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Microarray analysis of differentially expressed genes in inner cell mass and trophectoderm of parthenogenetic embryos

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Abstract: Mouse parthenogenetic (PG) embryos do not survive beyond day 9.5 of pregnancy. In this study, to understand the molecular mechanisms underlying the failure of parthenogenesis at the early developmental stage, we performed global gene expression profiling of the PG inner cell mass (ICM) and trophectoderm (TE) using microarray analysis, compared the results with those from *in vitro*-fertilized embryos, and identified genes whose expression levels showed more than a 4-fold change as a cutoff. Eighty probe sets were up-regulated and 59 probe sets down-regulated in the PG ICM, while 169 and 43 probe sets were respectively up-regulated and down-regulated in PG TE. We selected two genes (*Sfmbt2* and *Gab1*) that were down-regulated in both the PG ICM and TE, one gene (*Nat1*) that was down-regulated in the PG ICM, and one gene (*Lysmd2*) that was up-regulated in the PG TE, and analyzed the gene expression levels using real-time PCR. The quantitative expression levels of these four genes were confirmed by real-time PCR. In the present study, we identified differentially expressed genes in PG embryos and also identified those that were ICM- or TE-specific in PG embryos.
Key words: Parthenogenetic embryo, ICM, TE, Microarray

Introduction

Mouse parthenogenetic (PG) embryos, which have the entire maternal genome, do not survive beyond day 9.5 of pregnancy. PG embryos can develop up to the 25-somite stage, but with very limited development of extra-embryonic tissues [1, 2]. After implantation, PG embryos have been shown to produce trophoblast giant cells that express *Pl-1*, one of the trophoblast giant cell-specific genes, after three days of blastocyst outgrowth; however, fewer trophoblast giant cells were present in these embryos than in the fertilized embryos [3]. These results indicate that the maternal genome inhibits the proliferation and differentiation of the trophectoderm (TE) to extra-embryonic tissues. To confirm the characteristics of the PG TE, we attempted to establish trophoblast stem (TS) cells from PG embryos. However, TS cells from PG embryos could not be established, suggesting that the TE cells of PG embryos do not possess trophoblast stem cells [4].

PG embryos have developmental deficiency due to a lack of paternally expressed imprinted gene expression, or abnormal expression of genes on the X-chromosome [5]. To date, the molecular mechanisms underlying the failure of parthenogenesis have been detected by microarray analysis in PG embryos at E9.5 and 11.5 [6–9]. In these studies, more than 2000 genes were detected as abnormally expressed in PG embryos, and almost all of these genes were non-imprinted genes. Therefore, the aberrant expression of non-imprinted genes causes a deficiency in the PG fetus. However, the gene expression analysis of the PG placenta has not been examined, because placental formation in PG embryos fails. To understand the factors behind the deficiency of PG placenta formation, embryos at an earlier stage such as the blastocyst stage are suitable for microarray analysis.

In mammals, the blastocyst stage is the first step in the generation of differentiated cells. Blastocysts comprise two types of cells, namely, the inner cell mass (ICM) and TE [10]. After implantation, the ICM and TE differentiate into the fetus and the extra-embryonic tissues, respectively. In mouse blastocysts, the ICM and TE respectively express the major transcription factors, *Oct3/4* and *Cdx2* [11, 12]. In PG blastocysts, OCT3/4 and CDX2 are respectively localized in the ICM and TE, similar to their localization in fertilized blastocysts [4]. Therefore, the

©2016 Japan Society for Ova Research
Received: July 9, 2015
Accepted: August 19, 2015
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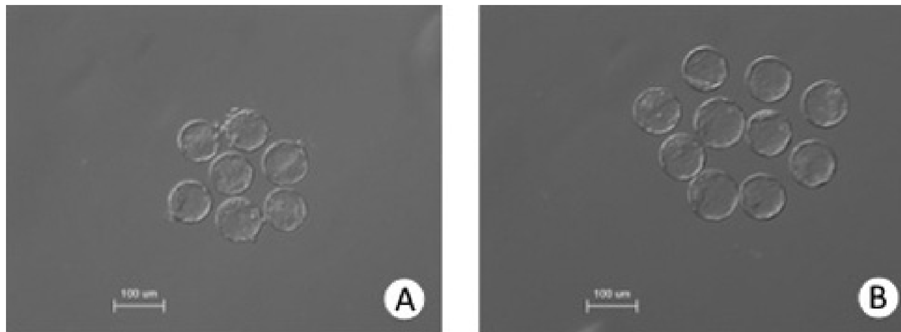

Fig. 1. Morphologies of expanded blastocysts of fertilized (A) and parthenogenetic (B) embryos. Blastocysts that were about 100 μm in diameter were used in this study.

failure of PG embryo development might be due to the abnormal expression of other factors in the ICM and TE.

In the present study, to understand the molecular mechanisms underlying the failure of parthenogenesis in the early stages of development, we used microarray analysis to perform global gene expression profiling of PG ICM and TE, and compared the results with those from *in vitro*-fertilized embryos.

