt3a* and *Dnmt3b* are essential for de novo methylation. Because de novo methylation of DNAs was observed specifically in the ICM but not in the trophectoderm of the blastocyst (Santos et al., 2002) and, especially, *Dnmt3b* protein is localized in ICM (Watanabe et al., 2002), it has been speculated that de novo methylation may contribute to the differentiation of the ICM and TE at the blastocyst stage. Furthermore, it has been suggested that the 8-cell stage could be a key period for DNA methylation, because *Dnmt1o* protein shifts from the cytoplasm to the nucleus only in 8-cell embryos (Howell et al., 2001). Taken together, our microarray data on DNA methyltransferases are consistent with the notion that DNA methylation is critical for later stages of preimplantation development.

Genes Activated from 8-Cell Embryos to Morula

A relatively small number of transcripts (511 genes) increased from the 8-cell to morula stage for the first time during preimplantation stages (Figure 2D). MAPPFinder identified the following GO terms as the ones with major changes: "GTP binding," "ATP binding," "adherent junction," "small GTPase-mediated signal transduction," "microtubule cytoskeleton," and "electron transport" (Supplemental Table S1E). The "microtubule cytoskeleton," "ATP binding," and "electron transport" categories are perhaps related to the dramatic morphological changes and energy requirements of compaction and cavitation. The "adherent junction" category included *Ilk*, *Catna1*, and *Catnb*. It is well known that catenins have a critical role in blastocyst formation. Stable cell-to-cell contacts and adhesion plaques arise in trophectoderm through associations of *E-cadherin* with the actin cytoskeleton, mediated by interactions with catenins (Torres et al., 1997; Watson and Barcroft, 2001). The "small GTPase-mediated signal transduction" category included *Iqgap1*, *Rac1*, *Kras2*, and *cdc42*, all of which have been suggested to mediate the formation of E-cadherin-based adherent junctions (Matsui et al., 2002; Natale and Watson, 2002).

Interestingly, this gene list included *Glut1* and *Glut8* as well as their regulators, *Isr1* and *Igf1r*, which are known to regulate glucose transport by translocating Glut proteins. Simultaneous upregulation of these molecules suggests an increase of glucose uptake in the embryo at this stage. Furthermore, MAPPFinder also identified a GO term "glucose metabolism" to represent the gene expression changes from morula to blastocyst. This corresponds to reports of an energy source switch from the oxidation of lactate and pyruvate via the Krebs cycle (oxidative phosphorylation) to the anaerobic metabolism of glucose via glycolysis (Carayannopoulos et al., 2000; Martin and Leese, 1999). This switch is thought to be due to the biosynthetic and developmental demands placed on embryo as blastocyst creates the fluid-filled blastocoel and prepares for implantation. To check the expression changes of other genes involved in this pathway, the microarray data were projected onto the glycolysis and glyconeogenesis pathway map in GenMAPP based on the KEGG pathway database (Figure 4; Kanehisa et al., 2002). Genes involved in pyruvate metabolism were frequently assigned to the early activation pattern (ZGA: Clusters 1, 5, and 8), whereas genes involved in glucose metabolism were frequently assigned to the late activation pattern (Cluster 2, Cluster 3, Cluster 4, and Cluster 6a) (Figure 4). Taken together, the microarray analysis confirmed the previous findings of the energy source switch during preimplantation development.

Genes Activated from Morula to Blastocyst

Only 97 genes significantly increased their transcripts from the morula to blastocyst stage for the first time during preimplantation development. Half (45) of the listed genes overlapped with the 98 blastocyst-specific genes (Figure 1C and supplemental data). MAPPFinder characterized these genes as "skeletal development/morphogenesis (*Ank*, *Enpp1*, *Spp1/Osteopontin*, *Epb4.1*, *Igf2bp3*, and *Wnt6*)," "lipid binding (*Dbi*, *Fabp3*, and *Anxa3*)," "glucose metabolism/energy pathway (*Tpi*, *Gapd*, *Ugp2*, *Mod1*, *Cyb5*, and *mt-Co2*)," "actin binding/cytoskeleton organization and biogenesis (*Arpc2*, *Tmsb4x*, *Dstn*, and *Myh9*)," and "steroid metabolism (*Hmgcr* and *Soat1*)." The upregulation of tissue structure-related genes is consistent with the emergence of the first visible structures in embryos, consisting of at least two different cell types, i.e., ICM and TE. Further comparison of these genes with ES versus TS expression profiles (Tanaka et al., 2002) subgrouped them into those with higher expression in ES cells, such as *Spp1/Osteopontin*, *Sox2*, *N-myc*, and *Tmprss2*, and those with higher expression in TS cells, such as *Snx9*, *Gata2*, *Anxa3*, *Fos*, *Ndr1*, *Cldn3*, *Wnt6*, *Ppfibp1*, *Akap2*, *S100a10*, *Pawr*, and *Desrt*. Among these genes, *Sox2* has been shown as an essential gene for the first three lineages emerging in preimplantation embryos (Avilion et al., 2003), whereas *Gata2* is suggested to contribute to both positive and

