Identification of Differentially Expressed Genes in the *Dicer 1* Knock-down Mouse Embryos using Microarray

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ABSTRACT

Silencing of *Dicer1* by siRNA did not inhibit development up to the blastocyst stage, but decreased expression of selected transcription factors, including *Oct-4*, *Sox2* and *Nanog*, suggesting that *Dicer1* gene expression is associated with differentiation processes at the blastocyst stage (Cui *et al.*, 2007). In order to get insights into genes which may be linked with microRNA system, we compared gene expression profiles in *Gapdh* and *Dicer1* siRNA-microinjected blastocysts using the Applied Biosystem microarray technology. Our data showed that 397 and 737 out of 16354 genes were up- and down-regulated, respectively, following siRNA microinjection ($p<0.05$), including 24 up- and 28 down-regulated transcription factors. Identification of genes that are preferentially expressed at particular *Dicer1* knock down embryos provides insights into the complex gene regulatory networks that drive differentiation processes in embryos at blastocyst stage.

(Key words : Mouse, *Dicer1*, siRNA, Microarray)

INTRODUCTION

MicroRNAs (MiRNAs) are a recently discovered new class of RNAs that epigenetically regulate gene expression at post-transcriptional or translational level. miRNAs are expressed in a tissue-specific manner and are considered to play important roles in cell proliferation, apoptosis, and differentiation during mammalian development (Bernstein *et al.*, 2001; Ambros *et al.*, 2003; Kanellopoulou *et al.*, 2005). Currently, the role of miRNAs in preimplantation mammalian embryos is unclear. Deficiency of *Dicer* (an enzyme for the production of mature microRNAs) in mouse embryos derived from heterozygous mating causes reduced *Oct4* gene expression and death of the embryos before day 7.5 post-ovulation (Whitworth *et al.*, 2005). Our previous result also showed that silencing of *Dicer1* by siRNA did not inhibit mouse embryo development up to the blastocyst stage, but decreased expression of selected transcription factors, including *Oct-4*, *Sox2* and *Nanog* (Cui *et al.*, 2007). These results indicate that miRNAs play a role in early embryogenesis.

Despite clear evidence of developmental regulation, limited information is currently available on the expression and functions of microRNAs during early embryonic development in mammals. Further it was not clear that how microRNA system regulate gene control. To elucidate the role of microRNAs on the gene expression network in preimplantation embryos, we initially produce *Dicer1* gene knock down embryos. In order to identify the genes that are regulated by *Dicer1* in mouse embryos, we evaluate the global transcriptome of *Dicer1* knock down embryos. Identification of genes that are preferentially expressed at particular *Dicer1* knock down embryos provides insights into the complex gene regulatory networks that drive differentiation processes in embryos at blastocyst stage.

MATERIALS AND METHODS

Generation of Mouse Embryos

Six-week-old B6D2 F_1 female mice were induced to superovulate with 5 IU eCG (Sigma, St. Louis, MO), followed by 5 IU hCG (Sigma) 48 h later. The experiments were performed in accordance with the 'Guiding Principles for the Care and Use of Laboratory Animals'. Zygotes (1C) were collected 18 hr after hCG administration and cumulus cells were removed by brief exposure to medium containing 0.1% hyaluronida-

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se (Sigma) and provided for siRNA microinjection.

siRNA Microinjection and *In Vitro* Culture

Zygotes were collected and denuded of cumulus cells. We purchased siRNAs to silence mouse *Dicer1* (siRNA ID No., 173425, Ambion, Inc. Houston, TX, USA) or positive control (glyceraldeehyde 3-phosphate dehydrogenase, *Gapdh* siRNA, ID No. 407972, Ambion), negative control (Cat. No. 4611G, Ambion). The siRNA was diluted with RNase-free water (Ambion) to a final concentration of 100 μM and stored at −20℃. Approximately 10 pl of individual (*Dicer1*, *Gapdh*, negative control) siRNA was injected into the cytoplasm of the zygotes using an Eppendorf microinjector system (Eppendorf, Hamburg, Germany). Individual siRNA microinjected zygotes were then cultured in M16 (Sigma) medium at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air to development to the blastocyst stage.

