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A global view of gene expression in the preimplantation mouse embryo: morula versus blastocyst☆

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Abstract

As a first step to understand preimplantation development, we performed global gene-expression profiling of morula and blastocyst using the NIA 15k mouse cDNA microarray. Gene expression levels were measured four times for blastocyst and five times for morula. Student's *t*-test at the 5% significance level identified 428 genes upregulated and 748 downregulated in blastocyst compared to morula. This trend was consistent with semi-quantative RT-PCR analysis of sample genes. The upregulated genes known to be involved in critical regulatory processes, included *Mist1*, *Id2*, *Hd1*, and *Requiem*; the downregulated genes included *CREB-binding protein*, *Per3*, *zinc finger protein 217*, *Krox-25*, and *miwi1*. Such well-characterized genes and many novel genes provide markers for early stages in development and starting materials for further functional studies.
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Keywords: Preimplantation mouse embryos; cDNA microarray; Morula; Blastocyst

1. Introduction

Preimplantation development involves discrete, important biological processes (reviewed in [1–4]). First, preimplantation development is a major model to study the pluripotency of cells, because totipotent fertilized eggs successively lose totipotency and then pluripotency. Second, massive dynamic switches shift from a process governed by the activity of maternally stored RNA/proteins to one governed by the activity of zygotically activated genes [5]. Some maternal mRNAs are translated, but fertilization triggers bulk mRNA degradation [6]. Transcription from the zygotic genome begins at the late one- to two-cell stage in mouse, when the first zygotic nucleus is formed. Although it is well-established that this transition is regulated by a ''zygotic clock'' [7,8], it is not generally known what type(s) of genes is activated first, or how (though some information is available for Hsp70.1). Third, the first cell differentiation event in mammalian development, ''compaction,'' occurs during this period, at the late eight-cell stage. Cells that were previously loosely associated then become more adhesive and develop into the tightly organized cell mass of the morula. Assignment of cells as outer or inner occurs at 16-cell stage, followed by the differentiation of the inner cell mass (ICM), that is destined to contribute the embryo proper, and the trophectoderm (TE), later differentiates to form the placenta. Although several transcription factors appear to play important roles in the first cell differentiation event [9], the global network of gene expressions in the differentiation remains unknown. Here we report the first attempt of global gene-expression profiling between morula and blastocyst by cDNA microarrays.

2. Materials and methods

2.1. Embryos

Preimplantation embryos were obtained from superovulated C57BL/6 female mice mated with C57BL/6 male mice. One-cell embryos were harvested from ampullae in Brinster's medium (Invitrogen, Carlsbad, CA) and treated with 0.33 mg/ml hyarulonidase (Sigma, St. Louis, MO) to remove cumulus cells. Two-, four-, and eight-cell embryos and morulae were flushed out from oviducts in Brinster's medium. Blastocysts were flushed out from oviducts and collected from uterus in Brinster's medium. Unfertilized eggs were collected from ampullae of superovulated female without mating in a day after the hCG injection. Particularly

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for microarray hybridization of morula, eight-cell embryos were cultured in M16 medium for 10–12 h and only compacted morulae (in 85 h after hCG injection) were collected.

2.2. RNA

By using Quick Prep Micro mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ), poly(A) + RNAs were extracted from morulae (2510) and blastocysts (1149) for microarray hybridization, and from unfertilized eggs (278), one-cell (248), two-cell (289), four-cell (312), and eight-cell embryos (181), morulae (144) and blastocysts (175) for RT-PCR analysis.

2.3. Microarray hybridization

Two hundred fifty to 300 blastocysts-, or 500 morulae-equivalent purified RNAs were primed with oligo(dT)12-18 primer (Amersham Biosciences) and first strand cDNA labeled with ^{33}P-dCTP (Amersham Biosciences) was synthesized and used as a probe. Prehybridization was carried out at 65 °C for 4 h with MicroHyb solution (Invitrogen) supplemented with yeast tRNA and PolyA RNA, followed by hybridization at 65 °C for 20 h with same hybridization solution plus mouse Cot1 DNA, 8% Dextran Sulfate and heat-denatured probe as already described [10,11]. Washing of the membranes was done as follows; first 2× SSC, 0.1% SDS at RT for 30 min twice and then 2× SSC, 0.1% SDS at 65 °C for 15 min twice. Dried membranes were exposed to Phosphor Screen for 10 days at RT. Hybridization for each probe was repeated four times for blastocyst and five times for morula in a same manner.