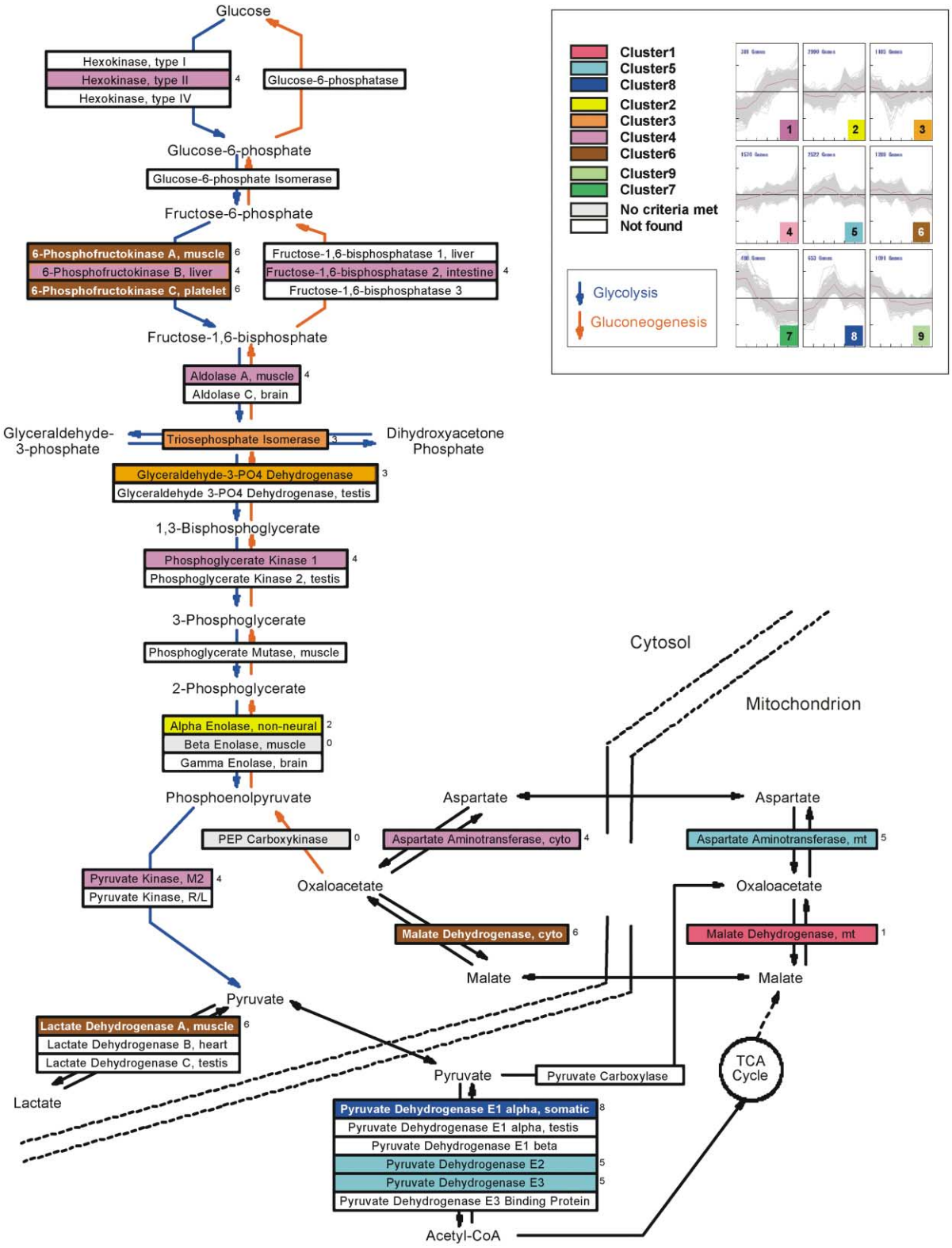


Figure 4. Expression Patterns of Genes Involved in Glycolysis and Gluconeogenesis in Preimplantation Embryos

Genes in the pathway were color coded by their expression patterns assigned to each cluster by the GenMAPP tool.

negative regulation of trophoblast giant cell-specific gene expression (Ma and Linzer, 2000).

Degradation of Maternally Stored RNAs

One of the unique features of preimplantation embryo development is the presence of maternally stored RNAs in unfertilized eggs (oocytes). Based on the analysis of a limited number of genes (Paynton et al., 1988) and radiolabeling experiments (Piko and Clegg, 1982), it has been thought that 90% of oocyte-stored RNAs are degraded by the 2-cell stage (Nothias et al., 1995; Schultz, 2002). The current microarray analysis indeed confirmed the massive maternal RNA degradation pattern (Figure 2A, Cluster 9). However, we observed three additional patterns of RNA degradation (Clusters 3, 6b, and 7). Not only was maternal RNA degradation observed by the 2-cell stage, but 68.7%, 70.5%, and 32.5% of the transcripts in Cluster 6, Cluster 7, and Cluster 9, respectively, showed significant reduction from the 4-cell to 8-cell stage. Cluster 6b also showed a gradual but moderate decrease of RNA levels throughout preimplantation development. Although the fourth expression pattern, Cluster 3, also started with the degradation of maternal RNAs, most of the genes were activated from the zygotic genome soon after the 2-cell stage. Thus, Cluster 3 genes were in sharp contrast to three patterns of maternal RNA degradation, i.e., most genes in Clusters 6b, 7, and 9 were not reactivated during the preimplantation development. This suggests the possibility that the genes in these clusters have specific functions either in oogenesis, oocyte maturation, fertilization, and/or the early phases of preimplantation development.

To examine this possibility further, we took advantage of our large preimplantation EST collections. We identified 196 genes that were recovered only from mouse unfertilized and fertilized egg cDNA libraries, but were not present in other analyzed tissues (Sharov et al., 2003). Of 196 genes, 154 genes were on the NIA 22k 60-mer microarray and 134 genes showed statistically significant expression changes during preimplantation stages (Figure 2E). Among those, 49 (36.6%) and 38 (28.4%) genes were placed in Cluster 7 and Cluster 9, respectively (Figure 2E). To look at the data in a different way, we counted a number of genes that have at least one EST in all cDNA libraries made from mouse unfertilized and fertilized eggs. Of 498 total genes in Cluster 7, 452 (90.8%) were indeed represented as ESTs more than once in unfertilized or fertilized egg libraries. Similarly, of the 1091 gene genes in Cluster 9, 892 (81.8%) were represented as ESTs more than once in unfertilized or fertilized egg libraries (Figure 2E). These analyses provided independent confirmation that genes grouped in Cluster 7 and 9 were expressed predominantly in oocytes and not expressed in later stage preimplantation embryos. Many genes are not expressed even in other tissues or cell types, suggesting their specific functions in oogenesis and/or early phases of preimplantation development.

To further characterize these genes from their functional categories, we used MAPPFinder and associated these genes to GO terms. Cluster 7 was characterized by "circadian rhythm," "small GTPase regulatory/interacting protein," and "M-phase of mitotic cell cycle," whereas Cluster 9 was characterized by "adherent junction," "Golgi apparatus/intracellular protein transport," "DNA replication," and "intracellular signaling cascade" (Supplemental Tables S1F and S1G: see supplemental data for examples of individual genes and the analysis of cytoplasmic polyadenylation element [CPE]).