Materials and Methods

Production of embryos

B6D2F1 (C57BL/6 X DBA2) mice were used in this study. The PG embryos were produced as previously described [4]. Fertilized embryos derived by IVF were used as controls. These embryos were cultured in M16 to the blastocyst stage. To obtain the same size of expanded blastocysts (less than 100 μm in diameter), PG embryos were collected at 98–102 h after activation, and control embryos were collected at 96–100 h after insemination (Fig. 1).

TE isolation with Bio-Cut Blade

The expanded blastocysts were washed several times in PBS (−) to remove bovine serum albumin contained in the culture medium. Then, the expanded blastosysts were divided into ICM with TE (Polar TE and ICM) and TE (Mural TE) with a Bio-Cut Blade (No. 715; Feather, Osaka, Japan) under an inverted microscope (Fig. 2A). The separated TE was washed in M2, and collected for cDNA synthesis.

ICM isolation by immunosurgery

The expanded blastocysts were incubated in acid tyrode for a few mins to remove the zona pellucida. The TE was removed by immunosurgery as follows. Zona pellucida-removed blastocysts were incubated in Anti-Mouse Serum antibody (Sigma-Aldrich, St. Louis, MO, USA) at 1: 10 in M2 for 1 h at 37 °C, and then in Complement Guinea Pig Serum (Merck, Darmstadt, Germany) at 1: 50 in M2 for 30 min at 37 °C. After Complement Guinea Pig Serum treatment, lysed trophectoderm was removed by pipetting and the ICM was isolated (Fig. 2B), and collected for cDNA synthesis.

Microarray analysis

Total RNA of each group (ICM and TE pooled from 20 expanded blastocysts) was extracted using an RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol. cRNA was synthesized using a Two-Cycle Eukaryotic Target Labeling Kit (Affymetrix) and hybridized with a GeneChip® Mouse Genome 430 2.0 Array (Affymetrix). Analysis was performed using GeneSpringX11.0 (Agilent Technologies). After global normalization, transcripts with a raw signal intensity greater than 100 for at least one sample were used in the analysis. To identify genes specifically expressed in the TE, the genes were subjected to the *t*-test with 5% false discovery rate (FDR) ($P < 0.05$), and screened for more than 4-fold changes. Genes specifically expressed in the ICM were identified by a comparable process ($P < 0.05$ with 5% FDR and more than 4-fold change).

Gene expression analysis by real-time PCR

Total RNA of each group (ICM and TE pooled from 20 expanded blastocysts) was extracted as described above. Total RNA was reverse-transcribed to cDNA using SuperScript III Transcriptase (Invitrogen). The quantitative gene expression analysis was performed by real-time PCR using a 7500 Real-time PCR System (Applied Biosystems, Carsbad, CA). PCR products were detected with SYBR Green using SYBR Green Master

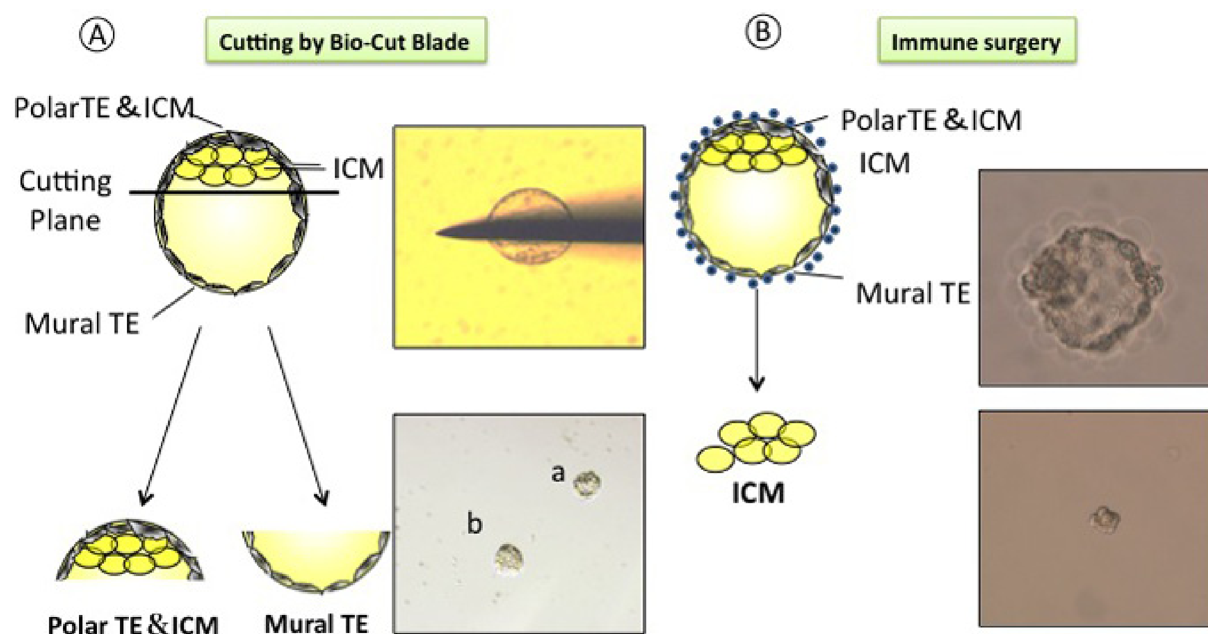

Fig. 2. Method of TE and ICM isolation from expanded blastocysts. (A) The expanded blastosyst was divided into ICM with TE (Polar TE and ICM) and TE (Mural TE) with a Bio-Cut Blade (a: Mural TE, b: Polar TE and ICM). (B) ICM isolation by immunosurgery using Anti-Mouse Serum antibody and Complement Guinea Pig Serum.

