

# Identification of Differentially Expressed Genes in Murine Embryos at the Blastocyst Stage Using Annealing Control Primer System

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**ABSTRACT** The identification of embryo-specific genes would provide insights into early embryonic development. However, the current methods employed to identify the genes that are expressed at a specific developmental stage are labor intensive and suffer from high rates of false positives. Here we employed a new and accurate reverse transcription-polymerase chain reaction (RT-PCR) method that involves annealing control primers (ACPs) to identify the genes that are specifically or prominently expressed in mouse blastocysts compared to 4-cell stage embryos. Using 120 ACPs, we identified and sequenced 74 of these differentially expressed genes (DEGs). Basic Local Alignment Search Tool (BLAST) searches revealed that 53 were known genes, 9 encoded ribosomal proteins, and 12 were unknown genes. Of the known genes, 14 were selected and further characterized using real-time quantitative PCR to assess their stage-specific expression in mouse embryos. This analysis suggests that the ACP system is a very good method for the identification of stage-specific genes in small numbers of mouse embryos. Further analysis of the differentially expressed blastocyst genes we have identified will provide insights into the molecular basis of preimplantation development. *Mol. Reprod. Dev.* 70: 278–287, 2005. © 2005 Wiley-Liss, Inc.

**Key Words:** gene regulation; mouse embryos; differential expressed genes; annealing control primer

## INTRODUCTION

In mammals, preimplantation development is characterized by various morphological and physiological transitions that occur after fertilization. These developmental events include the first cleavage division, the timing of which is known to be an indicator of the subsequent developmental potential of the embryo (Lonergan et al., 1999), the activation of the embryonic genome (Memili et al., 1998), the compaction of the morula, and the formation of the blastocyst. These morphological and physiological transitions in the preimplantation embryo are accompanied and regulated by the differential expression of developmentally important genes (Zimmermann and Schultz, 1994; Schultz et al., 1999).

Thousands of genes have to be expressed in a stage-specific manner to assure normal embryo development but to date only about 15 physiological functions and the expression of 60–70 genes have been studied (Niemann and Wrenzycki, 2000). To understand the molecular basis of preimplantation development, the differentially expressed genes (DEGs) that participate in mammalian embryogenesis will have to be identified and studied in detail. However, it is difficult to identify a DEG in a cell that functions during development, particularly within an embryo, because the gene is only expressed at low levels and its transcript is hard to detect in the abundant mRNA within a cell. To detect DEGs that are transcribed at low levels, highly specific polymerase chain reaction (PCR) amplification is required.

Several reverse transcription-polymerase chain reaction (RT-PCR) methods have been used to date to identify novel embryonic genes or expressed sequences. Differential display methods, which are based on PCR using short arbitrary primers, are simple and fast but suffer from high rates of false positives. They are also biased toward detecting more abundant transcripts. Recently, suppression subtractive hybridization has been used to identify genes in preimplantation mammalian embryos (Mohan et al., 2002; Zeng and Schultz, 2003) but it is labor-intensive and also prone to false positives.

Here we describe the identification of DEGs in murine 4-cell and blastocyst stage embryos by using a new differential display PCR method that is based on annealing control primers (ACPs) (Hwang et al., 2003; Kim et al., 2004). This is an easy technique without false positives that only allows real products to be amplified. We used it to analyze blastocyst-specific mRNA expression patterns and to isolate differentially expressed transcripts. We also quantified the gene expression of 14 selected blastocyst-specific DEGs. The possible roles

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these genes could play in preimplantation development are discussed.

## MATERIALS AND METHODS

### Generation of Embryos

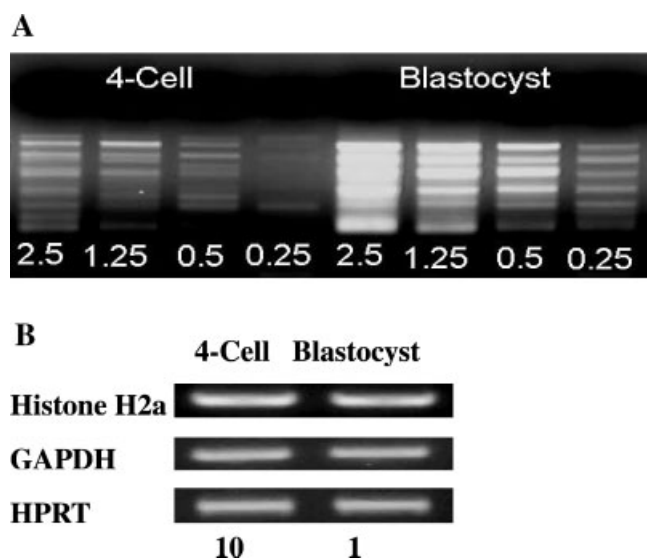
To obtain fertilized embryos, 5-week-old B6C3 F1 female mice (C57BL/6 female  $\times$  C3H/He male) were induced to superovulate by intraperitoneal injections of pregnant mare serum gonadotropin (PMSG, 5 IU) and human chorionic gonadotrophin (hCG, 5 IU) 48 h apart. The fertilized zygotes were harvested from the mated female mice 16 h after the hCG injection. The embryos were washed twice in M2, twice in M16 medium, and then cultured in M16 medium for 4-cell or blastocysts at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air. For other experiments, 1-cell, 2-cell, 4-cell, morula, and blastocyst stage embryos were harvested. The embryos were then washed in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS, snap frozen in liquid nitrogen, and stored at -70°C until use.