Gene Expression Profiles of In Vitro-Cultured Embryos from the Early 1-Cell to Late 2-Cell Stage with Inhibitors

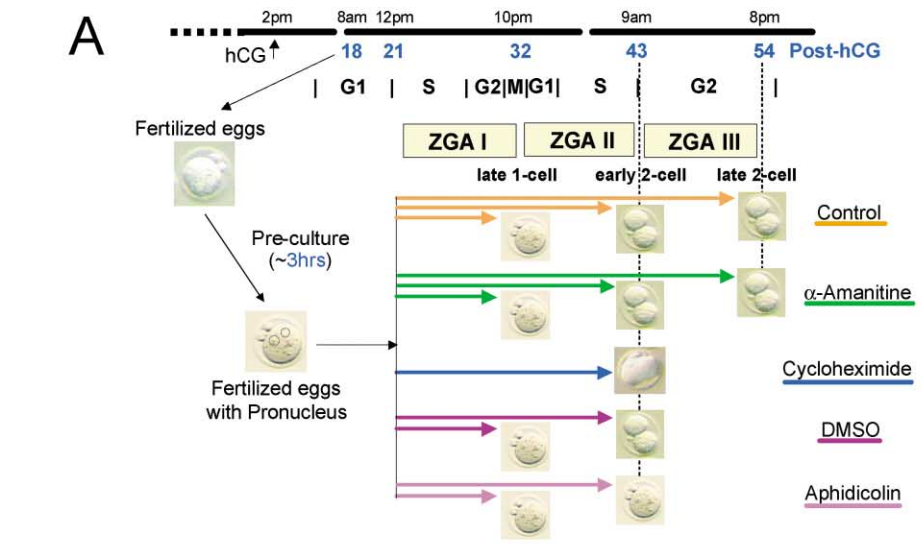
To investigate earlier events in more detail, we performed gene expression profiling of in vitro cultured embryos from the formation of pronuclei to the late 2-cell stage. In this way, the timing of earlier events such as ZGA can be more easily synchronized and studied (the details are in the Experimental Procedures section of the supplemental data). Furthermore, to interface the current microarray data to previous mechanistic studies (reviewed in Latham and Schultz, 2001; Schultz et al., 1999), we also performed microarray analysis after treating these embryos with inhibitors of transcription (α-AM), translation (CHX), and DNA replication (AC) (Figure 5A). We also examined DMSO-treated embryos, because the AC includes DMSO as a solvent.

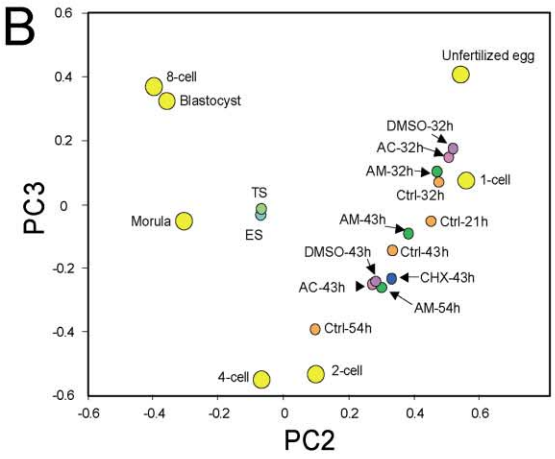
Eighteen hours (hr) after the stimulation of ovulation by hCG injection, eggs were collected and transferred into culture. To collect only fertilized eggs, after $\sim$3 hr incubation in culture, only eggs carrying both male and female pronuclei were selected as fertilized eggs (Aoki et al., 1997). At 22 hr after hCG, the inhibitors were added to the culture and incubated for 32 hr, 43 hr, and 54 hr, respectively (Figure 5A). These embryos were then harvested for the microarray analysis. To minimize the impact of sample-to-sample variations, each condition was replicated three times. Although the timing of events could be slightly different between in vitro and in vivo embryos, these periods correspond to those from the fertilized egg (early 1-cell) to 2-cell stage, and therefore correspond to the ZGA between Phase I and Phase II of the in vivo studies. Consistent with this view, the PCA of all the expression data placed these in vitro cultured and manipulated embryos along the line between 1-cell embryos and 2-cell embryos (Figures 5B and 5C). For brevity, we tentatively named these periods as follows: ZGA I, period from post-hCG 21 hr to post-hCG 32 hr; ZGA II, period from post-hCG 32 hr to post-hCG 43 hr; ZGA III, period from post-hCG 43 hr to post-hCG 54 hr (Figure 5A).

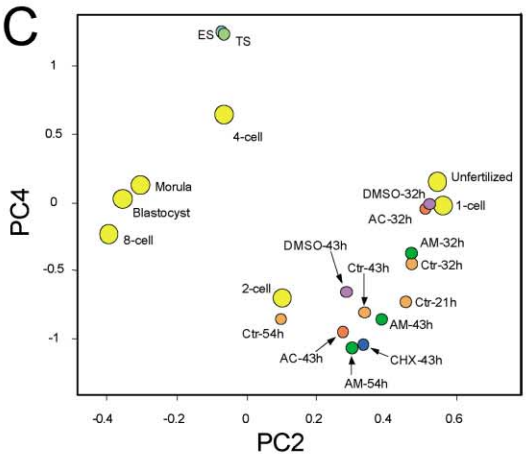
Expression Profiles of Control, DMSO-Treated Embryos, and AC-Treated Embryos

PCA showed that in vitro cultured control embryos followed the developmental track of in vivo embryos that are immediately used after harvest (Figures 5B and 5C). To look for more subtle differences in the in vitro cultured and manipulated embryos, we performed PCA without the in vivo embryo data (Figure 5D). The analysis identified clear trends of global gene expression profiles, revealing that DMSO- and AC-treated embryos showed similar expression patterns to the control embryos, whereas α-AM- and CHX-treated embryos showed dramatically different expression patterns (Figure 5D).

Although DMSO-treated embryos were examined as







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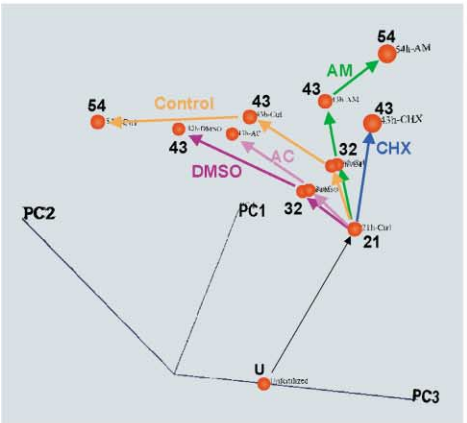


E

Pair-wise comparison	ZGA I 21h-32h	ZGA II 32h-43h	ZGA I+II 21h-43h	ZGA III 43h-54h
Significantly increased features in the control embryos	47	114	129	1733
Gene examples	Kpna2/ Importin α 2 Kanadaptin Brd4 IK cytokine Ddx21 Ube2a/Rad6 Cdc2l2 Camk2g	**Eif1a (X-isoform)** **Rex1/Zfp42** **Gata2** Ier2 Cbx8 Recql4 Pimt/Ncoa6ip **Sin** **Nsap1l** Sfrs7 Heab Pr1 Hoxa10 **Zfp52** **Oncostatin M** **Nme2/NM23** **Esx1/Spx1** **Spi-C** Gtf2e1 Rbbp2	**Eif1a (X-isoform)** **Rex1/Zfp42** **Gata2** Ier2 Cbx8 Recql4 Pimt/Ncoa6ip **Sin** **Nsap1l** Sfrs7 Heab Pr1 Zdhhc3 **Pdgfa** **Makorin1**	
No increase with α-Amanitine (%)	1 2.1%	101 88.6%	99 76.7%	1727 99.7%
No increase with Cycloheximide (%)	- -	- -	107 82.9%	- -
No increase with DMSO (%)	0 0.0%	9 7.9%	5 3.9%	- -
No increase with Aphidicolin (%)	0 0.0%	73 64.0%	55 42.6%	- -

Figure 5. Expression Profiling of In Vitro Cultured Embryos from the Early 1-Cell to Late 2-Cell Stage in the Absence or Presence of Various Drugs

(A) Experimental Design. See text and supplemental data for details.