Table 1. PCR conditions

Genes	Primer sequences	Annealing Temp (°C)	Cycles	Size (bp)
Gab1	F:5'-AGTTACGACATTCCGCCAAC-3' R:5'-ACACGTTTCCACAGGAGACC-3'	60	38	165
Sfmbt2	F:5'-TGTGAGGCCAATTCTTACCC-3' R:5'-GCAGCAGTACTTGGCATTGA-3'	60	38	192
Lysmd2	F:5'-GCCAGAGATTTCCTGCAAAG-3' R:5'-TTTAGGGTCCAGCCATTCTG-3'	63	38	171
Nat1	F:5'-GAAGCCATGCATCTGGATTT-3' R:5'-AAGGTGGACCATTTCACTGC-3'	50	38	189
GAPDH	F:5'-GTCGTGGAGTCTACTGGTGTC-3' R:5'-GAGCCCTTCCACAATGCCAAA-3'	60	38	240

Mix (Applied Biosystems). The PCR conditions are listed in Table 1.

Statistical analysis

The gene expression levels of *Gab1*, *Sfmbt2*, *Lysmd2* and *Nat1* in the ICM and TE of control and PG embryos were compared using Student's *t*-test; $P < 0.05$ was considered to indicate statistical difference.

Results

The evaluation of ICM and TE for microarray analysis

In mice blastocysts, *Oct4* and *Cdx2* are respectively expressed in the ICM and TE. In the present study, the expressions of *Oct4* and *Cdx2* were assayed in isolated TE and ICM by RT-PCR, as previously described [4]. Our results showed that *Oct4* and *Cdx2* were exclusively detected in the ICM and TE, respectively (data not shown), indicating that the ICM and TE used in this study were suitable for global gene expression analysis by microarray.

Table 2. Transcripts with increased expression in parthenogenetic ICM (FC≥4.0)

Gene Symbol	Probe Set ID	Fold Change	Chr	Gene Symbol	Probe Set ID	Fold Change	Chr
Il18r1	1421628_at	41.1	1	Tmem45a	1422587at	4.9	16
Fcgr2b	1435477_s_at	10.3	1	Snai2	1447643_x_at	4.8	16
Sgpp2	1457867_at	6.4	1	T	1419304_at	19.1	17
Stat4	1448713_at	5.4	1	1700097N02Rik	1436419_a_at	6.7	17
Gli2	1459211_at	4.3	1	Crispl	1416325_at	4.8	17
Slco4a1	1438160_x_at	6.4	2	Ttr	1459737_at	21.8	18
Cubn	1426990_at	4.5	2	Brunol4	1452240_at	5.9	18
Stmn2	1423281_at	5.7	3	Spink3	1415938_at	4.8	18
Car2	1448752_at	5.5	3	Il33	1416200_at	4.1	19
Pdgfc	1449351_s_at	4.6	3	Armcx3	1415780_a_at	23.6	X
Dad1	1421100_a_at	11.6	4	Armcx2	1456739_x_at	21.5	X
Tcea3	1424531_a_at	7.2	4	Rhox9	1449540_at	19.2	X
D630039A03Rik	1437085_at	4.7	4	Rhox6	1419018_at	11.8	X
Tpm2	1425028_a_at	4.6	4	Cited1	1449031_at	10	X
Sh3gl2	1418791_at	4	4	Nrk	1450079_at	9.3	X
Uchl1	1448260_at	4.1	5	AV320801	1449962_at	9.2	X
Trh	1418756_at	4.8	6	AU015836	1444038_at	7.3	X
Tcfec	1419537_at	4.5	6	100039003	1422617_at	7	X
Fgf15	1418376_at	4.9	7	100039003	14422618_x_at	4.7	X
Ndn	1455792_x_at	4.1	7	221001202O21Rik	1430538_at	6.4	X
Cd81	1416330_at	4.1	7	3830403N18Rik	1422011_s_at	6.1	X
Atp4a	1421286_a_at	4.1	7	6530401D17Rik	1451679_at	6	X
Kcnk1	1448690_at	8.2	8	Gm364	1440692_at	5.3	X
Zfp423	1419380_at	7.4	8	Pdzd4	1435457_at	5.3	X
Stox2	1447624_s_at	5.7	8	1700013H16Rik	1453331_at	4.8	X
Exoc3l	1436536_at	5	8	3830417A13Rik	1430162_at	4.7	X
Pcolce2	1451527_at	4.6	9	Armcx4	1427167_at	4.7	X
Sc5d	1434520_at	4.5	9	Glod5	1460454_at	4.6	X
Slc29a3	1451013_at	9.8	10	Enox2	1427043_s_at	4.6	X
Slc35d3	1428642_at	4.5	10	Lonrf3	1436200_at	4.4	X
Grb10	1425458_a_at	7.7	11	Eda	1449524_at	4.4	X
Notum	1451857_a_at	6.8	11	Pcyt1b	1437648_at	4.4	X
Spns2	1451601_a_at	4.1	11	Pcsk1n	1416965_at	4.4	X
Phf15	1455345_at	4.1	11	Gm1140	1446051_at	4.4	X
Ptprn2	1435968_at	16.6	12	Gm715	1445503_at	4.3	X
Nid2	1423516_a_at	5	14	Sh3bgrl	1436997_x_at	4.2	X
Pcdh21	1418304_at	4.4	14	Klhl13	1448269_a_at	4.2	X
Lgals2	1417079_s_at	11.3	15	Tro	1426016_a_at	4.2	X
Ccdc80	1424186_at	5.7	16	Pgk1	1439435_x_at	4.1	X
Fstl1	1448259_at	5.1	16	Bex2	1417388_at	4	X