2.4. Data analysis

Signal intensities of single spots were quantified by ImageQuant ver. 5.1. All the other calculations including statistical analysis and drawing pie charts were done by MS Excel. Scatter plots were used to see the global expression profiles for each probe. Scatter plots were done with Spotfire software.

2.5. Semi-quantitative RT-PCR

First strand cDNAs for each stage were synthesized essentially in the same manner as described, except that 10 mM dNTP mix (PE Biosystems, Foster City, CA) was used. Five, 2, 1, 1/5, 1/25, and 1/125 embryos-equivalent cDNAs for each stage were used for the templates of PCR. Reaction mixture with recombinant Taq polymerase (Invitrogen) was assembled into 15 μl according to manufacturer. PCR conditions are as follows: denature at 95 °C for 1 min, followed by 40 cycles of denature at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, and final extension at 72 °C for 3 min. Primer pairs used in this study have been described as follows, except CBP (303 bp product, forward 5′-CGGTCTGAGATGATGGAAGAG-3′, reverse 5′-CTGAGGATCTACAGGCTGACG-3′), Miwi1 (499 bp product, forward 5′-ACCAGTCTGTCACTGGAAACG-3′, reverse 5′-TCGATTCGGTAGGTACGATTG-3′), Otx2 (149 bp product, forward 5′-AAACAGCGAAGGGAGAGGAC-3′, reverse 5′-CGGCACTTAGCTCTTCGATTC-3′) and Requiem (304 bp product, forward 5′-CATGAAAGTTGGAAGCAGAGC-3′, reverse 5′-GTGGAGCACAACATGTGAATG-3′); β-actin [12], BRK1 [13], EF1α [14], G3PDH [15], gp130 [16], Grb7 [17], and Id2 [18]. Whole reaction mixtures were loaded onto 2% agarose gel and ran at 100 V for 20 min.

3. Results and discussion

Despite its importance, the molecular study of preimplantation development has been slow, mainly because of the scarcity of the materials for molecular biological/biochemical approaches [19]. In one approach to help to overcome this problem, we have produced dependable expression profiles with small amounts of RNA using nylon arrays and with ^{33}P-labeled probes. We carried out hybridization experiments on NIA mouse 15k cDNA microarray [10,11,20] with radio-labeled cDNAs, four experiments starting from 1149 blastocysts, and five experiments from 2510 morulae (Fig. 1). According to the reported estimate of total RNA amounts [6], 1149 blastocysts contain 1.69 μg of total RNAs and 2510 morulae contain 1.73 μg of total RNA. Student's *t*-test at 5% significance level identified 428 genes more highly expressed in blastocyst and 748 genes more highly expressed in morula (Fig. 2A). The identification of a cohort of genes that are differentially expressed at the morula to blastocyst transition, is consistent with 2D SDS-PAGE results finding protein spots that are differentially expressed in preimplantation embryos at different stages ([21–23], for review [24]) and in the ICM and TE [25].

In starting to assess possible biological significance, genes with more than two-fold expression difference between morula and blastocyst were selected and grouped by their putative functions (Fig. 2B). Overall functional groupings were similar (Fig. 2B), but we found more matrix/structural genes are expressed in blastocyst and more protein synthesis/transcriptional control genes are expressed in morula. This may reflect translational activities during the morula-blastocyst transition [26], which lead to the appearance of tissue-specific polypeptides in ICM and TE [25].

Tables 1 and 2 list genes with more than three-fold expression differences between morula and blastocyst (for more complete lists, see http://lgsun.grc.nia.nih.gov/microarray/data.html). Genes upregulated in the blastocyst include *Mist1*, *Id2*, *Hd1*, and *Requiem*, whereas genes downregulated in blastocyst include *CREB-binding protein* (*CBP*), *Per3*, *zinc finger protein 217*, *Krox-25*, and *miwi1*. Among these differentially expressed genes, 10 genes were