### ACP RT-PCR Analysis

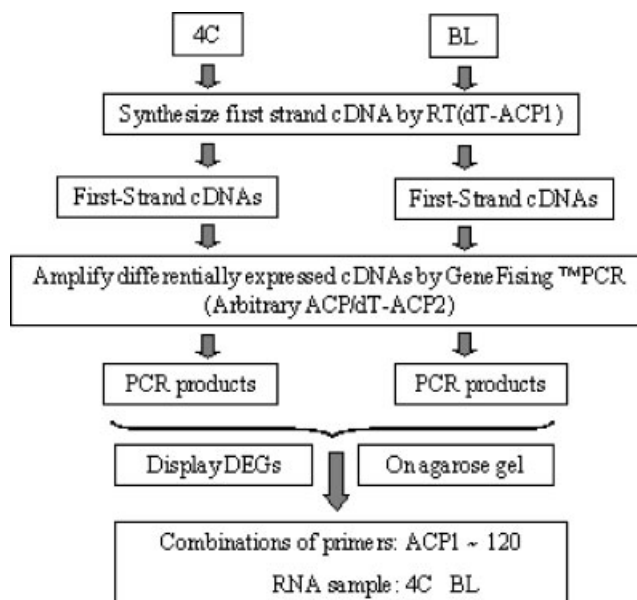
Blastocysts and 4-cell embryos were resuspended in 300  $\mu$ l lysis/binding buffer for 5 min, and the polyadenylated RNA was prepared by adding 50  $\mu$ l of washed Dynabeads from the Dynabeads mRNA Direct Kit (DynaL Asa, Oslo, Norway). The mRNAs were then subjected to ACP RT-PCR analysis. A control ACP RT-PCR experiment was performed to determine the optimal levels of blastocyst and 4-cell embryo mRNAs to use (Fig. 1A). This experiment revealed that the gel intensities were similar when the mRNAs of 2.5 4-

cell embryos and 0.25 blastocysts were used. This optimal 10:1 ratio of 4-cell embryo:blastocyst mRNAs was confirmed by analyzing the mRNAs of ten 4-cell embryos and one blastocyst for the expression of the housekeeping genes that encode histone H2a, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and hypoxanthine phosphoribosyltransferase (HPRT) by ACP RT-PCR, as equivalent levels were detected (Fig. 1B). The ACP RT-PCR method is depicted schematically in Figure 2. The first step is first-strand cDNA synthesis, which is performed using the dT-ACP1 primer (GeneFishing™ DEG kits, Seegene, Korea). The 3'-end core portion of this primer bears a hybridizing sequence that is complementary to the poly A region of mRNA transcripts. Thus, the purified mRNA was incubated with 1  $\mu$ l of dT-ACP1 (10 mM) at 80°C for 3 min after which the RT reaction was performed in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 6 mM MgCl<sub>2</sub>, 2 mM DTT, 1 mM of each dNTP, 20 U of RNase inhibitor, and 200 U of Superscript II (Invitrogen, Carlsbad, CA). The reaction mixture was incubated at 42°C for 90 min and then at 94°C for 2 min.

The cDNAs are then subjected to second-strand cDNA synthesis by random PCR amplification using dT-ACP2 and one of 120 arbitrary ACPs (GeneFishing™ DEG kits, Seegene, Korea) as primers. The amplification is conducted so the 3'-end core portion of the dT-ACP2 primer is prevented from annealing to the first-strand cDNAs and only the 3'-end core portion of the arbitrary ACP that bears a hybridizing sequence that is suffi-



**Fig. 1.** Optimization of the 4-cell and blastocyst mRNA amounts to be used in ACP RT-PCR. **A:** ACP RT-PCR using the arbitrary ACP primer (#6) was performed with mRNA from various numbers of 4-cell and blastocyst embryos. The numbers of embryos are presented at the bottom of the gel. Similar gel intensities were observed when 2.5 4-cell and 0.25 blastocyst embryos were used. **B:** The expression of the three house-keeping genes that express histone H2a, GAPDH, and HPRT in the mRNAs from ten 4-cell embryos and one blastocyst.



**Fig. 2.** Schematic depiction of the ACP RT-PCR GeneFishing Procedure. The mRNAs isolated from 4-cell (4C) and blastocyst (BL) embryos were used for the synthesis of first-strand cDNA using the dT-ACP1 primer. Second-strand cDNAs were then amplified during second-stage PCR by using a combination of dT-ACP2 (reverse primer) and one of 120 arbitrary ACP primers (forward primer). The products were then separated on an agarose gel to identify the DEGs that show greater expression in the blastocyst stage.

ciently complementary to a region on the first-strand cDNA can anneal. Thus, the PCRs were performed in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 10 pM of each primer, and 2.5 U of Taq DNA polymerase (Promega, Madison, WI) in a final volume of 25 µl. After incubation at 94°C for 3 min, 50°C for 3 min, and 72°C for 1 min, 40 cycles of 94°C for 40 sec, 65°C for 40 sec, and 72°C for 40 sec followed, after which a post-extension was performed at 72°C for 5 min. The PCR products were then subjected to electrophoresis on an 2% agarose gel and stained with 1 µg/ml ethidium bromide (Fig. 2).

### Cloning and Analysis of cDNA

After the RT-PCR subtraction procedure described above, the amplified products were cloned into the pCR<sup>®</sup> 2.1-TOPO vector of the TA cloning kit (Invitrogen Co., Grand Island, NY) and used to transform competent TOP10 *Escherichia coli* cells. The colonies were grown for 16–18 hr at 37°C on Luria broth agar plates containing ampicillin, X-gal (5-bromo 4-chloro 3-indoyl-b-D-galactopyranoside), and isopropyl-b-D-thiogalactopyranoside for blue/white colony selection. The plasmids were extracted and the inserts were subjected to dideoxy chain termination sequencing (Applied Biosystems,

Model 373A Automated Sequencer, Oklahoma State University Recombinant DNA/Protein Resource Facility). The identity of each product was confirmed by sequence homology analysis using the Basic Local Alignment Search Tool (BLAST, Altschul et al., 1990).