Underlined gene symbols are the maternally expressed imprinted genes.

Genes with abnormal expression in the PG ICM

The gene expression analysis showed that the number of probe sets with significantly differential expression in PG and control ICM was 139 (up-regulated 80; down-regulated 59 in PG ICM) (Table 2 and 3). Among the 80 up-regulated probe sets, there were 2 probe sets of maternally expressed imprinted genes (2 genes: *Cd81* and *Grb10*), and 31 probe sets of X-chromosome-located genes. Among the 59 down-regulated probe sets, there were 16 probe sets of paternally expressed imprinted genes (9 genes: *Sfmbt2*, *Mcts2*, *Mest*, *Snrpn*, *Peg3*, *Kcnq1ot1*, *Plagl1*, *Zrsr1* and *Slc38a4*).

Table 3. Transcripts with decreased expression in parthenogenetic ICM (FC≥4.0)

Gene Symbol	Probe Set ID	Fold Change	Chr	Gene Symbol	Probe Set ID	Fold Change	Chr
Rxrg	1418782_at	11.7	1	D7Ertd715e	1455087_at	118.5	7
Khdc1b	1434164_s_at	5.8	1	Kcnq1ot1	1457781_at	26.3	7
	1436459_at	4.7	1	Kcnq1ot1	1458161_at	14.7	7
Sfmbt2	1434353_at	39.9	2	Gab1	1417693_a_at	7.3	8
Sfmbt2	1443176_at	5.9	2	Gab1	1417694_at	10.8	8
	1456752_at	5.8	2	Nat1	1421758_at	17.1	8
	1444837_at	29	2	Nat2	1449981_a_at	7.6	8
Dnajc1	1459791_at	8.5	2	Apoa1	1419233_x_at	9.3	9
Mcts2	1451058_at	7.5	2	Apoa1	1455201_x_at	6.3	9
Etohi1	1429712_at	5.6	2	Ubtfl1	1439670_at	7.2	9
Fabp2	1418438_at	4.5	3	Tbx20	1453351_at	7.5	9
Phf17	1426752_at	4.4	3	Plagl1	1426208_x_at	15.8	10
Phf17	1426753_at	5.4	3	Zrsr1	1449354_at	17.7	11
Phf17	1452179_at	5.2	3	Zfp454	1456507_at	6.6	11
C430002E04Rik	1441279_at	9.8	3	Akr1c14	1418979_at	6.1	13
	1456570_at	5.4	4	Mboat1	1435323_a_at	4.9	13
Oas1e	1424536_at	15	5		1443564_at	10.4	13
Fkbp6	1425101_a_at	12.5	5		1447021_at	9.2	13
Mest	1423294_at	9.6	6	Mbnl2	1433754_at	5.9	14
Slc6a13	1424338_at	11.8	6	Mbnl2	1455827_at	6.3	14
Ppm1k	1441988_at	4.4	6	5830407P18Rik	1453512_at	11.4	14
Snrpn	1415895_at	5.6	7	Gml	1422400_a_at	9.8	15
Snrpn	1415896_x_at	42.6	7	Slc38a4	1428111_at	128.9	15
Snrpn	1432096_at	30.7	7	Slc38a4	1448889_at	374	15
Snrpn	1435716_x_at	54.9	7		1439732_at	4.5	15
Snrpn	1441905_x_at	48.1	7	Slc38a1	1454764_s_at	13.1	15
Peg3	1417355_at	29.9	7	Septin12	1438298_a_at	4.4	16
Snrpn	1421063_s_at	11.1	7	Septin12	1460497_a_at	4.3	16
	1433924_at	27.3	7	Fermt3	1456014_s_at	7.1	19
D7Ertd715e	1436964_at	54.3	7				

Underlined gene symbols are the paternally expressed imprinted genes.