(B–C) Principal component analysis of gene expression levels. All the gene expression data, including the data from Figure 1 and in vitro cultured embryos, were analyzed. PC2 and PC3 axes were shown in (B), and PC2 and PC4 axes were shown in (C).

(D) 3D-representation of principal component analysis. Gene expression levels of only in vitro cultured embryos were analyzed.

(E) Representative genes upregulated between each time point in control embryos and their suppression by α-AM, CHX, DMSO, and AC. AC did not suppress the upregulation of genes (shown in bold) observed in control embryos.

a control for AC-treated embryos, they also showed interesting expression patterns, suggesting an acceleration of development by DMSO (Figure 5D). The mode of DMSO action is not clear at this point, but this may be related to a previous observation that the addition of 1% (v/v) DMSO to the medium significantly improves the success rate of development to blastocysts in mouse cloned embryos (Wakayama and Yanagimachi, 2001).

AC blocks DNA replication by inhibiting DNA polymerase α and δ. As a result, cell division was blocked and embryos at 43 hr, which would normally be at the early 2-cell stage, appeared to be 1-cell embryos (Figure 5A). Interestingly, however, there was only a small difference between the expression profiles of AC-treated embryo and the control embryos at 43 hr (Figure 5D). For example, only 33 transcripts and one transcript showed higher expression in AC-treated embryos than in control embryos and DMSO-treated embryos, respectively. Similarly, only two transcripts each showed lower expression in AC-treated embryos than in control and DMSO-treated embryos.

These results indicate that all developmental programs, at least in terms of transcripts, proceed normally up to the 2-cell stage, even without DNA replication and cell division, providing strong support to previous reports that a "zygotic clock" initiates the minor ZGA regardless of DNA replication and cell cycle (Nothias et al., 1995). On the other hand, our observation that a small fraction of ZGA in control embryos (55 [42.6%] transcripts in Figure 5E), including *Recql4*, *Cbx8*, *Sfrs7*, *Pimt/Ncoa6ip*, *Ier2*, and *Arx*, were inhibited by AC also supports the previous report that the first round of DNA replication in male and female pronuclei is required for some of ZGA genes (Aoki et al., 1997; Davis et al., 1996; Davis and Schultz, 1997; Forlani et al., 1998; Schultz et al., 1999).

Expression Profiles of Embryos Treated with α-AM: Precise Timing and Mode of ZGA

The goal of analyzing the expression profiles of α-AM-treated embryos was to investigate the precise timing and mode of ZGA. It has been well documented that α-AM inhibits RNA polymerase II selectively (Kidder et al., 1985; Warner and Hearn, 1977). By comparing the expression profiles between α-AM-treated embryos and control embryos, it should be possible to identify genes that begin to be transcribed for the first time from zygotic genomes.

During ZGA I, only 47 transcripts were upregulated in control embryos. Therefore, this seems to be the beginning of ZGA. However, α-AM was able to suppress the upregulation of only one of these genes (Figure 5E), and the global expression patterns of α-AM-treated embryos were very similar to those of control embryos at post-hCG 32 hr (Figure 5D). Because we used a high enough concentration of α-AM to suppress mRNA transcription (Kidder et al., 1985), insensitivity to α-AM during ZGA I can be explained only by changes in mRNA stability or changes in the length of poly(A) tails (Rambhatla et al., 1995; Wang and Latham, 1997). Transcripts with short poly(A) tails cannot be efficiently detected here, because labeling is specific for polyadenylated transcripts, but we can at least establish that de novo transcription from the zygotic genome did not start during ZGA I (by post-hCG 32 hr).

In contrast, 114 transcripts were upregulated during ZGA II, and the majority of them were inhibited by α-AM (Figure 5E). Therefore, the increase in transcription during ZGA II should be the first zygotic de novo transcription. Although both ZGA I and ZGA II are sometimes called minor ZGA or early ZGA, these results indicate that only ZGA II is truly a "minor ZGA." It has also been demonstrated by RT-PCR that apparent changes in the abundance of *G6pd* and *Eif1a* mRNAs during the 1-cell stage (ZGA I) were insensitive to α-AM (Wang and Latham, 1997).

During ZGA III, 1733 transcripts were upregulated, but the majority (99.7%) of them were suppressed by α-AM (Figure 5E). This α-AM-sensitive major burst of transcription indeed corresponds to the major ZGA previously reported (reviewed in Latham and Schultz, 2001; Nothias et al., 1995; Schultz et al., 1999; Thompson et al., 1998).

Expression Profiles of Embryos Treated with CHX: Mechanism of ZGA

The goal of analyzing the expression profiles of CHX-treated embryos was to examine whether proteins newly translated from maternally stored RNAs affect the level of transcripts. The CHX-treated embryos showed significant differences in their global expression profiles from the control embryos at 43 hr, with the upregulation of 1189 genes and the downregulation of 778 genes. These 778 downregulated genes included 49 (43.0%) of the 114 genes that were upregulated in control embryos during ZGA II, supporting the previous report that CHX can suppress the minor ZGA (Wang and Latham, 1997). Similarly, the 1189 upregulated genes included 221 genes from the 1416 genes downregulated in control embryos during ZGA II, indicating that CHX can also suppress the degradation of maternal transcripts. This suggests that protein synthesis from either maternally stored RNAs or newly transcribed RNAs are required for both the ZGA and the degradation of maternal transcripts.

To investigate these data from a different angle, we analyzed the 129 transcripts that showed significant increase in the control embryos during ZGA I+II (minor ZGA) and found that the majority (82.9%) were suppressed by CHX to a similar extent by α-AM (76.7%) (Figure 5E), indicating that either the translation of maternal transcripts at the 1-cell stage or translation of ZGA transcripts is essential for the minor ZGA.