Real Time Reverse Transcriptase-Polymerase Chain Reaction (Real Time RT-PCR)

Frozen-thawed embryos were used to quantify mRNA levels. Messenger RNA was extracted using the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway) according to the manufacturer's instructions. First, standard cDNA synthesis was achieved by reverse transcription of the RNA by using the Oligo $(dT)_{12-18}$ primer and the Superscript reverse transcriptase enzyme (Invitrogen Co., Grand Island, NY).

Real time RT-PCR was performed using the 10 primer sets shown in Table 1. The threshold cycle (Ct) value represents the cycle number at which sample fluorescence is statistically higher than background. The reactions were conducted according to the protocol of the DyNAmo SYBR green qPCR kit that contains modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5 mM $MgCl_2$, and a dNTP mix that includes dUTP (Finnzyme, Espoo, Finland). The PCR protocol involved a denaturation program (95℃ for 10 min) followed by an amplification and quantification program repeated 40 times (95℃ for 15 sec, 55/60℃ for 15 sec, 72℃ for 15 sec with a single fluorescence measurement), a melting curve program (65~95℃, with a heating rate of 0.2℃ per second and continuous fluorescence measurement), and finally a cooling step to 4℃. Fluorescence data was acquired after the extension step during PCR reactions that contained SYBR Green. The PCR products were then analyzed by generating a melting curve. Because the melting curve of a product is sequence-specific, it can be used to distinguish between non-specific and specific PCR products. To use the mathematical model, it is necessary to determine the crossing points (CP) for each transcript. Crossing point is defined as the point at which the fluorescence rises appreciably above the backgro-

Table 1. List of primers used for real time RT-PCR

Genes	Genbank acc. No.	Primer sequence	Ann. tem.	Base pairs
Dicer1	NM_148948	F: ggtggtctggcaggtgtact R: cctgaggctggttagctttg	60℃	272
Cox5a	NM_007747	F: gggtcacacgagacagatga R: acfacctccaagatgcgaac	60℃	206
Cox5b	NM_009942	F: gatgaggagcaggctactgg R: cagccaaaccagatgata	55℃	198
Cdc42	NM_009861	F: ttgttggtgatggtgctgtt R: aatcctcttgccctgcagta	60℃	168
Par3	DQ082726	F: cagactcaaggcaggagacc R: gggtgtgagaacaacgtgct	60℃	232
Par6	AF070970	F: tgacagtagcgatgacagca R: agaggctgaatccgctaaca	60℃	212
Rac1	NM_009007	F: tatgggacacagctggacaa R: acagtggtgtcgcacttcag	60℃	152
Hmgb1	NM_010439	F: ccattggtgatgttgcaaag R: ctttttcgctgcatcaggtt	60℃	158
Gapdh	NM_008084	F: aactttggcattgtggaagg R: acacattgggggtaggaaca	60℃	223
H2a	X16495	F: acaacaagaagacccgcatc R: cttggccttgtggtgactct	60℃	167

und fluorescence. The relative quantification of gene expression was analyzed by the 2-ddCt method (Livak and Schmittgen, 2001). In all experiments, Histone H2a (*H2a*) mRNA was used as an internal standard, and rabbit Globin mRNA was used as an external reference for the analyses of relative mRNA expression levels of *Dicer1* in various developmental stages of oocytes and embryos. The sizes of the real time RT-PCR products were confirmed by gel electrophoresis on a standard 2% agarose gel, stained with ethidium bromide, and visualized by exposure to ultraviolet light.

Applied Biosystems Expression Array Analysis

Applied Biosystems Mouse Genome Survey Arrays (Applied Biosystems Inc., Foster City, CA USA) were used to analyze the transcriptional profiles of all samples, following acute morphine exposure. The Applied Biosystems mouse Genome Survey Array contains 32,996 60-mer oligonucleotide probes representing a set of 32,381 individual mouse genes and more than 3,000 control probes. Sequences used for microarray probe design are from curated transcripts from the Celera Genomics Mouse Genome Database (http://www.celera.com, Celera Discovery System), RefSeq transcripts that have been structurally curated from the LocusLink public database (http://ncbi.nlm.nih.bov/LocusLink/refseq.html), high-quality cDNA sequences from the Mammalian Gene Collection (MGC) (http://mgc.nci.nih.gov) and transcripts that were experimentally validated at Applied Biosystems. Array hybridization (three arrays per sample), chemiluminescence detection, image acquisition and analysis were performed using Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer (version 1.1.0) following the manufacturer's protocol. The captured images were auto-gridded and the chemiluminescent signals were quantified corrected for background, and differences in microarray spot intensities were normalized and grouped using Avadis Prophetic 3.3 version (Strand Genomics Ltd. India).