Genes with abnormal expression in the PG TE

The gene expression analysis revealed that the number of probe sets showing significantly different expression between PG and control TE was 212 (up-regulated 169; down-regulated 43 in PG TE) (Table 4 and 5). Eight of the 212 up-regulated probe sets were of maternally expressed imprinted genes (6 genes: *Cdkn1c*, *Slc22a18*, *Phlda2*, *Ascl2*, *H19* and *Grb10*), and 44 were of X-chromosome-located genes. Fifteen of the 43 down-regulated probe sets were of paternally expressed imprinted genes (7 genes: *Mcts2*, *Sfmbt2*, *Kcnq1ot1*, *Snrpn*, *Plag1*, *Zrsr1* and *Slc38a4*).

Genes with abnormal expression in ICM and TE of PG embryos

Among the 59 and 43 probe sets respectively down-regulated in the ICM and TE of PG embryos (Table 3 and 5), 30 probe sets showed significantly repressed expression in both the ICM and TE of PG embryos compared with their expression in control embryos. In these 30 probe sets, there were 14 probe sets (7 genes) of paternally expressed imprinted genes. The expressions of two paternally expressed imprinted genes (*Mest* and *Peg3*) were significantly repressed only in the ICM of PG embryos. Among the 80 and 169 probe sets respectively up-regulated in ICM and TE of PG embryos (Table 2 and 4), 12 probe sets showed significantly activated expression in both the ICM and TE of PG embryos. Of these 12 probe sets, 1 probe set (*Grb10*) was a maternally expressed imprinted gene, and 10 probe sets were localized on the X-chromosome. The expressions of one (*Cd81*) and five (*Cdkn1c*, *Slc22a18*, *Phlda2*, *Ascl2* and *H19*) maternally expressed imprinted genes were significantly activated in the ICM and TE of PG embryos, respectively.

Table 4. Transcripts with increased expression in parthenogenetic TE (FC≥4.0)