Discussion

With the NIA 60-mer oligo microarray platform and linear amplification labeling procedures, we have performed rigorous experimental tests and shown that starting with a small amount of material, we tend to lose detection sensitivity for expression changes ($\sim$60% detection), but the false positive rate is very low ($\sim$4%) using a stringent statistical test (False Discovery Rate: FDR). Based on extensive validation by quantitative real-time reverse-transcription polymerase chain reaction (Q-PCR), the array results with this rigorous standard have been

confirmed for essentially all genes tested (Carter et al., 2003). Therefore, as long as the same statistical parameters are applied in the current study, the validation of microarray results by Q-PCR is not required. On the other hand, the current statistical analysis is so stringent that there will be genes that had biologically significant expression changes but were not detected. Staging and embryo collection have been done by one skilled researcher (T.H.) with extreme care, and contamination by other tissues has been carefully avoided. As shown by the comparison to the EST frequency data set, the current array results are consistent with those data, suggesting that the microarray data are accurate and reliable.

Mode of Gene Activation

One of the most salient findings is the observation of many genes showing wave-like activation patterns (Figure 6A). We found at least two major activations followed by two minor activations. The first major wave is the ZGA, which peaks at the 2-cell to 4-cell embryo stages. The second major wave is the MGA, which peaks at the 8-cell embryo stage. The third wave peaks at the morula stage, and the fourth at the blastocyst stage. Each wave consists of distinct sets of genes, most of which are immediately downregulated. This may imply that not only the activated genes are shut down, but also their transcripts are actively degraded. Considering a rapid downregulation of the transcripts, some of the protein products of these transcripts may actively suppress their own transcription and/or degrade their own transcripts by a negative feedback mechanism.

It has already been demonstrated that some ZGA genes, such as *Eif1a* and *U2afbp-rs*, show transient increase at the 2-cell stage (Davis et al., 1996; Temeles et al., 1994; Wang and Latham, 1997, 2000). Global EST analysis also identified genes that showed wave-like expression at every stage of preimplantation development (Ko et al., 2000). All these reports indicated stage-specifically expressed genes exist, and hinted at their fundamental importance during preimplantation development. Here, we extended such studies to assess the global scope of this phenomenon. The microarray results reported here decisively imply that a large number of genes show wave-like expression patterns, which can be equated with stage-specific expression during preimplantation development (Figure 1C).

Why Waves of Gene Activation?

Transient expression waves of a large number of genes have not been detected in other systems. Synchronized changes of cellular status among the blastomeres during preimplantation development may make it easier to detect such an expression pattern. Programmed waves of gene activation during preimplantation development may serve to satisfy the developing embryo's requirement for specific classes of proteins at specific times. Genes that have completed their function during early sates of development may be harmful at later stages, and thus the timing of gene inactivation is as important as the timing of activation.

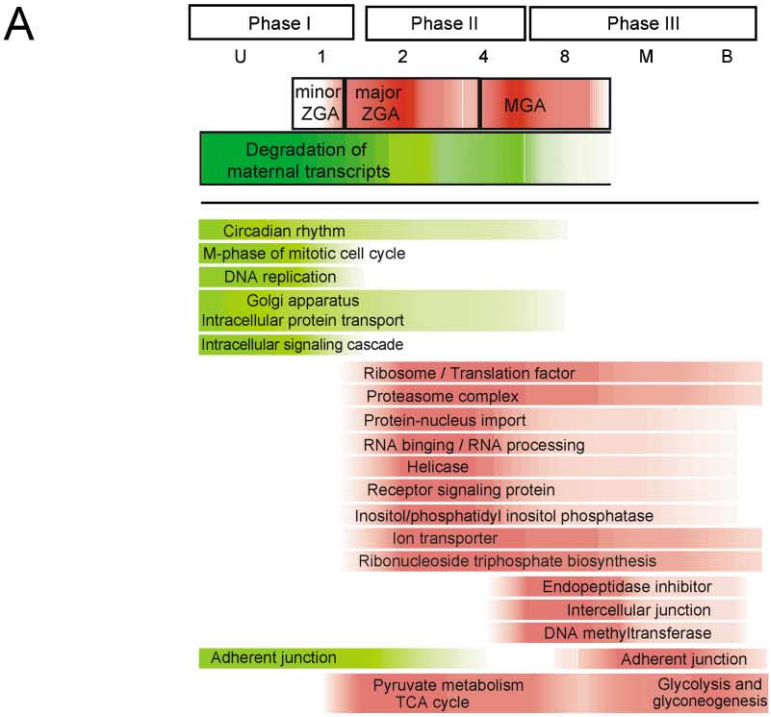
For example, the annotation of genes in MGA (Figure 6A) suggests important functions of these genes for blastocyst formation, such as establishment of intercellular junctions and DNA methylation (Howell et al., 2001; Santos et al., 2002; Watanabe et al., 2002; Watson and Barcroft, 2001). Another example is the switch from pyruvate-dependency to glucose-dependency during preimplantation development (Figure 4). The activities of such proteins seem to be required at specific times, but not before or after. Because we measured RNAs, which need several hours to be translated, actual gene actions should take place some time later. Thus, the MGA genes whose expression peaks in 8-cell embryos are translated and functions by the morula stage, leading to the formation of blastocysts. Therefore, the timing of MGA seems to be compatible with the morphological and functional changes of the embryos.

Mechanism for Waves of Gene Activation

The first wave of activation (ZGA) is timed by the "Zygotic Clock." But what times the second, third, and fourth waves of activation? One possibility is that a clock-like mechanism times activations throughout preimplantation development. A second possibility is that cell cycles or cell numbers time the activations. A third possibility is that the zygotic transcription occurs in a stepwise manner: maternal proteins translated from maternal transcripts during the 1-cell stage trigger ZGA; maternal proteins and ZGA proteins initiate the MGA (Figure 6B). We favor this model over the others, because this is the simplest, forgoing the need for an ad hoc mechanism such as a clock throughout preimplantation development. This also seems to be consistent with observations accumulated about ZGA in mammals (reviewed in Kanka, 2003) and our results with inhibitor-treated embryos, as follows.

It is known that maternally stored RNAs are actively translated during ZGA I+II, but nascent RNAs (ZGA RNAs) are not. In other words, translation of ZGA transcripts is delayed until the 2-cell stage. This is called uncoupling of transcription and translation during ZGA (Nothias et al., 1995; Schultz, 2002). Because only maternally stored RNAs and proteins are present in oocytes before fertilization, the majority of proteins translated during ZGA I+II are apparently the product of maternally stored RNAs but not of nascent ZGA transcripts. If so, the nascent proteins translated from maternally stored RNAs are required for and most likely trigger the initiation of ZGA, because blocking translation by CHX during ZGA I+II blocked the majority (82.9%) of ZGA transcription at this stage. This has also been suggested by a previous study (Wang and Latham, 1997) and is consistent with the "wave of activation" hypothesis for ZGA transcription.