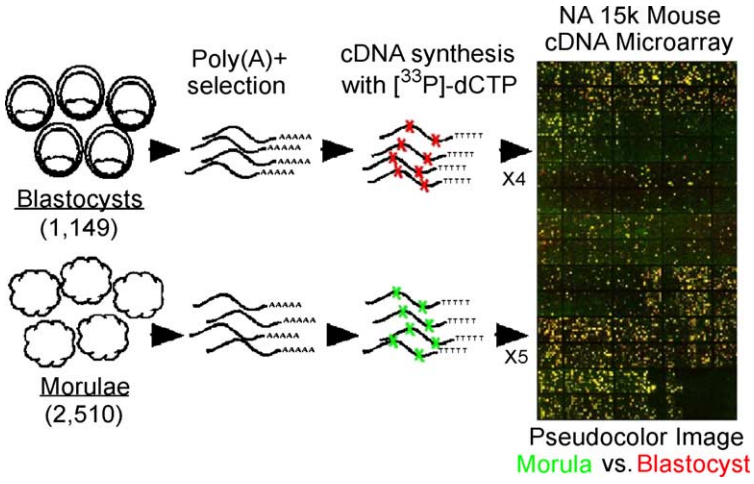

Fig. 1. Experimental strategy.

subjected to semi-quantative RT-PCR analysis and seven showed consistent expression levels with those obtained from the microarray analysis (Fig. 3). The concordant results confirm that although these experiments are limited by the extremely small amounts of RNA available, the bulk of the results are reliable; but confirmatory tests must be done on any particular gene that will be studied further.

Genes identified in this manner suggest some possible features of development. For example higher expression of *Id2* in blastocyst may be related to the separation of ICM and TE, because *Id2* is an inhibitor of bHLH-type transcription factors like *I-mfa*, *Mash2*, and *eHand*, all of which are implicated in trophoblast and/or placenta formation. Furthermore, higher expression of *CREB-binding protein* in morula than in blastocyst may reflect chromatin-remodeling that leads to the first cell differentiation event.

A second example of potential interest is the mouse *piwi*-like gene 1 (*miwi1*, also called as *mili*). *piwi* plays an important role in the maintenance of self-renewal of germline stem cell in Drosophila ovary [27]. Two *piwi*-related