Gene Symbol	Probe Set ID	Fold Change	Chr	Gene Symbol	Probe Set ID	Fold Change	Chr
Slc40a1	1417061_at	7.5	1	Krt19	1417156_at	6.3	11
Tmem37	1417611_at	5.5	1	Myl4	1422580_at	4.4	11
Ier5	1417612_at	6	1	Egfr	1424932_at	4.6	11
Mosc1	1428005_at	5.3	1	Grb10	1425458_a_at	5.6	11
Itpkb	1435272_at	5	1	Socs3	1455899_x_at	8.4	11
Slc40a1	1448566_at	15.1	1	Septin9	1417038_at	5.2	11
Atf3	1449363_at	4.5	1	Socs3	1456212_x_at	7.1	11
Batf3	1453076_at	4.8	1	Egfr	1435888_at	4.9	11
Igsf8	1460675_at	8.2	1		1439965_at	5.9	11
Mfsd7b	1434424_at	6.4	1	Atp10b	1453352_at	24.5	11
Iqca	1435992_at	4.2	1	Itgb3	1455257_at	19.9	11
ENSMUSG00000073738	1439816_at	5.2	1	Oog1	1427977_x_at	4.2	12
	1447227_at	5.5	1	Emb	1415857_at	5	13
Rabgap1l	1453365_at	5.6	1	Pfkp	1416069_at	8.1	13
Ada	1417976_at	4	2	Foxc1	1419486_at	20.6	13
Nelf	1436959_x_at	5.8	2	2310051M13Rik	1421684_at	92.3	13
Bfsp1	1450571_a_at	5	2	F2r	1437308_s_at	4.9	13
Nostrin	1441075_at	4.6	2	Ssbp2	1449815_a_at	4.9	13
Gstm5	1416842_at	6.6	3	F2r	1450852_s_at	4.3	13
Vav3	1417122_at	22.2	3	Arrdc3	1454617_at	4.3	13
F3	1417408_at	11.8	3	Erc2	1434582_at	6.1	14
Gbp2	1418240_at	5.4	3	Lrch1	1438032_at	5.4	14
Gbp3	1418392_a_at	5.7	3	Ppp1r1a	1422605_at	4.7	15
Gbp2	1435906_x_at	5.2	3	Nipal2	1424612_at	4.4	15
Pld1	1437113_s_at	7.6	3	Fam134b	1424683_at	4.1	15
Vav3	1448600_s_at	14.8	3	Prlr	1448556_at	11.3	15
Car2	1448752_at	10.1	3	Prlr	1437397_at	17.2	15
Csf1	1448914_a_at	6.8	3	Ehhadh	1448382_at	5.2	16
Csf1	1460220_a_at	9.1	3	Igsf5	1451407_at	5.3	16
Sgms2	1428663_at	11.2	3	Plcxd2	1455324_at	4.2	16
Lhfp	1433776_at	4.4	3	Zdhhc14	1437614_x_at	4.5	17
Slc25a31	1453133_at	5	3	Zdhhc14	1438619_x_at	8	17
Glipr2	1428492_at	4.1	4	Tnfrsf21	1450731_s_at	5.5	17
Plk3	1434496_at	4.5	4	Tulp1	1451582_at	8.9	17
Fam92a	1451570_a_at	4.2	4	Fhod3	1435551_at	12.9	18
Hcrtr1	1436295_at	7.4	4	Csnk1a1	1430529_at	4.2	18
Ccng2	1416488_at	4.9	5	Dsc3	1434534_at	6.1	18
9130213B05Rik	1428891_at	4.4	5	Slc15a3	1420697_at	54.2	19
	1434379_at	5.1	5	Anxa1	1448213_at	4.1	19
Il6	1450297_at	5	5	Afap1l2	1436870_s_at	5.6	19
Tmem116	1453710_at	4.5	5	Klhl13	1416242_at	4.5	X
Nat8l	1435842_at	4.2	5	Sytl4	1417336_a_at	4.4	X
Cmklr1	1456887_at	5.3	5	Fhl1	1417872_at	10.4	X
Mgst1	1415897_a_at	8.6	6	Rhox6	1419018_at	28	X
Olr1	1419534_at	7.1	6	Rhox4a	1419229_at	13.9	X
St8sia1	1419695_at	4.2	6	Slc6a14	1420503_at	4.4	X
Eps8///LOC632638	1422823_at	12.6	6	3830403N18Rik	1422011_at	6.3	X
Eps8	1422824_s_at	7.5	6	100039003	1422617_at	4.1	X
Itpr2	1424834_s_at	10.3	6	Phka1	1422743_at	11.2	X
St8sia1	1455695_at	5.2	6	Zic3	1423424_at	12.7	X
Cdkn1c	1417649_at	8.9	7	LOC100046560	1426306_a_at	4.3	X
Slc22a18	1417809_at	26.8	7	Il13ra1	1427164_at	14.5	X
Phlda2	1417837_at	187.3	7	Luzp4	1427346_at	4.6	X
Nucb2	1418355_at	5.6	7	Spin4	1427985_at	4	X
Nupr1	1419665_a_at	11.8	7	Sh3bgrl	1428107_at	6.3	X
Nupr1	1419666_x_at	10.6	7	2900062L11Rik	1428333_at	4.4	X
Fgf4	1420086_at	13.3	7	Dusp9	1433844_a_at	7.7	X
Ascl2	1422396_s_at	12.1	7	Dusp9	1433845_x_at	7.5	X
Ceacam1	1425538_x_at	4.8	7	Morc4	1434436_at	7.2	X
Ascl2	1432018_at	15.3	7	Sh3bgrl	1436997_x_at	8.7	X
H19	1448194_a_at	12.2	7	Klhl13	1448269_a_at	8.2	X
Saa3	1450826_a_at	4.3	7	Heph	1448696_at	55.8	X
Ascl2	1460514_s_at	8.1	7	Rhox9	1449540_at	11	X
Tmem86a	1428758_at	6.6	7	Nrk	1450078_at	23.1	X
Ctsc	1437939_s_at	4.1	7	Nrk	1450079_at	12.6	X
Ctsc	1446834_at	5.2	7	6530401D17Rik	1451679_at	4.3	X
Hpgd	1419905_s_at	20.9	8	Il13ra1	1451775_s_at	6.3	X
Mfhas1	1429005_at	6	8	Arhgap6	1451867_x_at	7.5	X
Nfix	1436363_a_at	4.4	8	Dusp9	1454737_at	6.9	X
Kcnk1	1448690_at	12.9	8	Il13ra1	1454783_at	10.6	X
Atp2c2	1452326_at	4.6	8	Rps6ka6	1429759_at	7.2	X
	1444020_at	5.8	8	3830417A13Rik	1430162_at	14.5	X
Snap91	1416688_at	25.9	9	Ammecr1	1430697_at	4.4	X
Ntm	1426282_at	26.7	9	4932442L08Rik	1431809_at	16.8	X
Lrrc2	1428626_at	4.3	9	AU022751	1433456_at	4.5	X
Lysmd2	1433434_at	4.1	9	Wdr40b	1434170_at	4.2	X
AW551984	1458492_x_at	7.9	9	Zxda	1439077_at	4.4	X
Sorl1	1416527_at	13.4	10	Wnk3	1443924_at	6	X
Ntm	1418393_a_at	6.6	10	AU015836	1444038_at	9.6	X
Rab32	1430128_a_at	5.3	10	Reps2	1446652_at	4.1	X
Itga7	1439260_a_at	12.7	10	Wdr40b	1454869_at	7	X
Reep6	1448765_at	5.7	10	Gpr50	1455498_at	13.2	X
Enpp3	1434252_at	4	10	Zxdb	1455817_x_at	4.4	X
Fyn	1435554_at	5.7	10	EG668339	1456942_x_at	24.5	X
Ppm1h	1455737_at	7.2	10				

Underlined gene symbols are the maternally expressed imprinted genes.