### Semi-Quantitative RT-PCR

Messenger RNA was prepared from ten 4-cell embryos and a blastocyst embryo using the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway) according to the manufacturer's instructions. Standard cDNA synthesis by RT of the RNA was then performed using the Oligo (dT)<sub>12–18</sub> primer and the Superscript reverse transcriptase enzyme (Invitrogen Co., Grand Island, NY). The mRNAs of several genes were then detected by RT-PCR with specific primer pairs (Table 1) using reagents supplied with a Taq DNA polymerase kit (Takara Korea Biomedical, Inc., Seoul, Korea). The PCR products were visualized under ultraviolet light on 1.2% agarose (Invitrogen Co., Grand Island, NY) gels in 1× TAE buffer containing 1 µg/ml ethidium bromide (Sigma).

### Real-Time RT-PCR

The mRNAs of the same numbers of 1-cell (1C)-, 2-cell (2C)-, 4-cell (4C)-, morula (Mo)-, and blastocyst (BL)-

**TABLE 1. Primer Sequences and Cycling Conditions Used in Semi-Quantitative RT-PCR and/or Real-Time RT-PCR**

Genes	GenBank acc. no.	Primer sequence	Annealing temperature (°C)	RT-PCR cycle no.	Base pairs
<i>MT-1</i>	AK018727	5' tcc tga gta cct tct cct ca 3' gta gaa aac ggg ggt tta gt'	60	38	339
<i>Ckb</i>	M74149	5' gac ttt cct ggt gtg gat ta 3' aaa taa act cta ccc agg gc	60	35	623
<i>Psap</i>	XM_192668	5' atg tga ttt tat gcc aga cc 3' ctt tga gca gtg cag ata ca	60	38	303
<i>Glut3</i>	BC034122	5' ttc tca tct cca ttg tcc tc 3' agt aac agc gaa atc gtc at	55	38	256
<i>Gm2a</i>	NM_010299	5' tgg tcc ctg gag atg tag tc 3' agg gta gag aac cgt agg ag	59	35	522
<i>Ndufa7</i>	BC055698	5' cta ctc gcg tta tcc aaa ag 3' ttg tga gga cat gat gat tg	55	38	289
<i>Calpactin</i>	BC025044	5' agg ttt cga cag act ctt ca 3' ccc ttc tgc ttc atg ttt ac	55	35	306
<i>Lamc1</i>	J03484	5' gtg acc gga gtc acg aag tc 3' gca cgt aag tga tgt caa aa	55	38	202
<i>Dppa1</i>	NM_178247	5' cct tca agt cct cat ttc ag 3' ggc aac tgg aac ttt gat aa	55	35	351
<i>Cn2</i>	BC005532	5' ctg act tca acc atc ctc at 3' gca ttt tgg ttc ctt cta tg	55	35	228
<i>Apoa1bp</i>	AJ344092	5' tca gtg tca gaa aat gga ca 3' tat cgg cca gta aag tga gt	60	35	297
<i>Ass1</i>	M31690	5' ccc aga tgt cct tga gat ag 3' cta aat gag cgt ggt aaa gg	60	35	230
<i>Sparc</i>	X04017	5' aga tag tgg agg agg aaa cc 3' gtc gaa ggt ctt gtt gtc at	60	35	278
<i>Gsta4</i>	AK009668	5' cag gat tga cat gta tgc ag 3' gag ttc ttc cac cat caa aa	55	35	241
<i>Histone H2a</i>	BF703857	5' taa cgg cgg aga tcc tgg ag 3' tgg ctc tcc gtc ttc ttg gg	60	32	196
<i>GAPDH</i>	M32599	5' gtg aag gtc ggt gtg aac gg 3' gat gca ggg atg atg ttc tg	60	35	284
<i>HPRT</i>	J00423	5' ctt gct cga gat gtc atg aag 3' gtt tgc att gtt tta cca gtg	60	35	290

stage embryos were prepared as described above and subjected to RT as described above. Histone H2a mRNA was used as an internal standard. The cDNAs of several genes and histone H2a were then detected by real-time RT-PCR using the specific primer pairs shown in Table 1 (apart from the GAPDH and HPRT primers). The PCR reaction was performed according to the real-time PCR machine manufacturer's instructions (DNA Engine Opticon 2 fluorescence detection system, MJ Research, MA).

The threshold cycle (Ct) value represents the cycle number at which sample fluorescence rises statistically above background. The reactions were conducted according to the protocol of the DyNAmo SYBR green qPCR kit (Finnzyme, Finland) that contains a modified Taq DNA polymerase, SYBR Green, optimized PCR buffer, 5 mM MgCl<sub>2</sub>, and a dNTP mix that includes dUTP. The PCR involved a preliminary denaturation program (95°C for 10 min), followed by 45 cycles of amplification and quantification (95°C for 30 sec, 55°C/59°C/60°C for 30 sec, 72°C for 30 sec with a single fluorescence measurement), after which a melting curve program was employed (65~95°C, with a heating rate of 0.2°C per second and continuous fluorescence measurement). The products were then cooled to 4°C.

The fluorescence data were acquired after the extension step during PCR reactions containing SYBR Green 1. Thereafter, PCR products were analyzed by generating a melting curve. Since the melting curve of a product is sequence-specific, it can be used to distinguish between nonspecific and specific PCR products. For the mathematical model, it is necessary to determine the crossing points (CP) for each transcript. CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence.