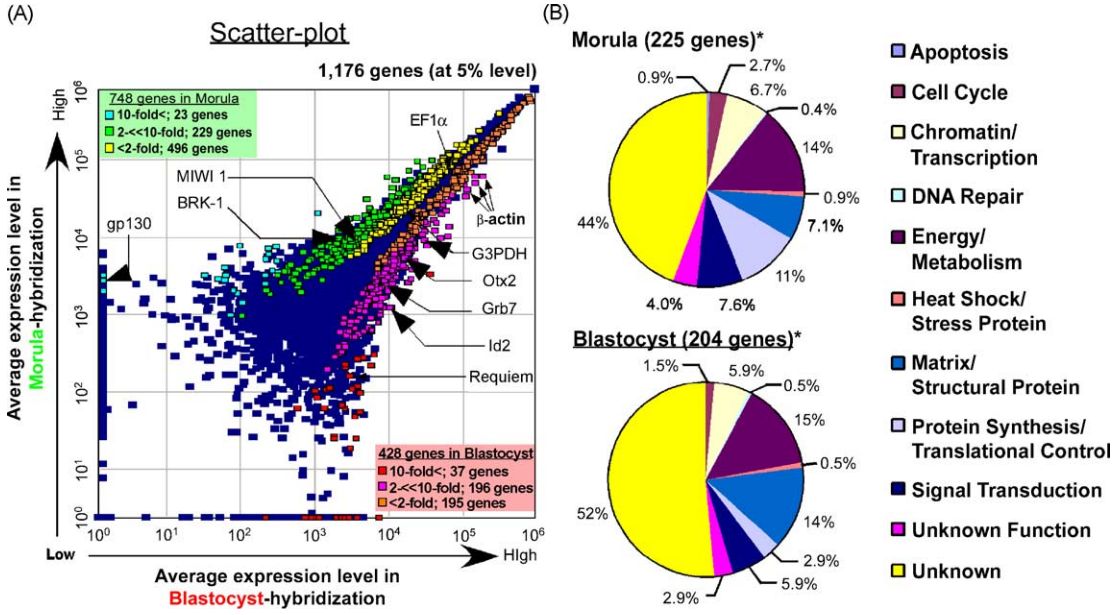

Fig. 2. (A) Scatter plot. Average expression levels (arbitrary units) of each gene were calculated from four independent hybridizations for blastocyst and five for morula, respectively, and displayed on a scatter plot. Genes that show significantly different expression levels between morula and blastocyst at the 5% significance level ($P < 0.05$) are displayed as colored spots, and the other genes are displayed as dark blue spots. For genes that are expressed higher in morula than in blastocyst; blue indicates genes expressed greater than 10-fold, green indicates those expressed between 2- and 10-fold, and yellow indicates those expressed less than two-fold. For genes that are expressed higher in blastocyst than in morula; red indicates genes expressed greater than 10-fold, pink indicates those expressed between 2- and 10-fold, and orange indicates those expressed less than two-fold. Ten genes whose expression levels were verified by semi-quantitative RT-PCR are indicated. (B) Functional classification of genes expressed differentially. Only sequence-verified genes showing greater than two-fold difference of expression level are classified.

Table 1
List of genes upregulated in blastocyst (greater than three-fold)

Function	Clone ID	Clone description	Fold	Average expression level	
				Morula	Blastocyst
Cell cycle	H3025B08	Putative human proliferating-cell nucleolar antigen p120	3	3,100	8,900
	H3106D07	Mouse Cde 42 homolog	3	2,800	7,800
Chromatin/transcription	H3094E11	Rat bIILII transcription factor Mist1	792	2	1,900
	H3032II03	Mouse special AT-rich sequence binding protein Satb1	43	60	2,600
	H3100G09	Mouse ubi-d4/requiem	19	160	3,000
	H3059F07	Human CCR4-NOT transcription complex subunit 2, clone MGC:730	14	80	1,100
	H3098G05	Mouse Id2	9	1,200	11,000
	H3026F10	Mouse IIMG-1	3	2,300	7,500
	H3106G12	Human C3II-type zinc finger protein (MBLL)	3	2,800	8,900
	H3102E09	Mouse putative histone deacetylase (IID1)	3	890	2,600
	H3031B10	Mouse upstream transcription factor 2 (Usf2)	3	6,000	16,400
	H3028D09	Mouse SWI/SNF complex 60 kDa subunit (BAF60a)	3	1,800	4,900
	H3030II12	Mouse Otx2	3	6,200	16,100
	H3034A04	Mouse metal response element binding transcription factor 2 (Mtf2)	3	2,400	6,000
Energy/metabolism	H3040C06	Human serine hydroxymethyltransferase 1 SIIMT1	1,083	2	2,600
	H3042C06	Mouse progressive ankylosis (ank)	21	300	6,300
	H3040B03	Mouse solute carrier family 9 (sodium/hydrogen exchanger) 3 regulator 1	10	600	5,700