Microarray Data Analysis

Applied Biosystems Expression System software was used to extract assay signal, and assay signal to noise ratio values from the microarray images. Bad spots flagged (>100) by the software were removed from the analysis. To select deferentially expressed genes, the remaining set of genes was further filtered by Standard Expression Array System signal to noise threshold (S/N greater than 3 in at least three samples). The Filtered genes were normalized to Lowess Regression normalization method. Significant genes were calculated using 2-sample local-pooled-error (LPE) ($p<0.05$) and fold change method ($|\text{Fold change}| \geq 2$). Fold changes (IVH vs. IVT, IVT vs. buffer and buffer vs. siDicer1 assay signal ratio) were calculated from each replicated sample's average intensities. To minimize multiple testing problems, we performed Benjamini-Hochberg's FDR (Benjamini and Hochberg, 1995). Functional categories were classified significant genes using panther classification. (https://panther.appliedbiosystems. com)

Statistical Analysis

The general linear models (GLM) procedure in the SAS program (Cary, 1985) was used to analyze the data from all experiments. Significant differences were determined using Tukey's multiple range test (Steel and Torrie, 1980) and $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Effects of *Dicer1* siRNA on Target and Non-Target Genes Expression

As a control experiment, we injected zygotes with *Dicer1* siRNA, *Gapdh* siRNA (ID No. 407972, Ambion) as a positive control, a negative control (Cat. No. 4611G, Ambion) or left them untreated (Non). The mRNA levels were then measured at the blastocyst stage by real time RT-PCR. *Dicer1* mRNA expression in-*Dicer1* siRNA injected group was shown similar decreases ($p<0.001$, Fig. 1B) compared to the *Gapdh* mRNA expression ($p<0.001$, Fig. 1A) following *Gapdh* siRNA injection, which were significantly lower than those in other groups. Additionally, mRNA expression following the injection of negative control (Negative) and non-injected (Non) groups, which showed no reduction in *Gapdh* or *Dicer1* transcript levels.

Seven non-target genes in blastocysts were analyzed by real-time RT-PCR. Three replicates of ten embryos each were assayed from each group and *H2a* was used as an internal standard. After *Dicer1* siRNA microinjected into zygotes, transcription of *Cox5a, Cox5b, Cdc42, Par3, Par6, Rac1* and *Hmgb1* were unchanged at the blastocyst stage (Fig. 1C). These data suggested that *Dicer1* or *Gapdh* can successfully down regulate their target gene expression and did not affect non- target genes. Then, in this study, we used *Gapdh* siRNA as the control.

Effects of *Dicer1* siRNA on Embryo Development

Based on control experiment, *Dicer1* (treatment) or *Gapdh* (control) siRNA was injected into zygote and *in vitro* cultured. Development of zygotes to the cleavage (treatment, 96.3±5.8% vs. control, 98.6±5.6%, Table 2) were no difference between the control and *Dicer1* siRNA injected group. Similarly, *Dicer1* siRNA did not affect development of zygote to the morula (treatment, 91.4±