The relative quantification of gene expression was analyzed by the 2-ddCt method (Livak and Schmittgen, 2001). The sizes of PCR products were confirmed by gel electrophoresis on a standard 1.2% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light.

### Statistical Analysis

The general linear models (GLM) procedure in the Statistical Analysis System (SAS User's Guide, 1985, Statistical Analysis System, Inc., Cary, NC) was used to analyze the data from all experiments. Significant differences were determined using Tukey's Multiple Range Test (Steel and Torrie, 1980), and *P*-values of <0.05 were considered significant.

## RESULTS

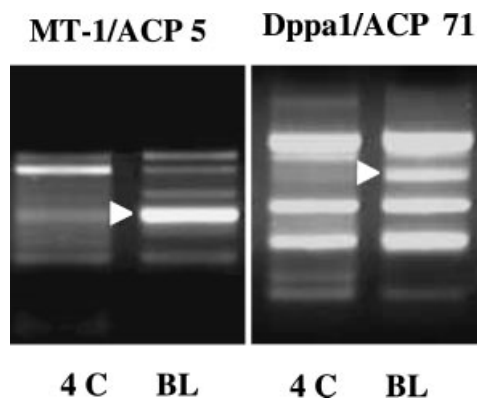
### DEGs in Murine Blastocysts

To identify genes that are specifically or predominantly expressed at the blastocyst stage, we compared the mRNA expression profiles of murine 4-cell and blastocyst stage embryos. To do this, the mRNAs from both types of embryos were extracted and subjected to ACP RT-PCR analysis using a combination of 120

arbitrary primers and two anchored oligo(dT) primers (dT-ACP 1 and dT-ACP 2). This method is depicted schematically in Figure 2 and is described in the Materials and Methods. The analysis generated about 800 amplicons, 74 of which were only found in blastocysts or were markedly upregulated in blastocysts compared to the 4-cell embryos. Examples of the identification of two blastocyst DEGs (metallothionein 1 [MT-1] and developmental pluripotency associated 1 [Dppa1]) by this analysis are shown in Figure 3. Of the 74 blastocyst DEGs isolated, 53 are known genes (Table 2), 9 are ribosomal proteins (Table 3) and 12 are unknown genes (Table 4). These differential display patterns between 4-cell and blastocyst stage embryos as assessed by ACP RT-PCR were very reproducible.

### Confirmation of ACP Observations by Semi-Quantitative RT-PCR

To confirm the results of the ACP RT-PCR analysis, mRNAs were isolated from murine 4-cell and blastocyst stage embryos and subjected to semi-quantitative RT-PCR using specific primers for 14 selected DEGs (Table 1). These genes encode MT-I, creatine kinase B (Ckb), prosaposin (Psap), solute carrier family 2 (facilitated glucose transporter) member 3 (Glut3), GM2 ganglioside activator protein (Gm2a), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex (Ndufa7), S100 calcium-binding protein A10 (Calpactin), laminin B2 (Lamc1), Dppa1, PoA-I-binding protein (Apoa1bp), cytosolic nonspecific dipeptidase (Cn2), argininosuccinate synthetase (Ass1), acidic cysteine-rich glycoprotein (Sparc), and glutathione S-transferase alpha 4 (Gsta4). The primer sequences, the number of PCR cycles, and the annealing temperatures used for the different DEGs are summarized in Table 1. This RT-PCR analysis revealed that all the DEGs were expressed at much higher levels in the blastocysts than in the 4-cell embryos (Fig. 4), which confirms the results of the ACP differential display analysis.



**Fig. 3.** Examples of gel photographs that show the differential banding patterns that allow the identification of DEGs at the 4-cell (4C) and blastocyst (BL) stage mouse embryos. The arrows indicate the DEGs that show higher expression in the blastocyst stage. The left blot shows the identification of MT-1 using the ACP5 primer, while the right gel shows the identification of Dppa1 using the ACP 71 primer.

**TABLE 2. Identity and Size of Murine DEGs That Are Prominently Expressed at the Blastocyst Stage and That Encode Known Proteins, and Their Homology to Known Sequences in GenBank**