	H3040C04	Putative NADP dependent leukotriene b4 12-hydroxydehydrogenase	7	2,000	14,000
	H3035D04	Mouse GM2 activator protein (Gm2a)	7	2,600	18,000
	H3116D11	Mouse cytochrome *c* oxidase VIIa 3 (Cox7a3)	5	660	3,500
	H3158F09	Rat F1-ATPase epsilon subunit nuclear gene encoding mitochondrial protein	5	1,500	7,600
	H3028II10	Mouse peptidylprolyl isomerase C (Ppic)	5	1,300	6,900
	H3102A07	Mouse phosphatidylserine synthase 1 (Ptdss1)	4	740	3,000
	H3026F02	Mouse glutathione peroxidase 1 (Gpx1)	4	5,000	18,500
	H3025F10	Mouse succinate dehydrogenase Ip	4	4,200	15,400
	H3027E08	Mouse alpha-enolase (2-phospho-D-glycerate hydrolase)	3	4,000	13,200
	H3031E11	Mouse glyceraldehyde-3-phosphate dehydrogenase (Gapd)	3	9,200	30,200
	H3025D10	Mouse Aga, aspartylglucosaminidase	3	2,600	8,100
	H3137E02	Human NADII dehydrogenase (ubiquinone) Fe–S protein 2 NDUFS2	3	6,500	20,000
	H3151D11	Mouse prosaposin (psap\SGP-1)	3	15,200	44,000
	H3025D11	Mouse phosphofructokinase B	3	3,200	8,600
	H3158D09	Human 1-acylglycerol-3-phosphate *O*-acyltransferase 3	3	2,400	6,300
Heat shock/stress	H3158F10	Mouse Ceth chaperonin containing TCP-1 eta	5	900	4,100
Matrix/structural proteins	H3034II10	Human transmembrane 4 superfamily member 3	3,083	2	7,400
	H3038D05	Rat major vault protein	68	60	4,100
	H3058II08	Mouse vitamin K-dependent protein S precursor	7	280	1,900
	H3058D08	Rat nonmuscle myosin heavy chain-B	6	2,200	12,100
	H3014II12	Mouse endoA cytokeratin	6	3,800	20,900
	H3040E04	Human Rer1 protein	6	380	2,100
	H3007G06	Mouse endoB cytokeratin	5	13,400	65,000
	H3009D05	Mouse galactose binding lectin soluble 1 (Lga1s1)	5	4,900	22,300
	H3096G05	Mouse alpha-2-macroglobin precursor ALPHA2M	4	600	2,600
	H3003F11	Rat myosin I heavy chain Myr3	4	1,600	6,000
	H3031C01	Mouse endoA cytokeratin	3	12,100	41,200
	H3027II10	Human nucleolar protein NOP56	3	5,200	16,900
	H3072C08	Chinese hamster TRIP, TAR RNA interacting protein	3	2,100	6,600
	H3018D10	Mouse beta-actin	3	59,700	184,200
	H3027D08	Rat nucleoporin p58	3	6,100	18,100
	H3042II06	Mouse alpha actinin 4 (Actn4)	3	3,600	10,600
	H3046II04	Mouse WD40 repeat protein 1 (Wdr1)	3	1,200	3,400
	H3018D09	Mouse beta-actin	3	49,900	145,400
	H3034E05	Mouse ribophorin (Rpn), ER-specific membrane protein	3	2,100	5,900
	H3036A06	Mouse coated vesicle protein alpha-adaptin C	3	2,800	7,900
	H3010D02	Mouse cytoskeletal beta actin	3	41,200	113,600
	H3027II08	Rat brain myosin II	3	10,800	27,600
	H3143A02	Mouse prothymosin beta 4 (Ptmb-4)/actin monomer-sequestering proteins	3	7,700	19,300