Table 5. Transcripts with decreased expression in parthenogenetic TE (FC≥4.0)

Gene Symbol	Probe Set ID	Fold Change	Chr	Gene Symbol	Probe Set ID	Fold Change	Chr
	1456752_at	14.6	2	Snrpn	1421063_s_at	52.7	7
Mcts2	1451058_at	15.4	2	Kcnn4	1421038_a_at	7.2	7
Sfmbt2	1444837_at	144.6	2	Snrpn	1415895_at	4.6	7
Wfdc15a	1443176_at	20.7	2	Snrpn	1415896_x_at	8.7	7
Sfmbt2	1436288_at	4.2	2	Mt4	1450645_at	5	8
Phf17	1434353_at	28.9	2	Gab1	1417694_at	10	8
C430002E04Rik	1452179_at	4.1	3	Gab1	1417693_a_at	10	8
Lrrc66	1441279_at	11.5	3	Plagl1	1426208_x_at	19.3	10
Fkbp6	1442096_at	5.7	5	Zrsr1	1449354_at	10	11
	1425101_a_at	8.9	5	Mgl1	1419605_at	4.4	11
Nanog	1420312_s_at	4.1	5		1447021_at	177.7	13
Slc6a13	1429388_at	4.5	6	Akr1c14	1418979_at	6.9	13
Kcnq1ot1	1424338_at	8.9	6	5830407P18Rik	1453512_at	6.5	14
Kcnq1ot1	1458161_at	10.4	7	2410012E07Rik	1432118_at	4	14
D7Ertd715e	1457781_at	10.2	7	Slc38a1	1454764_s_at	24.8	15
Kcnq1ot1	1455087_at	25.3	7	Slc38a4	1448889_at	106.2	15
Snrpn	1442029_at	11.2	7	Slc38a4	1428111_at	88.3	15
D7Ertd715e	1441905_x_at	50.2	7	Slc38a1	1415903_at	4.8	15
Snrpn	1436964_at	106.8	7		1457356_at	18.1	17
Snrpn	1435716_x_at	7.4	7	Zfp799	1437873_at	4.1	17
Slc6a13	1433924_at	14.2	7	BC051142	1437264_at	5.3	17
Ppm1k	1432096_at	10	7				

Underlined gene symbols are the paternally expressed imprinted genes.

Quantitative gene expression of Gab1, Sfmbt2, Lysmd2 and Nat1 in the ICM and TE

To confirm the gene expression levels in ICM and TE, quantitative gene expression analysis by real-time PCR was carried out on four genes (*Gab1*, *Sfmbt2*, *Lysmd2* and *Nat1*) (Fig. 3). The results of the microarray analysis show that *Gab1* and *Sfmbt2* were significantly repressed in both the ICM and TE of PG embryos, while *Nat1* was repressed only in the ICM of PG embryos. The expression levels of *Gab1* in both the ICM and TE of PG embryos were 10% of those of control embryos. Sfmbt2 was not detected in either the ICM or TE of PG embryos. *Lysmd2* was expressed at higher levels in the PG TE than in the control TE. In contrast, the expression of *Nat1* was significantly repressed in the ICM of PG embryos compared with its expression in control embryos, but showed no difference in the TE.

Discussion

In mouse embryos, *Oct4* and *Cdx2* are respectively expressed in the ICM and TE. *Cdx2* is localized to the outer cells in the morula, and is completely restricted to the TE during blastocyst formation [13]. However, *Oct4* is expressed in all blastomeres, and its expression gradually declines in the TE [14]. Consequently, the TE of early blastocyst expresses both *Oct4* and *Cdx2*. In the present study, to assess the isolation of ICM and TE by the expression of *Oct4* and *Cdx2*, we first attempted to determine the suitable stage of blastocyst to use for microarray analysis. The results indicated that expanded blastocysts (about 100 μm in diameter) are suitable for isolation of the ICM and TE since they respectively expressed *Oct4* and *Cdx2*.

Global gene expression profiling of PG blastocysts in comparison with those of blastocysts from fertilized embryos has previously been performed [15]. It was demonstrated that four maternally expressed imprinted genes (*H19*, *Phlda2*, *Grb10*, and *Cdkn1c*) that affect placental growth showed increased expression in parthenotes [16]. In the present study, three of these four genes (*H19*, *Phlda2*, and *Cdkn1c*) showed increased expression in the TE, but not in the ICM of PG blastocysts. In addition, we found that *Ascl2* also showed increased expression only in the TE of PG blastocysts. *Ascl2* was the first transcription factor shown to play a critical part in the development of the mammalian trophoblast lineage [17], and is especially essential for the differentiation of the ectopla-