Identity	Clone #	Base pairs sequenced	GenBank acc. no.	Homology (%) <sup>a</sup>
Annexin A11	3	900	BC012875	403/419 (96) +242/242 (100)
Testin	4	650	X78989	240/240 (100)
Dorz1	5	750	AB073619	91/113 (80) +96/122 (78)
Stathmin 1	7	700	BC054396	409/410 (99)
Metallothionein 1 (MT-I)	8	700	AK018727	398/400 (99)
Sphingomyelin phosphodiesterase 2	15	550	BC010978	465/470 (98)
Creatine kinase B (Ckb)	16	300	BC015271	189/223 (84)
Tumor rejection antigen P1A (Trap1a)	19	800	NM_011635	290/296 (97)
Prosaposin (Psap)	20	350	XM_192668	284/287 (98)
Diphosphoinositol (Nudt4)	21	700	AK048062	554/559 (99)
Glutamate oxaloacetate transaminase 1, soluble (Got1)	22	350	BC002057	288/296 (97)
Transcription factor IIIH polypeptide 2 (Gtf2h2)	24	300	AF242432	198/203 (97)
Solute carrier family 2 (facilitated glucose transporter), member 3 (Glut3)	25	900	BC034122	726/741 (97)
GM2 ganglioside activator protein (Gm2a)	26	900	NM_010299	631/650 (97)
Aflatoxin aldehyde reductase (Afar)	29	400	BK000393	302/302 (100)
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex (Ndufa7)	32	600	BC055698	484/484 (100)
Complement component 1, q subcomponent binding protein (C1qbp)	34	400	BC038075	309/316 (97)
S100 calcium-binding protein A10 (calpactin)	35	700	BC025044	523/547 (95)
Survivin40 (TIAP)	36	450	AB013819	368/370 (99)
Insulin-like growth factor-binding protein 7 (Igfbp7)	37	400	NM_008048	336/339 (99)
Mitochondrial cytochrome c oxidase subunit Vb	39	350	X53157	149/156 (95)
FBP-interacting repressor (FIR)	40	400	AF217197	151/166 (90)
Proliferation-related acidic leucine-rich protein PAL31 (PAL31)	41	800	AB025582	463/476 (97)
Phosphatidylinositol 4-kinase, catalytic, alpha polypeptide	42	600	BC049252	526/530 (99)
Chromodomain helicase DNA-binding protein 1-like (Chd11)	43	500	NM_026539	359/365 (98)
Laminin receptor	44	1,100	J02870	687/719 (95)
Nucleolar protein family A, member 3 (Nola3)	45	450	NM_025403	328/333 (98)
Laminin B2 (Lamc1)	46	850	J03484	740/754 (98)
Endothelial precursor protein B9 (Eppb9)	48	850	AK010355	739/754 (98)
Developmental pluripotency-associated 1 (Dppa1)	50	400	NM_178247	245/245 (100) +52/52 (100)
Thymosin, beta 4, X chromosome (Tmsb4x)	51	500	BC018286	381/398 (95)
H19	53	600	X58196	364/365 (99) +75/76 (98)
Phosphatidylinositol transfer protein, beta (Pitpnb)	54	800	NM_019640	556/556 (100)
N-myc downstream-regulated 1 (Ndr1)	55	500	BC015282	447/448 (99)
Filamin, beta	57	1,250	BC023873	753/764 (98)
E-cadherin	58	650	X06115	345/442 (78)
ATP synthase, H <sup>+</sup> -transporting, mitochondrial F0 complex, subunit F (Atp5j)	59	550	NM_016755	465/471 (98)
Cytosolic nonspecific dipeptidase (Cn2)	60	1,100	NM_023149	721/744 (96)
Eukaryotic translation elongation factor 1 alpha 1 (Eef1a1)	61	1,200	XM_203909	807/823 (98)
Adenine nucleotide translocator	62	700	X70847	570/570 (100)
Carboxyl terminal LIM domain protein (Clim1)	63	850	AF053367	704/711 (99)
Peptidyl-prolyl cis-trans isomerase A (PPIase)	64	800	XM_122180	682/686 (99)
PoA-I-binding protein (Apoa1bp)	65	400	AJ344092	221/255 (86)
Myosin heavy chain IX (Myh9)	67	450	NM_022410	198/206 (96) +126/126 (100)
Argininosuccinate synthetase (Ass1)	68	900	M31690	594/607 (97) +77/77 (100)
Acidic cysteine-rich glycoprotein (Sparc)	70	1,000	NM_009242	665/679 (97)
Annexin A6 (Anxa6)	71	600	XM_193625	566/576 (98)
Calpain, small subunit 1 (Capns1)	72	800	NM_009795	569/586 (97)
Aldo-keto reductase family 1, member B8 (Akr1b8)	74	400	NM_008012	235/236 (99)
Thymosin, beta 4, X chromosome (Tmsb4x)	76	300	AL646092	168/168 (100)
Plastin 2, L	78	1,000	AK009211	495/509 (97)
Calponin 3, acidic (Cnn3)	79	900	BC055711	374/391 (95)
Glutathione S-transferase, alpha 4 (Gsta4)	80	1,000	NM_010357	695/711 (97)

<sup>a</sup>The percentages are based on BLAST searches of the GenBank database. The other numbers in this column show the number of bases (query/subject) that were compared.

**TABLE 3. Identity and Size of Murine DEGs That Encode Ribosomal Proteins, and Their Homology to Known Sequences in GenBank.**

Identity	Clone #	Base pairs sequenced	GenBank acc. no.	Homology (%) <sup>a</sup>
Ribosomal protein L12 (Rpl2)	14	700	BC018321	564/609 (92)
Ribosomal protein S18 (Rps18)	18	450	NM_011296	346/358 (96)
Ribosomal protein S7 (Rps7)	23	650	BC002014	585/587 (99)
Ribosomal protein L31 (Rpl31)	24	300	BC008223	202/203 (99)
Ribosomal protein S20 (Rps20)	27	400	BC011323	166/166 (100)
Ribosomal protein L37a (Rpl37a)	30	650	NM_009084	353/353 (100)
Ribosomal protein L23 (Rpl23)	47	500	BC025918	415/418 (99)
Ribosomal protein S3a (Rps3a)	49	1,000	BC039659	741/760 (97)
Ribosomal protein L8 (Rpl8)	75	800	NM_012053	340/362 (93)

<sup>a</sup>The percentages are based on BLAST searches of the GenBank database. The other numbers in the column show the number of bases (query/subject) that were compared.

### Confirmation of ACP Observations by Real-Time RT-PCR

We further analyzed the expression patterns of the 14 selected genes described above during the early preimplantation period (from the 1-cell embryo through to the blastocyst stage) by using real-time RT-PCR (Table 1). To normalize the RT-PCR reaction efficiency, histone H2a was used as an internal standard. After normalization with histone H2a mRNA levels, the mRNA expression of all the genes at the blastocyst stage was significantly higher than at the 4-cell stage ( $P < 0.01$ , Fig. 5). Psap, Ndufa7, Calpactin, Dppa1, Ass1, and Sparc were similar in that they were only highly expressed at the blastocyst stage. Glut3 and Cn2 were highly expressed from the morula stage onwards and were equivalently abundant at the blastocyst stage. Lamc1, Apo1bp, and Gsta4 also had similar patterns, namely, the expression of maternal-derived mRNAs at the 1-cell stage, the degradation of these mRNAs after genomic activation, and then high expression in blastocyst stage embryos. The expression patterns of MT-1

and Ckb are also similar as they show higher expression at the 2-cell stage followed by a decrease in the 4-cell stage and then increasing expression. Only Gm2a expression is dependent on the number of blastomeres as its expression increased only gradually with development.