Table 1 (*Continued*)

Function	Clone ID	Clone description	Fold	Average expression level	
				Morula	Blastocyst
Protein synthesis/ translational control	H3094C11	Putative ubiquitin carboxyl-terminal hydrolase 64E	155	20	3,100
	H3028F01	Human BAP1, BRCA1 associated ubiquitin carboxy-terminal hydrolase	3	3,000	9,600
	H3027C08	Human ribosomal protein S6 kinase A6	3	3,800	11,900
	H3014G12	Mouse ubiquitin conjugating enzyme 2e (Ubc2e)	3	6,700	20,100

Table 2
List of verified known genes downregulated in blastocyst (greater than three-fold)

Function	Clone ID	Clone description	Fold	Average expression level	
				Morula	Blastocyst
Apoptosis	H3054F12	Mouse TR2L	3	7,900	2,500
Cell cycle	H3047H05	Drosophila Crn, crooked neck protein	7	10,100	1,400
	H3136B07	Mouse nucleophosmin 1 (Npm 1)	4	71,000	18,400
	H3088C10	Human CDC28 protein kinase 2 (CKS2)	4	9,500	2,700
	H3148E01	Mouse ING1	3	8,300	2,800
Chromatin/transcription	H3047G08	Mouse CREB-binding protein	9	3,600	390
	H3044B12	Human pituitary tumor-transforming 1 interacting protein (PTTG1IP)	5	8,200	1,600
	H3054G12	Rat Hbp1, HMG-box containing protein 1	3	6,800	2,100
	H3134A06	Mouse period 3 (Per3), circadian oscillators	3	8,400	2,700
	H3092A10	Human zinc finger protein 217	3	7,400	2,400
	H3020B07	Mouse nucleosome assembly protein 1-like 1 (Nap1l1)	3	19,500	7,000
	H3106E01	Mouse Kruppel-type zinc finger protein KROX-25	3	21,400	8,100
	H3112B01	Mouse Sin3-associated polypeptide 18 (Sap18)	3	9,100	3,500
	H3117E04	Mouse cellular nucleic acid binding protein	3	18,900	7,500
Energy/metabolism	H3045D07	Human NAALADase II	24	7,500	310
	H3047B07	Mouse tripeptidyl peptidase II (Tpp2)	24	6,000	250
	H3047C09	Putative beta-ketoacyl-ACP synthase II, KAS II	14	3,700	270
	H3088E05	Mouse alpha-galactosidase A	13	1,400	110
	H3045A08	Mouse alkaline phosphatase 5, embryonic precursor	6	59,500	9,600
	H3081H08	Human glycine cleavage system protein H (GCSH)	6	17,000	2,900
	H3101D05	Mouse phosphatidylethannolamine *N*-methyltransferase	5	12,900	2,400
	H3130D04	Rat outer mitochondrial membrane import receptor rTOM20	5	17,500	3,700
	H3045B03	Putative rabbit cytochrome p450 2B5 (CYPHB5)	5	13,400	2,900
	H3111F09	Rat Y-b3 glutathione-S-transferase	4	3,000	780
	H3105C03	Putative yeast diphthine synthase	3	4,200	1,400
	H3033G02	Mouse betaine-homocysteine methyltransferase (Bhmf)	3	43,200	15,100
	H3078F02	Mouse ornithine decarboxylase	3	36,400	13,700
	H3087A12	Mouse uterine lactotransferrin	3	15,500	6,100
	H3058A12	Mouse phosphodiesterase 8 (Pde8)	3	11,400	4,500
Heat shock/stress	H3024D07	Mouse mSTI1	3	18,300	6,700
Matrix/structural proteins	H3105H07	Rat kidney injury molecule-1 (KIM-1)	18	19,900	1,100
	H3109D11	Mouse coagulation factor C homolog (Coch)	11	2,500	220
	H3109E03	Mouse Ma MHC class II alpha chain	9	4,400	470
	H3109D03	Mouse lysosomal membrane glycoprotein type B (1gp-B)	6	3,800	610
	H3036C05	Rat kidney injury molecule-1 (KIM-1)	6	30,400	5,000
	H3106D01	Rat microtubule-associated proteins 1A/1B light chain 3	4	42,600	11,300
	H3074A10	Rat kidney injury molecule-1 (KIM-1)	3	53,400	19,600
	H3014H05	Mouse adhesion regulating molecule 1 (Arm1)	3	16,800	6,400
Protein synthesis/ translational control	H3043B02	Mouse homolog of Drosophila ariadne 2 (Arih2)	2,194	3,200	1
	H3046D07	Human SUPV3L1 putative ATP-dependent mitochondrial RNA helicase	107	3,200	30
	H3038H08	Human DNA-directed RNA polymerase III C11	77	7,700	100
	H3045D06	Mouse 26S proteasome (prosome, macropain) non-ATPase 7 (Psmd7)	7	6,500	1,000
	H3136B08	Mouse nucleophosmin 1 (Npm-1)	4	94,800	24,300

Table 2 (*Continued*)

Function	Clone ID	Clone description	Fold	Average expression level	
				Morula	Blastocyst
	H3134G10	Mouse eukaryotic translation initiation factor 4E Eif4e	3	10,700	3,400
	H3054H12	Rat putative splicing factor YT521-B	3	7,700	2,500
	H3022G02	Rat proteasomal ATPase (MSS1)	3	13,000	5,200
Signal transduction	H3086G09	Mouse interleukin 6 signal transducer (II6st)/gpl30	1,851	2,700	1
	H3060A10	Mouse arachidonate epidermis-type 12(S)-lipoxygenase	10	4,200	440
	H3047D07	Mouse liver receptor homologous protein (LRH-1)	9	6,400	700
	H3132F12	Mouse Krit1	6	5,100	890
	H3080A09	Rat vasopressin-activated calcium-mobilizing receptor	5	7,400	1,600
	H3105G07	Mouse type I receptor BRK-1/Alk3, BMPR-1A	5	9,600	2,100
	H3041D03	Mouse myo-inositol 1-phosphate synthase A1 (IsynA1)	4	43,500	11,800
	H3074B05	Mouse serine/threonine kinase Stk25	3	21,100	6,300
	H3087C02	Putative guanine nucleotide-binding protein beta-like	3	6,000	1,800
	H3150E09	Mouse bone morphotic protein 2 (BMP-2)	3	5,700	1,800
	H3058B12	Mouse ganglioside-included differentiation-associated protein 1 (Gdap1)	3	20,200	7,100

genes, *miwi* and *mili* have been identified in mouse [28] and *miwi* has been shown to play an important role in spermatogenesis [29]. Since *miwi1* is expressed more highly in morula than in blastocyst, and also showed testis-specific expression by northern hybridization (data not shown), one can formulate testable notion that Miwi1 may be involved in the maintenance of ICM and germline in mammalian embryos.