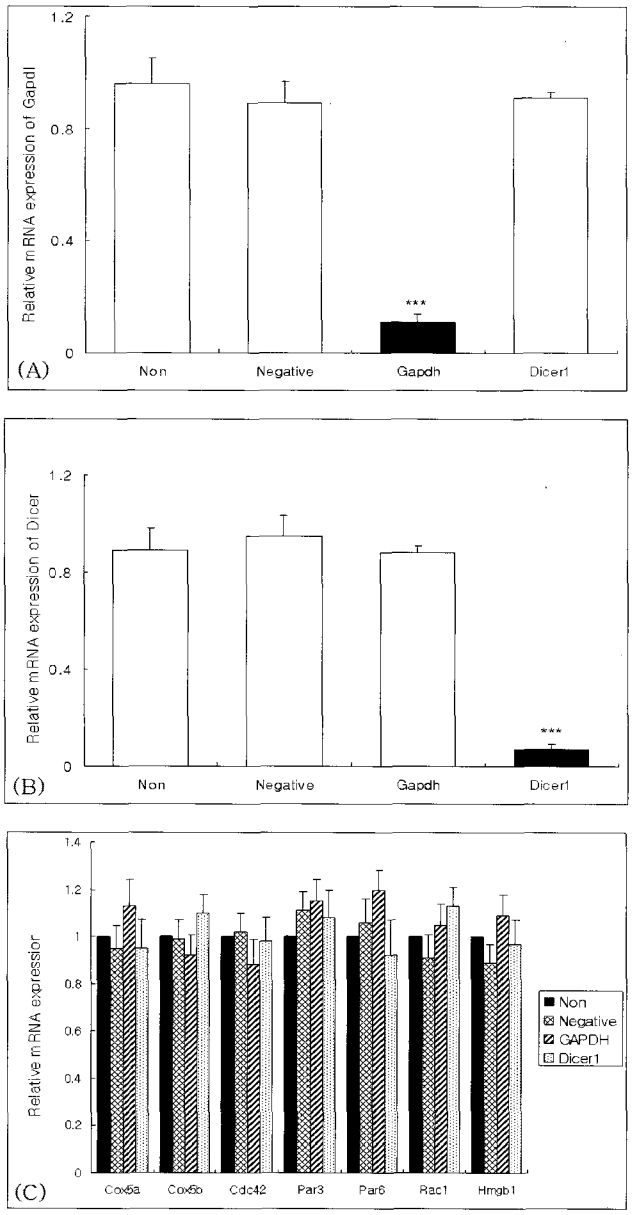

Fig. 1. Relative mRNA levels in blastocyst stage embryos were examined by real-time RT-PCR. Relative mRNA expression of *Gapdh* (A), *Dicer1* (B) or several non-target genes (C) were analyzed from non injected (Non) or negative control (Negative), positive control (*Gapdh*, *Gapdh* siRNA) and *Dicer1* siRNA (*Dicer1*) injected blastocyst stage embryos. Three replicates of ten embryos were assayed from each group and *H2a* was used as an internal standard. Statistically significant differences are indicated: *** $p<0.001$. Values are the mean ± SEM of three separate experiments.

5.8% vs. control, 92.3±4.5%) and blastocyst (treatment, 89.6±5.1% vs. control, 90.0±4.8%) stages. Data indicated that *Dicer1* siRNA did not influence development of zygote to the blastocyst stage, however, whether *Dicer-1* siRNA affect development of post-implantation in the mouse embryo, it is required more studies.

Microarray Results

Previously, transcription profiling during preimplantation mouse embryo development had been accomplished using a short oligonucleotide microarray (Affymetrix oligonucleotide, Wang *et al.*, 2004; Zeng *et al.*, 2004) or a long oligonucleotide-based microarray (NIA 60-mer oligo microarray, Hamatani *et al.*, 2004; Tanaka and Ko, 2004). With the NIA 60-mer oligo microarray platform, Hamanati *et al.* (2004) obtained a very low (~4%) false positive rate using a stringent statistical test (False Discovery Rate: FDR). Similarly, we have identified differentially expressed genes and analyzed our results by ANOVA with Benjamini-Hochberg's FDR of 0.05 (Benjamini and Hochberg, 1995). In the present study, a gene transcription profile has been identified in mouse *Dicer1* knock down embryos by Applied Biosystems mouse Genome Survey Array, which contains 32,996 60-mer oligonucleotide probes. Because preimplantation mouse embryos provide very little RNA for evaluation, we have linearly amplified extracted RNA for two rounds using RiboAmp HS RNA Amplification Kit (Arcturus, USA).

To identify the transcripts affected by *Dicer1* siRNA, we compared the transcriptomes of the *Gapdh* and *Dicer1* siRNA-treated blastocysts. In total, 397 and 737 of 1,134 genes were up- and down-regulated, respectively, following *Dicer1* siRNA microinjection, compared to the *Gapdh* siRNA-injected control group. Moreover, 52 of these 1,134, including 24 up- and 28 down-regulated genes were identified as transcription factors using the PANTHER classification system.