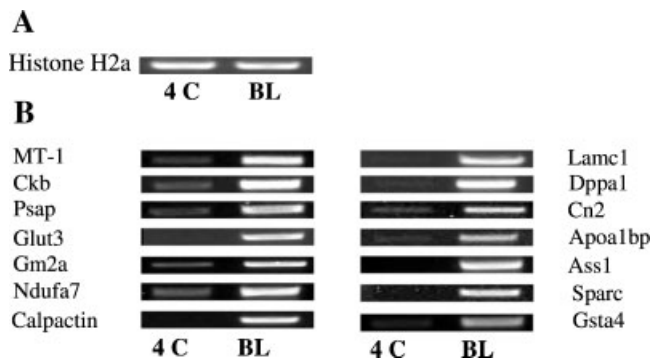
### DISCUSSION

The specificity and sensitivity with which a primer anneals to its target sequence are the most critical factors in determining the success of PCR amplification. In this study, we employed a new differential display RT-PCR technique (Hwang et al., 2003; Kim et al., 2004) to compare the gene expression in 4-cell and blastocyst stage embryos. The ACP-based RT-PCR method involves an ACP that has a unique tripartite structure in that its distinct 3'- and 5'-end portions are separated by a regulator. This ACP-based RT-PCR system is easy and accurate and lacks false positives. With this technique, we identified 74 DEGs that are specifically or more prominently expressed in murine blastocysts compared to the 4-cell stage.

**TABLE 4. Identity and Size of Murine DEGs That Encode Unknown Proteins, and Their Homology to Sequences in GenBank**

Identity	Clone #	Base pairs sequenced	GenBank acc. no.	Homology (%) <sup>a</sup>
Hypothetical domain of unknown function DUF59-containing protein	2	650	AK003830	462/463 (99)
Hypothetical protein	6	500	AK003984	435/438 (99)
CGI-128	10	550	NM_016062	294/342 (85)
Hypothetical protein	12	600	AK021193	515/520 (99)
Hypothetical YjeF-like structure-containing protein	13	250	AK090005	116/143 (81)
Hypothetical thioredoxin-containing protein	17	700	AK076425	561/581 (96)
Musculus expressed sequence AA517853	28	1,300	BC031725	674/706 (95)
Hypothetical protein C130073F10 (C130073F10)	31	1,000	NM_177731	790/803 (98)
Hypothetical S-adenosyl-L-methionine-dependent methyltransferases structure-containing protein	38	500	AK089212	275/299 (91)
Early transposon (ETn)	66	900	M16515	455/481 (94) +110/114 (96)
Hypothetical protein	69	1,100	AK076104	809/809 (100)
Hypothetical protein	77	600	26185849	359/360 (99)

<sup>a</sup>The percentages are based on BLAST searches of the GenBank database. The other numbers in the column show the number of bases (query/subject) that were compared.



**Fig. 4.** Confirmation by semi-quantitative RT-PCR of the differential mRNA expression patterns of 14 genes that were identified by ACP RT-PCR as being DEGs. **A:** The mouse histone H2a gene was used as a control to confirm the integrity of the mRNA samples. **B:** Comparison of the expression patterns of the 14 DEGs in 4-cell (4C) and blastocysts (BL) by semi-quantitative RT-PCR blastocysts. The amplified DNA products were separated on an 1.2% agarose gel and stained with ethidium bromide.

Of the 74 DEGs, we selected 14 and assessed their expression during preimplantation development in more detail by employing quantitative RT-PCR. The expression levels of these genes in 1-cell, 2-cell, 4-cell, morula, and blastocyst stage embryos were normalized relative to the histone H2a mRNA levels to reveal how their expression changes during development. We found that the mRNA expression of the histone H2a-encoding gene increased in a proportional manner from the zygote to blastocyst stage. In mammalian embryos, unlike somatic cells, the majority of the genes, even house-keeping genes, are expressed in a stage-specific manner and show two major patterns of expression. In one, expression commences after the onset of genomic activity. In the other, the genes are expressed before and after the onset of embryonic transcription, which indicate maternal and embryonic activity (Mohan et al., 2002). With regard to the 14 DEGs we identified, we could also categorize them according to their gene expression patterns during preimplantation embryo development.

The first important morphological transition during preimplantation embryo development is compaction, which occurs between the 8-cell and 16-cell stages of development in the mouse and which is accompanied by changes in cell structure and developmental fate. Changes at the cellular level include an increase in the level of cell adhesion between the blastomeres of the embryo (Gumbiner and McCrea, 1993). One of the genes that we found was differentially expressed at the blastocyst stage was E-cadherin (Table 2). E-cadherin (uvomorulin) is a well-known principle molecular component of the adherent junction. Stable cell-to-cell contacts and adhesion plaques arise through associations of E-cadherin with the actin cytoskeleton that are mediated by interactions with  $\beta$ -catenin,  $\alpha$ -catenin, and  $\gamma$ -catenin (Nagafuchi et al., 1987; Gumbiner and McCrea, 1993). Blastocyst formation is an interactive process that requires the coordinated expression of