Similarly, ZGA transcripts and their protein products seem to be required for the progression of embryos beyond the 4-cell stage. This has been suggested by the findings that added α-AM results in cleavage arrest, usually within one cleavage division, even in the presence of maternally stored proteins and the products newly translated from maternal transcripts (Schultz et al., 1999). We have no direct evidence to link this to the second wave of transcripts, i.e., MGA transcripts, but the association of MGA transcripts with the timing of progression from 4-cell to 8-cell embryos is consistent



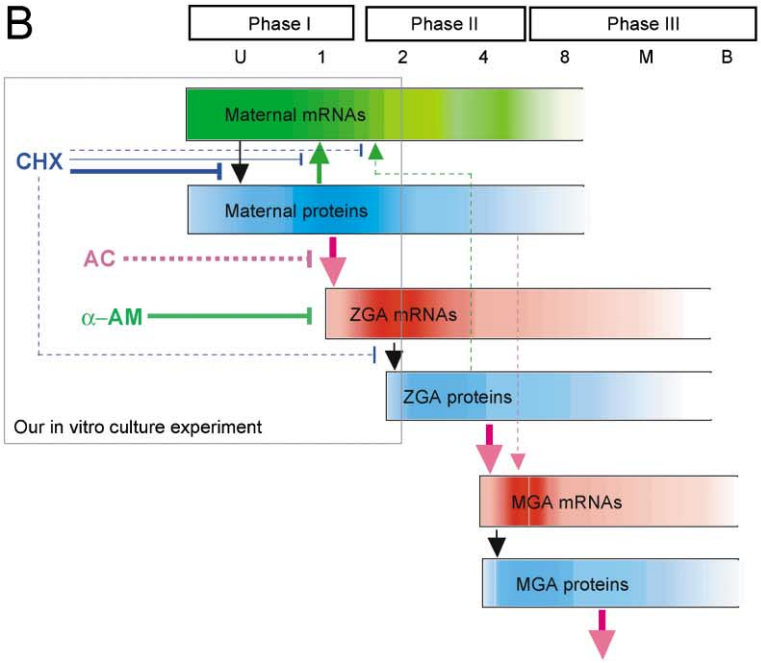


Figure 6. Major Functional Characteristics of Preimplantation Development and a Model of Its Mechanisms

(A) Major trends of gene expression changes during preimplantation development. Up- and downregulation of genes were plotted with their major functional characteristic by GO terms. Green boxes represent the transcript levels of maternally stored RNAs, whereas red boxes represent those activated from the zygotic genomes.

(B) Model for the mechanism underlying the waves of zygotic genome activations. The zygotic transcription occurs in a stepwise manner: maternal proteins translated from maternal transcripts during the 1-cell stage trigger ZGA, and then ZGA proteins initiate the MGA. The lines from reagents represent their inhibitory effects, but the broken lines from AC and CHX mean incomplete inhibition and uncertain inhibition, respectively. The downward arrows from proteins indicate activation of transcription, but the broken one was not clarified in this study. The upward arrow from maternal proteins represents a role in the degradation of maternal mRNAs, whereas the broken one from ZGA proteins represents a possible role. Maternal proteins shown in this panel include both ones derived from oocyte-stored proteins and ones de novo synthesized from maternal mRNAs after fertilization.

with triggering of MGA transcripts by ZGA transcripts and their protein products. Further tests of this hypothesis should be done by blocking the transcription or translation for specific periods of time or specific genes and observing whether downstream waves of activation are affected. It will also be interesting to compare the regulatory sequences of these coordinately activated genes. Such analysis may be able to find common regulatory elements for these genes and provide better insights into the mechanisms of these gene activations.

Perspectives

Preimplantation embryos offer a relatively homogeneous biological system that is well adapted to expression profiling studies. Global expression data have now elucidated clearly discernible major trends in preimplantation development. This feature will be advantageous for further meta-analysis of the data by theoretical biologists and computer scientists to test computer simulations of gene regulatory networks. Initial examination of the data already hints at some oscillations in the waves

of gene activations; i.e., a large wave tends to be followed by a smaller wave. This could be a sign of dynamic gene expression networks.

The comprehensive expression profiles of the majority of genes in normal mouse preimplantation development provided here should give a baseline for the analysis of abnormal mouse early development in transgenic, knockout, knockdown, cloning, and other mutant mouse models, and for comparative analysis of preimplantation development in other mammalian species, including human.

Experimental Procedures

Embryo Collection

Details are available as supplemental data (http://www.developmentalcell.com/cgi/content/full/6/1/117/DC1). In brief, embryos were collected from superovulated C57BL/6J mice by the standard method (Hogan et al., 1994). For in vitro study, fertilized eggs were harvested from superovulated C57BL/6J mice and cultured in the presence or absence of drugs: α-AM (0.1 mg/ml, Sigma), CHX (20 μg/ml, Sigma), AC (3 μg/ml, Sigma), and DMSO (0.3% (v/v), Sigma).

RNA Extraction, Labeling, and Hybridization on the NIA 22K 60-Mer Oligo Microarray

Details are available as supplemental data. In brief, mRNAs were extracted using a Quickprep micro poly-A RNA Extraction Kit (Amersham Biosciences, NJ) and linear acrylamide as a carrier (Ambion, TX). Aliquots of mRNAs were labeled with Cy3-dye by two-round linear amplification using a Fluorescent Linear Amplification Kit (Agilent Technologies, CA) (Carter et al., 2003). Universal mouse reference RNA (Stratagene, CA) was labeled with Cy5-dye by one-round linear amplification. A set of cRNA targets from embryo and universal reference was assembled into a hybridization reaction on the NIA 22K 60-mer oligo microarray (Carter et al., 2003).

Microarray Data Analysis

Details are available as supplemental data. In brief, intensity of 21,939 gene features per array was extracted from scanned microarray images using Feature Extraction 5.1.1 software (Agilent Technologies), which performs background subtractions and dye normalization. This normalization method is targeted at detecting changes in relative expression of individual genes rather than global expression. Global expression change would require external normalization controls (van de Peppel et al., 2003). The data were analyzed by the NIA microarray analysis tool (Carter et al., 2003; http://lgsun.grc.nia.nih.gov/ANOVA/), TIGR Multiple Experiment Viewer (MEV) (Saeed et al., 2003), MATLAB (The MathWorks, MA), and GenMAPP/MAPPFinder (Doniger et al., 2003) with GO terms (Ashburner et al., 2000).