In summary, we compared gene expression profiles in control and *Dicer1* siRNA-microinjected blastocysts using the Applied Biosystem microarray technology. Our data showed that 397 and 737 out of 16,354 genes were up- and downregulated, respectively, following siRNA microinjection ($p<0.05$), including 52 transcription factors. Identification of genes that are preferentially expressed at particular *Dicer1* knock down embryos provides insights into the complex gene regu-

Table 2. *In vitro* development of mouse embryos after *Dicer1* siRNA microinjection

	Zygote (n)	Embryos develop to (%)			
		2-cell	4-cell	Morula	Blastocyst
Control	305 (6)	98.6±5.6	95.6±5.0	92.3±4.5	90.0±4.8
Dicer1 SiRNA	310 (6)	96.3±5.8	94.3±5.1	91.4±5.8	89.6±5.1

Table 3. Transcription factors up-regulated in the blastocystes following *Dicer1* siRNA microinjection

Gene ID	P Value	Fold (siD./Con)	Gene symbol	Gene name	RefSeq NM	Chr. no.
Low Level Unclassified						
mCG10368.1	0.0000	2.9175506	*Fcho1*	FCH domain only 1	NM_028715.2	8
Basic helix-loop-helix transcription factor						
mCG23627.2	0.0259	2.2892709	*Scx*	Scleraxis	NM_198885.2	15
Homeobox transcription factor						
mCG15669.2	0.0108	2.1522923	*Hoxc4*	Homeo box C4	NM_013553.1	15
mCG121166.1	0.0141	2.6113877	*Evx1*	Even skipped homeotic gene 1 homolog	NM_007966.1	6
Other transcription factor						
mCG19735.1	0.0003	4.477069	*Prkcdbp*	Protein kinase C, delta binding protein	NM_028444.1	7
mCG15194.1	0.0032	2.11786	*Btbd3*	BTB (POZ) domain containing 3	NM_145534.1	2
mCG10299.2	0.0047	2.4748476	*Nfkb2*	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	NM_019408.1	19
mCG5146.3	0.0235	2.3247912	*E2f2;9230110J10*	E2F transcription factor 2;hypothetical protein 9230110J10	NM_183301.1	4
mCG20753.1	0.0276	1.6692067	*1700018B24Rik*	RIKEN cDNA 1700018B24 gene	NM_025493.2	3
mCG3689.2	0.0280	2.871601	*E2f1*	E2F transcription factor 1	NM_007891	2
mCG22347.2	0.0281	1.7896678	*Nfkbia*	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	BC046754.1	12
Transcription cofactor						
mCG13508.2	0.0485	1.7999438	*Cited4*	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	NM_019563.1	4
Zinc finger transcription factor						
mCG21513.2	0.0001	2.747454	*BC026432*	cDNA sequence BC026432	NM_146202.1	7
mCG50868.1	0.0002	2.4186766	*Zfp341*	Zinc finger protein 341	NM_199304.1	2
mCG141840.1	0.0006	2.0872536	*G630024C07Rik*	RIKEN cDNA G630024C07 gene	NM_177362.2	7
mCG123808.1	0.0010	1.9255544	*Zfp100*	Zinc finger protein 100	NM_009541.1	4
mCG1338.1	0.0037	1.9739103	*Hic1*	Hypermethylated in cancer 1	NM_010430.1	11
mCG18709.2	0.0073	1.9422789	*Zfp219*	Zinc finger protein 219	NM_027248.1	14
mCG131388	0.0081	2.0252264	*Zfpm1*	Zinc finger protein, multitype 1	NM_009569.1	8
mCG117906.1	0.0083	3.2354393	*Uncha.*	Mus musculus 10 days embryo whole body cDNA, RIKEN full-length enriched library, clone: 2610008E11 product:unclassifiable	AK011343.1	10
mCG10285.2	0.0224	1.7941225	*Trim8*	Tripartite motif protein 8	NM_053100.1	19
mCG11762.2	0.0278	1.8167628	*Zfpl1*	Zinc finger like protein 1	NM_024231.1	19
mCG4870.1	0.0283	1.7140045	*Zfp296*	Zinc finger protein 296	NM_022409.1	7
mCG123556.1	0.0474	1.8655382	*Klf13*	Kruppel-like factor 13	NM_021366.2	7

Table 4. Transcription factors down-regulated in the blastocystes following *Dicer1* siRNA microinjection