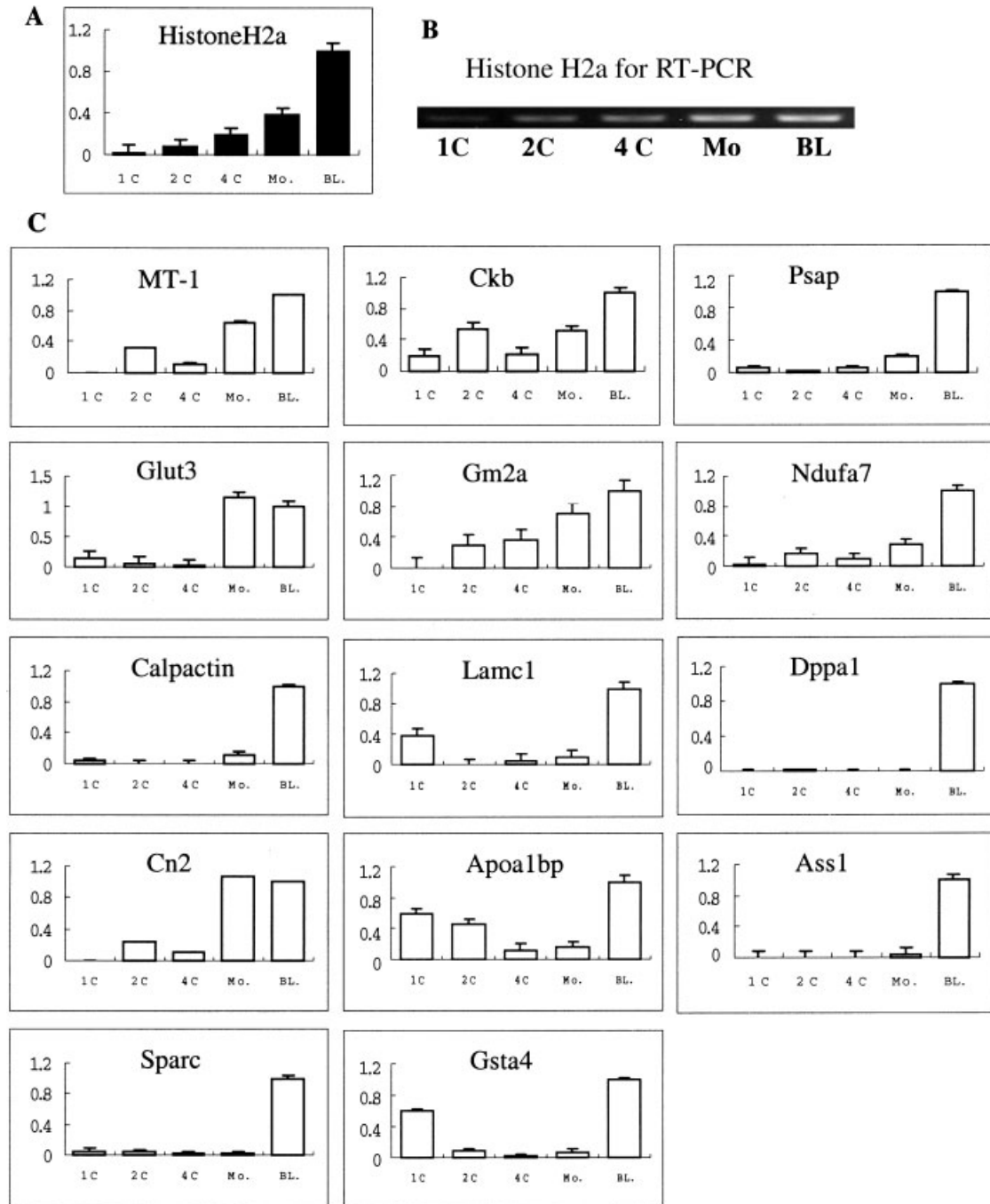
several gene products, including Na/K-ATPase subunits, E-cadherin, and tight junction components. The events of blastocyst formation are initiated during compaction, which is mediated by the appearance of E-cadherin-mediated cell-to-cell adhesion and the formation of focal tight junctions (Watson et al., 1999). Thus, our identification of E-cadherin as a blastocyst DEG supports the validity of our ACP RT-PCR analysis.

We found that MT-1 is expressed significantly higher at the morula and blastocyst stages than at the 4-cell embryo stage. Andrews et al. (1991) also reported that in fertilized (1-cell) eggs and 2-cell embryos, the MT-I gene was not detectably responsive to metal ions, whereas in later cleavage stage embryos (4-cell and 8-cell), the MT-I gene was detectably responsive to metals in some blastomeres of some of the embryos. Moreover, after the third cleavage (morula/blastocyst stage), this gene was highly metal-inducible in essentially all cells of the embryo. Thus, the preimplantation mouse embryo may develop a capacity to respond to metals in the environmental milieu by inducing MT gene expression at about the third cleavage.

We also found that Ckb is expressed at high levels in blastocyst stage embryos. Lyons et al. (1991) have reported that Ckb is also expressed abundantly in developing striated muscle and is an early marker for skeletal myogenesis. Ckb has been found to bear the binding site of the POU domain of Oct-4 (Ritchie et al., 1991; Wiebe et al., 2000), which suggests that it may be involved in early differentiation processes in cooperation with Oct-4.