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References

Aoki, F., Worrad, D.M., and Schultz, R.M. (1997). Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. Dev. Biol. *181*, 296–307.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., et al. (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. *25*, 25–29.

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. *17*, 126–140.

Bachvarova, R. (1985). Gene expression during oogenesis and oocyte development in mammals. Dev. Biol. (NY1985) *1*, 453–524.

Carayannopoulos, M.O., Chi, M.M., Cui, Y., Pingsterhaus, J.M., McKnight, R.A., Mueckler, M., Devaskar, S.U., and Moley, K.H. (2000). GLUT8 is a glucose transporter responsible for insulin-stimulated glucose uptake in the blastocyst. Proc. Natl. Acad. Sci. USA *97*, 7313–7318.

Carter, M.G., Hamatani, T., Sharov, A.A., Carmack, C.E., Qian, Y., Aiba, K., Ko, N.T., Dudekula, D.B., Brzoska, P.M., Hwang, S.S., and Ko, M.S.H. (2003). In situ-synthesized novel microarray optimized for mouse stem cell and early developmental expression profiling. Genome Res. *13*, 1011–1021.

Davis, W., Jr., and Schultz, R.M. (1997). Role of the first round of DNA replication in reprogramming gene expression in the preimplantation mouse embryo. Mol. Reprod. Dev. *47*, 430–434.

Davis, W., Jr., De Sousa, P.A., and Schultz, R.M. (1996). Transient expression of translation initiation factor eIF-4C during the 2-cell stage of the preimplantation mouse embryo: identification by mRNA differential display and the role of DNA replication in zygotic gene activation. Dev. Biol. *174*, 190–201.

Doniger, S.W., Salomonis, N., Dahlquist, K.D., Vranizan, K., Lawlor, S.C., and Conklin, B.R. (2003). MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. Genome Biol. *4*, R7.

Edwards, R.G. (2003). Aspects of the molecular regulation of early mammalian development. Reprod. Biomed. Online *6*, 97–113.

Forlani, S., Bonnerot, C., Capgras, S., and Nicolas, J.F. (1998). Relief of a repressed gene expression state in the mouse 1-cell embryo requires DNA replication. Development *125*, 3153–3166.

Hogan, B.L.M., Beddington, R., Costantini, F., and Elizabeth, L. (1994). Manipulating the Mouse Embryo: A Laboratoty Manual, 2nd ed. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Houghton, F.D., Barr, K.J., Walter, G., Gabriel, H.D., Grummer, R., Traub, O., Leese, H.J., Winterhager, E., and Kidder, G.M. (2002). Functional significance of gap junctional coupling in preimplantation development. Biol. Reprod. *66*, 1403–1412.

Howell, C.Y., Bestor, T.H., Ding, F., Latham, K.E., Mertineit, C., Trasler, J.M., and Chaillet, J.R. (2001). Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene. Cell *104*, 829–838.

Hwang, S.Y., Oh, B., Knowles, B.B., Solter, D., and Lee, J.S. (2001). Expression of genes involved in mammalian meiosis during the transition from egg to embryo. Mol. Reprod. Dev. *59*, 144–158.

Kanehisa, M., Goto, S., Kawashima, S., and Nakaya, A. (2002). The KEGG databases at GenomeNet. Nucleic Acids Res. *30*, 42–46.

Kanka, J. (2003). Gene expression and chromatin structure in the pre-implantation embryo. Theriogenology *59*, 3–19.

Kidder, G.M., Green, A.F., and McLachlin, J.R. (1985). On the use of alpha-amanitin as a transcriptional blocking agent in mouse embryos: a cautionary note. J. Exp. Zool. *233*, 155–159.

Knowles, B.B., Evsikov, A.V., De Vries, W.N., Peaston, A.E., and Solter, D. (2003). Molecular control of the oocyte to embryo transition. Philos. Trans. R. Soc. Lond. B Biol. Sci. *358*, 1381–1388.

Ko, M.S.H. (2001). Embryogenomics: developmental biology meets genomics. Trends Biotechnol. *19*, 511–518.

Ko, M.S.H., Kitchen, J.R., Wang, X., Threat, T.A., Hasegawa, A., Sun, T., Grahovac, M.J., Kargul, G.J., Lim, M.K., Cui, Y., et al. (2000). Large-scale cDNA analysis reveals phased gene expression patterns during preimplantation mouse development. Development *127*, 1737–1749.

Latham, K.E., and Schultz, R.M. (2001). Embryonic genome activation. Front. Biosci. *6*, D748–D759.

Latham, K.E., Garrels, J.I., Chang, C., and Solter, D. (1991). Quantitative analysis of protein synthesis in mouse embryos. I. Extensive reprogramming at the one- and two-cell stages. Development *112*, 921–932.

Ma, G.T., and Linzer, D.I. (2000). GATA-2 restricts prolactin-like protein A expression to secondary trophoblast giant cells in the mouse. Biol. Reprod. *63*, 570–574.

MacPhee, D.J., Jones, D.H., Barr, K.J., Betts, D.H., Watson, A.J., and Kidder, G.M. (2000). Differential involvement of Na(+),K(+)-ATPase isozymes in preimplantation development of the mouse. Dev. Biol. *222*, 486–498.

Marra, M., Hillier, L., Kucaba, T., Allen, M., Barstead, R., Beck, C., Blistain, A., Bonaldo, M., Bowers, Y., Bowles, L., et al. (1999). An encyclopedia of mouse genes. Nat. Genet. *21*, 191–194.

Martin, K.L., and Leese, H.J. (1999). Role of developmental factors in the switch from pyruvate to glucose as the major exogenous energy substrate in the preimplantation mouse embryo. Reprod. Fertil. Dev. *11*, 425–433.

Matsui, T., Kinoshita, T., Morikawa, Y., Tohya, K., Katsuki, M., Ito, Y., Kamiya, A., and Miyajima, A. (2002). K-Ras mediates cytokine-induced formation of E-cadherin-based adherens junctions during liver development. EMBO J. *21*, 1021–1030.

Natale, D.R., and Watson, A.J. (2002). Rac-1 and IQGAP are potential regulators of E-cadherin-catenin interactions during murine preimplantation development. Gene Expr. Patterns *2*, 17–22.