Gene ID	P Value	Fold (siD./Con)	Gene symbol	Gene name	RefSeq NM	Chr. no.
Low Level Unclassified						
mCG113712.1	0.0012	−2.5479426	*Tceb1*	Transcription elongation factor B (SIII), polypeptide 1	NM_026456.2	1
Basal transcription factor						
mCG128240.1	0.0000	−3.226732	*Gtf2e1*	General transcription factor II E, polypeptide 1 (alpha subunit)	NM_028812.1	16
mCG2938.1	0.0004	−2.239989	*Gtf2a2*	General transcription factor II A, 2	NM_199151.1	9
mCG67427.2	0.0004	−2.5778248	*Tcea1*	Transcription elongation factor A (SII) 1	NM_011541.3	1
mCG116162.1	0.0010	−2.3067095	*Gtf2h2*	General transcription factor II H, polypeptide 2	NM_022011.2	13
mCG16405.2	0.0157	−2.5327284	*Mnat1*	Menage a trois 1	NM_008612.1	12
mCG7669.2	0.0374	−2.2328947	*Supt4h2;Supt4h*	Suppressor of Ty 4 homolog 2 (S. cerevisiae); suppressor of Ty 4 homolog (S. cerevisiae)	NM_011509.1	11
mCG113797	0.0447	−4.154462	*Uncha.*	Adult male testis cDNA, RIKEN full-length enriched library, clone: 4932409F03 product:weakly similar to transcription initiation factor TFIID 105 kDa subunit (TAFII-105) (TAFII105) (FRAGMENT) [Homo sapiens]	AK029943.1	18
HMG box transcription factor						
mCG10155.2	0.0062	−2.2472572	*Hmgb3*	High mobility group box 3	NM_008253.2	X
mCG15247.1	0.0257	−4.136312	*Sox2*	SRY-box containing gene 2	NM_011443.2	3
Other transcription factor						
mCG114122.1	0.0002	−2.8695939	*Cops5*	COP9 (constitutive photomorphogenic) homolog, subunit 5 (Arabidopsis thaliana)	NM_013715.1	1
mCG19061.1	0.0005	−2.7664034	*4732497O03Rik*	RIKEN cDNA 4732497O03 gene	NM_144826.2	11
mCG5634.2	0.0404	−2.143738	*Sin3b*	Transcriptional regulator, SIN3B (yeast)	NM_009188.1	8
mCG19145.2	0.0487	−1.649655	*Smad2*	MAD homolog 2 (Drosophila)	NM_010754.2	18
Transcription cofactor						
mCG7934.1	0.0000	−2.9634058	*Med6*	Mediator of RNA polymerase II transcription, subunit 6 homolog (yeast)	NM_027213.2	12
mCG12679.1	0.0017	−2.0407076	*Crsp9*	Cofactor required for Sp1 transcriptional activation, subunit 9	NM_025426.2	11
mCG13098.1	0.0028	−5.192982	*Uncha.*	Hypothetical protein	AK037666.1	3
mCG20952.2	0.0368	−1.622274	*Uncha.*	Thyroid hormone receptor interactor 13,	AK010336.1	13
Zinc finger transcription factor						
mCG1291.2	0.0000	−5.148244	*Rnf128*	Ring finger protein 128	NM_023270.3	X
mCG20802.2	0.0000	−3.7698817	*Rnf130*	Ring finger protein 130	NM_021540.2	11
mCG121593.1	0.0000	−2.9277542	*Znrf2*	Zinc finger/RING finger 2	NM_199143.1	6
mCG17256.2	0.0002	−2.533838	*2700038I16Rik*	RIKEN cDNA 2700038I16 gene	NM_028298.2	5
mCG8439.2	0.0004	−3.2375152	*Uncha.*	Adult female placenta cDNA, RIKEN full-length enriched library, clone:1600023E10 product:unclassifiable, full insert sequence	AK005523.1	1
mCG140432.1	0.0183	−2.0326452	*Uncha.*	Hypothetical protein	AK049289.1	7
mCG125469.2	0.0325	−2.3587568	*Zfp472*	Zinc finger protein 472	NM_153063.1	17
mCG127287.1	0.0351	−3.8045337	*Uncha.*	Jumonji domain containing 1A, transcript variant 1	BC059264.1	6
mCG134261	0.0444	−2.0924206	*Uncha.*	cDNA clone IMAGE:6811166, partial cds	BC064812.1	10
mCG123156.2	0.0477	−2.55405	*Rnf138*	Ring finger protein 138	NM_207623.1	18

latory networks that drive differentiation processes in embryos at blastocyst stage.

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