We observed that GLUT3 is degraded at the 4-cell stage embryo and is dramatically re-expressed at the morula stage. Glucose is the main energy substrate in most cells, including preimplantation embryos. The GLUT isoforms that have been identified as being responsible for glucose uptake in the early mouse embryo are GLUT1, GLUT2, and, more recently, GLUT3 and GLUT8 (Hogan et al., 1991; Aghayan et al., 1992; Dan-Goor et al., 1997; Pantaleon et al., 1997; Carayannopoulos et al., 2000). GLUT3 expression has been localized on the apical membranes of the polarized cells of the morula and the apical membranes of the trophectoderm cells of the blastocyst (Wiebe et al., 2000). In contrast, GLUT1 expression is restricted to the basolateral membranes of the outer trophectoderm cells in both morulae and blastocysts (Pantaleon et al., 1997). A glucose transporter (GLUT8) responsible for insulin-stimulated glucose uptake in the mouse blastocyst has also been identified recently (Carayannopoulos et al., 2000). More functional studies on the glucose transporters are required to understand embryo physiology as well as to determine the nutrient requirements of embryos that develop in vitro.

We found that Lamc1 is degraded from the point of genomic activation until the morula stage, and that it is highly expressed at the blastocyst stage. It is known that Lamc1 chain mRNAs first appear at the morula stage (Shim et al., 1996). Interestingly, it was shown that when embryos were flushed at the morula stage and



**Fig. 5. A, B:** Expression of histone H2a increased as development progressed due to the growing levels of the RNA population in the developing embryonic cells. Relative expression (A) and representative agarose gel (B) in an 1-cell (1C), 2-cell (2C), 4-cell (4C), morula (Mo), and blastocyst (BL). **C:** Relative expression levels of 14 DEGs after

normalization with histone H2a mRNA levels throughout preimplantation development as determined by real-time RT-PCR. mRNAs from the same number of 1-cell (1C), 2-cell (2C), 4-cell (4C), and morula (Mo); and blastocyst (BL) stages were reverse-transcribed and subjected to real-time quantitative PCR using transcript-specific primers (Table 1).

cultured *in vitro*, all laminin chain mRNA levels were decreased or not changed during blastocoele expansion. In contrast, when embryos at different stages were flushed and cultured, laminin chain mRNA levels increased during blastocoele expansion (Shim et al., 1996). These results indicate that laminin expression, which is probably regulated by the uterine environment,

may not be directly involved in the regulation of blastocoele expansion. Rather, it may be involved in the maternal and embryonic communication during the periimplantation period.

We also found Dppa1 expression is upregulated at the blastocyst transition (Table 2). This gene was identified as a developmental pluripotency associated

gene based on Oct-3/4-like temporal expression pattern and reproducibility of detection in mouse blastocyst (Bortvin et al., 2003). Structurally, Dppa1 is a type 1 membrane protein containing immunoglobulin SF and single transmembrane domain (Bortvin et al., 2003). The domain is similar with rat KIM-1 that is expressed in proliferating bromodeoxyuridine-positive and de-differentiated vimentin-positive cells in regenerating proximal tubules (Ichimura et al., 1998; Bortvin et al., 2003). Taken together, Dppa-1 is an embryonic stem cell specific gene controlled by Oct3/4.

We observed that Apoa1bp is expressed at the 1-cell and 2-cell stages but that expression levels decrease until a dramatic re-expression at the blastocyst stage. Apolipoproteins of the various lipoproteins regulate lipoprotein metabolism and determine the unique roles that these lipoproteins play in lipid metabolism. In mammals, a group of eight exchangeable and soluble apolipoproteins (apoC-I, apoC-II, apoC-III, apoC-IV, apoA-I, apoA-II, apoA-IV, and apoE) is characterized by the occurrence of repeated amphipathic helical regions that are considered to be structural units essential for lipid-binding properties (Beckstead et al., 2003). Little information is available on the role of apolipoproteins during embryo development. Previously, Babin et al. (1997) demonstrated highly expression of apoE and apoA in the yolk syncytial layer, an extra-embryonic structure that is implicated in the embryonic and larval nutrition of zebrafish embryos. ApoE transcripts were also observed in the deep cell layer during the blastula stage, in numerous ectodermal derivatives after gastrulation, and after 3 days of development in a limited number of cells both in the brain and the eyes.

Sparc, which is first expressed at the blastocyst stage, is a matricellular protein that modulates cell adhesion and proliferation and is thought to function in tissue remodeling and angiogenesis (Kupprion et al., 1998). Sparc inhibits cell cycle progression from G1 to S phase (Funk and Sage, 1991). Studies on human fibroblasts have shown that Sparc regulates cellular proliferation through an interaction with PDGF-AB and -BB and subsequent inhibition of binding to cognate cell-surface receptors (Raines et al., 1992). Sparc also reduces the proliferation- and migration-promoting effects of bFGF on endothelial cells. A direct interaction between bFGF and Sparc was also demonstrated (Hasselaar and Sage, 1992), which suggests that it participates in early embryonic development.

We found that many of the DEGs in blastocysts express ribosomal proteins. Similarly, in bovine blastocysts, several different types of ribosomal RNA have been observed (Mohan et al., 2002). It is known that the transcription of the rRNA genes is first detectable late in the third cell cycle in pigs and cattle (Viuff et al., 1998, 2002). The initiation of RNA synthesis is associated with a characteristic remodeling of the nucleolar architecture (Kopečný et al., 1996). Although it is not clear what role ribosomal RNA plays in specific protein translation, the expression of various types of rRNA early in preimplantation development suggests that

they may play critical roles in specific protein synthesis for early development.

In conclusion, the ACP-based strategy we used to identify DEGs in mouse embryos is easy, and lacks false positive and yields reproducible results. With this method we detected 74 genes that are differentially expressed in blastocyst stage embryos. Although the detailed functions of these genes and their products remain to be determined, their identification in this study provides insights into the molecular mechanisms involved in early development and the differentiation of mammalian embryos.

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