In previous work, we performed large-scale sequencing of cDNAs from each stage of mouse preimplantation embryos, and identified many genes that show stage-specific expression patterns during preimplantation mouse development [30]. For example genes were identified that are transiently upregulated at Morula stage, but downregulated at Blastocyst stage. However, those results were obtained by counting the frequency of expressed sequence tag (EST), which provides only rough estimate of gene expression levels (Ko, 2001). In contrast, the results reported here provide more accurate gene expression changes, supporting previous results by pointing to genes indeed downregulated from morula to blastocyst. As discussed in the previous work (Ko et al., 2000), the presence of genes expressed

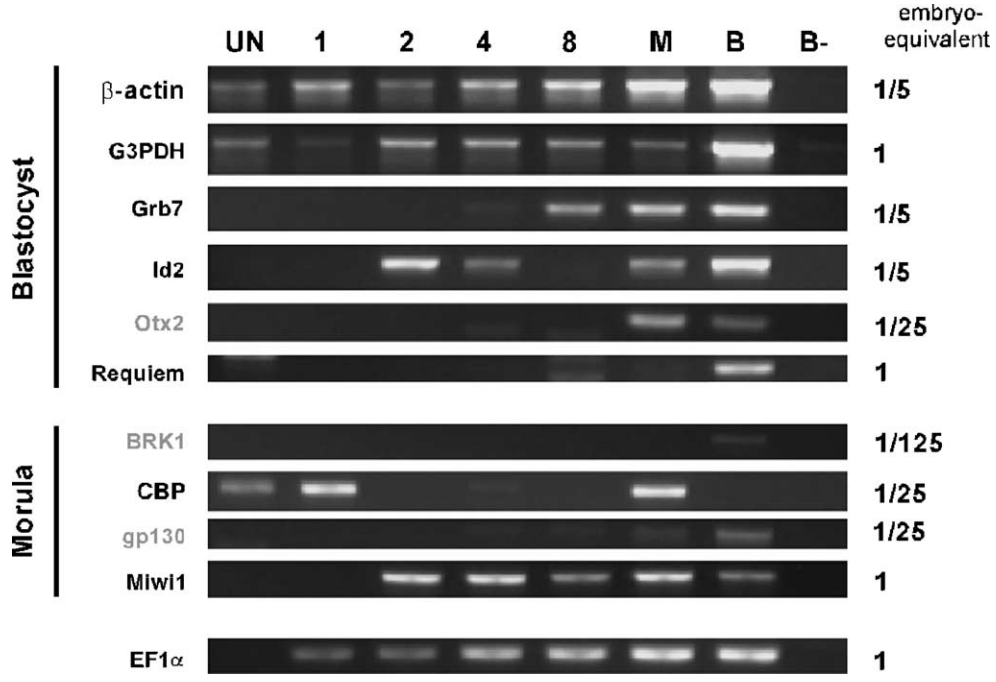

Fig. 3. Semi-quantitative RT-PCR. Six genes expressed highly in blastocyst based on microarray analysis and four genes in morula were subjected to semi-quantitative RT-PCR analysis. Template cDNAs equivalent to 5, 2, 1, 1/5, 1/25, and 1/125-embryos at each stage were used and only representative results were shown. EF1α was used as a standard. UN, unfertilized eggs; 1, one-cell embryos; 2, two-cell embryos; 4, four-cell embryos; 8, eight-cell embryos; M, morulae; B, blastocysts; and B−, reaction for blastocysts without reverse transcriptase.

stage-specifically implies sharp shifts in gene expression during preimplantation development, presumably part of the drive to advance preimplantation development. Both the new and known genes identified in this study provide starting materials to investigate the process further through.

4. Condensation

Microarray analysis identified genes expressed differentially between morula and blastocyst.

Note added in proof

The paper was prepared for the proceedings of the meeting in the fall of 2002. While this paper is being processed for the publication, the microarray-based gene expression profiling of mouse preimplantation embryos has been published by Hamatani et al. (Developmental Cell, Vol. 6, 117–131, 2004) and Wang et al. (Developmental Cell, Vol. 6, 133–144, 2004).

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