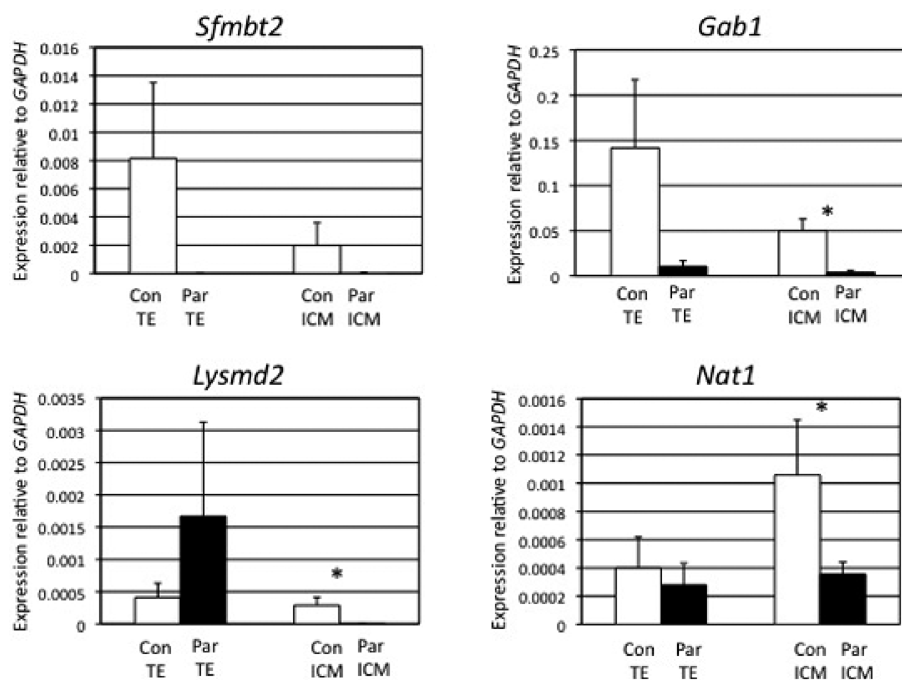

Fig. 3. Quantitative mRNA expression of Sfmbt2, Gab1, Lysmd2 and Nat1 in ICM and TE of control and PG blastocysts. The amounts of transcripts for Sfmbt2, Gab1, Lysmd2 and Nat1 genes are expressed relative to GAPDH values. The shown expression levels of the ICM and TE represent the means ±SEM of three trials. Asterisks indicate a significant difference from control embryo expression ($P<0.05$).

cental cone in the establishment of junctional zone cell types [18]. Thus, the samples of ICM and TE isolated in this study can be considered suitable for the detection of ICM- and /or TE-specific differentially expressed genes in PG blastocysts.

Mitogen-activated protein kinase (MAPK) signaling is reduced in PG blastocysts [15]. This pathway is involved in regulating cell growth and differentiation [19]. In the present study, expression of *Gab1*, the key gene for MAPK signaling, was decreased in both the ICM and TE of PG blastocysts. *Gab1* is involved in the signal transduction of many growth factors and interleukin [20–23]; therefore, this protein plays a crucial role in transmitting key signals that control cell growth, differentiation and function from multiple receptors [20]. These results suggest that PG embryos fail to develop due to the reduction of MAPK signal transduction.

Lysmd2 is lysine motif (LysM) and putative peptidoglycan-binding, domain containing 2. Peptidoglycan is a key component of the bacterial cell wall in both Gram-negative and Gram-positive bacteria and is important for maintaining the structural integrity of the plasma membrane [24]. Carbohydrate recognition is essential for growth, cell adhesion and signaling in all living organisms. LysM, a highly conserved carbohydrate binding module, is found in proteins from viruses, bacteria, fungi, plants and mammals [25]. The function of Lysmd2 has not been determined in mammals; however, the upregulation of Lysmd2 in PG TE might have an effect on growth or implantation.

N-acetyltransferase (NAT) is an enzyme that catalyzes the transfer of acetyl groups from acetyl CoA to arylamines [26]. Three NAT isozymes are present in mice, NAT1, NAT2 and NAT3 [27]. In humans, NAT1 and NAT2 are capable of detoxifying a range of potential arylamine carcinogens to innocuous metabolites via N-acetylation [27]. Mouse NAT1 exhibits a substrate profile similar to the human NAT2 isoenzyme, namely a higher catalytic activity for sulfamethazine (SMZ) and isoniazid [28]. Mouse NAT2 has been shown to metabolize p-aminosalicylic acid (PAS), p-aminobenzoic acid (PABA), and the folate catabolite p-aminobenzoyl-L-glutamate [28, 29]. Moreover, mouse NAT2 is present early in development, suggesting a possible link to folate metabolism and neural tube development [30, 31]. In this study, expressions of Nat1 and Nat2 were significantly reduced in PG ICM. These results indicate that acetylation has a crucial role in early mouse development.

In conclusion, PG blastocysts show up-regulated maternally expressed imprinted genes and down-regulated paternally expressed imprinted genes, and possibly reduced MAPK signal transduction via *Gab1*. These results are similar to those reported previously [15]. The majority of gene expressions reduced in PG blastocysts are down-regulated in both the ICM and TE of PG blastocysts. On the other hand, almost all the genes differentially induced in PG blastocysts are present only in the ICM or TE of PG blastocysts. These results suggest that gene expression profiles are quite different between the ICM and TE of PG blastocysts, and provide new information about the reasons for the lethality of PG embryos. In humans and mice, blastocysts invade the endometrium to complete the implantation, suggesting that the gene expression profile in the blastocyst may be similar between these two species. Therefore, the results of this study might be valuable information for the determination of the quality of human blastocysts.

Acknowledgment

The authors would like to thank Dr. A. Fukuda and Mr. Y. Kishi for assisting with the microarray analysis.

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