Nothias, J.Y., Majumder, S., Kaneko, K.J., and DePamphilis, M.L. (1995). Regulation of gene expression at the beginning of mammalian development. J. Biol. Chem. *270*, 22077–22080.

Okazaki, Y., Furuno, M., Kasukawa, T., Adachi, J., Bono, H., Kondo, S., Nikaido, I., Osato, N., Saito, R., Suzuki, H., et al. (2002). Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature *420*, 563–573.

Paria, B.C., Ma, W., Tan, J., Raja, S., Das, S.K., Dey, S.K., and Hogan, B.L. (2001). Cellular and molecular responses of the uterus to embryo implantation can be elicited by locally applied growth factors. Proc. Natl. Acad. Sci. USA *98*, 1047–1052.

Paynton, B.V., Rempel, R., and Bachvarova, R. (1988). Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. Dev. Biol. *129*, 304–314.

Peng, X., Wood, C.L., Blalock, E.M., Chen, K.C., Landfield, P.W., and Stromberg, A.J. (2003). Statistical implications of pooling NA samples for microarray experiments. BMC Bioinformatics *4*, 26.

Piko, L., and Clegg, K.B. (1982). Quantitative changes in total RNA, total poly(A), and ribosomes in early mouse embryos. Dev. Biol. *89*, 362–378.

Rambhatla, L., Patel, B., Dhanasekaran, N., and Latham, K.E. (1995). Analysis of G protein alpha subunit mRNA abundance in preimplantation mouse embryos using a rapid, quantitative RT-PCR approach. Mol. Reprod. Dev. *41*, 314–324.

Rothstein, J.L., Johnson, D., DeLoia, J.A., Skowronski, J., Solter, D., and Knowles, B. (1992). Gene expression during preimplantation mouse development. Genes Dev. *6*, 1190–1201.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., et al. (2003). TM4: a free, open-source system for microarray data management and analysis. Biotechniques *34*, 374–378.

Santos, F., Hendrich, B., Reik, W., and Dean, W. (2002). Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev. Biol. *241*, 172–182.

Sasaki, N., Nagaoka, S., Itoh, M., Izawa, M., Konno, H., Carninci, P., Yoshiki, A., Kusakabe, M., Moriuchi, T., Muramatsu, M., et al. (1998). Characterization of gene expression in mouse blastocyst using single-pass sequencing of 3995 clones. Genomics *49*, 167–179.

Schena, M., Shalon, D., Davis, R.W., and Brown, P.O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science *270*, 467–470.

Schultz, R.M. (2002). The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. Hum. Reprod. Update *8*, 323–331.

Schultz, R.M., Davis, W., Jr., Stein, P., and Svoboda, P. (1999). Reprogramming of gene expression during preimplantation development. J. Exp. Zool. *285*, 276–282.

Sharov, A.A., Piao, Y., Matoba, R., Dudekula, D.B., Qian, Y., VanBuren, V., Falco, G., Martin, P.R., Stagg, C.A., Bassey, U.C., et al. (2003). Transcriptome analysis of mouse stem cells and early embryos. Plos. Biol., in press.

Sheth, B., Moran, B., Anderson, J.M., and Fleming, T.P. (2000). Post-translational control of occludin membrane assembly in mouse trophectoderm: a mechanism to regulate timing of tight junction biogenesis and blastocyst formation. Development *127*, 831–840.

Tanaka, T.S., Kunath, T., Kimber, W.L., Jaradat, S.A., Stagg, C.A., Usuda, M., Yokota, T., Niwa, H., Rossant, J., and Ko, M.S.H. (2002). Gene expression profiling of embryo-derived stem cells reveals candidate genes associated with pluripotency and lineage specificity. Genome Res. *12*, 1921–1928.

Temeles, G.L., Ram, P.T., Rothstein, J.L., and Schultz, R.M. (1994). Expression patterns of novel genes during mouse preimplantation embryogenesis. Mol. Reprod. Dev. *37*, 121–129.

Thompson, E.M., Legouy, E., and Renard, J.P. (1998). Mouse embryos do not wait for the MBT: chromatin and RNA polymerase remodeling in genome activation at the onset of development. Dev. Genet. *22*, 31–42.

Tong, Z.B., Gold, L., Pfeifer, K.E., Dorward, H., Lee, E., Bondy, C.A., Dean, J., and Nelson, L.M. (2000). Mater, a maternal effect gene required for early embryonic development in mice. Nat. Genet. *26*, 267–268.

Torres, M., Stoykova, A., Huber, O., Chowdhury, K., Bonaldo, P., Mansouri, A., Butz, S., Kemler, R., and Gruss, P. (1997). An alpha-E-catenin gene trap mutation defines its function in preimplantation development. Proc. Natl. Acad. Sci. USA *94*, 901–906.

van de Peppel, J., Kemmeren, P., van Bakel, H., Radonjic, M., van Leenen, D., and Holstege, F.C. (2003). Monitoring global messenger RNA changes in externally controlled microarray experiments. EMBO Rep. *4*, 387–393.

Wakayama, T., and Yanagimachi, R. (2001). Effect of cytokinesis inhibitors, DMSO and the timing of oocyte activation on mouse cloning using cumulus cell nuclei. Reproduction *122*, 49–60.

Wang, Q., and Latham, K.E. (1997). Requirement for protein synthesis during embryonic genome activation in mice. Mol. Reprod. Dev. *47*, 265–270.

Wang, Q., and Latham, K.E. (2000). Translation of maternal messenger ribonucleic acids encoding transcription factors during genome activation in early mouse embryos. Biol. Reprod. *62*, 969–978.

Warner, C.M., and Hearn, T.F. (1977). The effect of alpha-amanitin on nucleic acid synthesis in preimplantation mouse embryos. Differentiation *7*, 89–97.

Wassarman, P.M., and Kinloch, R.A. (1992). Gene expression during oogenesis in mice. Mutat. Res. *296*, 3–15.

Watanabe, D., Suetake, I., Tada, T., and Tajima, S. (2002). Stage- and cell-specific expression of Dnmt3a and Dnmt3b during embryogenesis. Mech. Dev. *118*, 187–190.

Watson, A.J., and Barcroft, L.C. (2001). Regulation of blastocyst formation. Front. Biosci. *6*, D708–D730.

Weil, M.R., Macatee, T., and Garner, H.R. (2002). Toward a universal standard: comparing two methods for standardizing spotted microarray data. Biotechniques *32*, 1310–1314.

Zeng, F., and Schultz, R.M. (2003). Gene expression in mouse oocytes and preimplantation embryos: use of suppression subtractive hybridization to identify oocyte- and embryo-specific genes. Biol. Reprod. *68*, 31–39.