Dynamism in the Gene Expression Profile after Oocyte Activation in Mice

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Abstract: *Maternal transcripts are indispensable and play an essential role in regulation of the resumption of meiosis, nuclear reprogramming and subsequent development. In this study, we conducted interactive subtraction analysis to investigate the dynamic changes in the gene expression profiles of mouse oocytes after artificial activation. To accomplish this, we compared the cDNA libraries of freshly ovulated metaphase II (MII) oocytes and pronuclear-stage eggs at 8 h after activation, which were obtained by interactive subtraction. After constructing the cDNA libraries, we sequenced 635 subtracted clones of differentially expressed genes and the identities of 515 of these genes were determined by online BLAST analysis. The analysis revealed that genes down-regulated after activation were mainly responsible for enzyme and receptor activities, and also for DNA, chromatin, ATP, protein, and ion binding. On the other hand, genes up-regulated after activation were mainly responsible for enzyme activities, ion binding, and the maintenance of cell structure. For further insight into the functions of the genes, gene network analysis was performed, and in order to validate differential gene expression, the gene expression levels were determined by using real-time quantitative PCR. The present analysis was capable of identifying the genes that changed dynamically following the resumption of meiosis, and these genes may control the developmental programs in oocytes and preimplantation embryos.*

Key words: *Oocytes, Activation, Gene expression profile, cDNA subtraction, Mouse*

Introduction

Mouse oocytes, which are ovulated at the metaphase II (MII) stage, resume meiosis at fertilization. Meiotic resumption is triggered by repeated Ca^{2+} oscillations that are generated by sperm derived phospholipase C (PLC) ζ, following which the fertilized eggs start to develop [1]. The oocytes also deposit various maternal genome-derived transcriptional products in their bulky cytoplasm during the growth period. These maternal transcripts are indispensable and also play an essential role in the regulation of meiotic resumption, nuclear reprogramming and the subsequent development. Once an oocyte is activated and starts developing, the maternal transcripts are selectively degraded [2–4]. Meanwhile, the synthesis of new embryonic genome-derived transcripts is initiated several hours after oocyte activation [5–8]. The temporal-spatio-specific dynamism involved in the synthesis of these transcriptional products, which is precisely regulated during development in the preimplantation stage, possibly plays an important role in postimplantation development.

To obtain an insight into the temporal-spatio-specific dynamism involved in the synthesis of the transcriptional products by oocytes and embryos, numerous comprehensive gene expression analyses have been conducted using mRNA differential display [5–8], cDNA microarray [8–10], and analysis of expressed sequence tags (EST) [7, 11]. From the large cohort of maternal transcripts identified by the global gene expression analysis, several important genes that are critical for the progress of the chain of events following meiotic resumption and early development have been identified for example, cellular Moloney murine sarcoma sequence (*c-mos*) [12–15] and tissue plasminogen activator (*Plat*) [16–19] that regulate meiotic resumption and the NLR family, the pyrin domain containing 5 (*Nlrp5*) [20–23], and zygote arrest 1 (*Zar1*) [24] genes that are required for development beyond the 2-cell stage. Although, considerable

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information has been accumulated regarding gene expression in very early stage embryos, many factors remain to be explained. We need to gather additional transcriptome information in order to understand the complete mechanism underlying the transition from maternal to embryonic regulation of development, which has long-term effects on full-term development. Furthermore, as the next step in studying the molecular mechanisms underlying the early development, it is important to select a smaller number of genes that are candidates for important roles in development.

Accordingly, in this study, we carried out subtraction analysis using mouse eggs at the following 2 stages: freshly ovulated MII oocytes and parthenogenetic eggs at 8 h after artificial activation. Here, we used artificially activated 1-cell embryos in order to eliminate transcripts derived from paternal genomes. In order to validate the dynamic changes in gene expression, we further assessed their gene expression levels quantitatively by using the real-time polymerase chain reaction (PCR). The present analysis was capable of identifying the gene expressions which were dynamically changed following meiotic resumption, and these genes may govern the developmental programs in oocytes and preimplantation embryos in mice.

Materials and Methods

Animals

For subtraction analysis, adult female B6C3F1 (C57BL/6NCrj × C3H/HeNJcl) mice were obtained from Clea Japan Inc. (Tokyo, Japan). For gene expression analysis, adult female CD-1 mice were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). During the course of the experiments, the mice were provided with food and water *ad libitum*, and maintained under controlled conditions of temperature (23°C ± 2°C), humidity (40%–60%) and a 12 h light and 12 h dark cycle. All the mice were maintained and used in accordance with the guidelines for the care and use of laboratory animals, as specified by the Japanese Association for Laboratory Animal Science and Tokyo University of Agriculture.

Oocyte and embryo collection

Ovulated MII oocytes were collected from mature B6C3F1 female mice after superovulation induced by consecutive injections of 5 IU equine chorionic gonadotrophin (eCG) and 5 IU human chorionic gonadotrophin (hCG) which were administered 48 h apart. Cumulus cells were removed by treatment with hyaluronidase (300 IU/ml) in M2 medium. For the production of diploid pronuclear stage eggs by artificial activation, the oocytes were cultured for 4 h in a drop of Ca^{2+}-free M16 medium containing 10 mM $SrCl_2$ (Sigma-Aldrich, MO) that was added to induce repeated intracytoplasmic Ca^{2+} oscillations and oocyte activation, and 5 μg/ml cytochalasin B (Sigma-Aldrich) that was added to induce diploidy by the inhibition of second polar body extrusion. After the first 4 h of culture in the M16 medium, only those activated eggs that formed 2 female pronuclei without polar body extrusion were selected and cultured further in potassium simplex-optimised medium (KSOM) [25] at 37°C for 4 h. The MII oocytes and activated eggs were then collected in a lysis buffer at 15–16 h and 24–25 h post-hCG injection, respectively. All *in vitro* cultures were performed under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

Total RNA preparation and cDNA subtraction analysis

Total RNA was isolated using an RNeasy® Mini Kit (Qiagen. K.K. Tokyo, Japan) from each of the approximately 3,000 MII oocytes and 1-cell embryos. The samples were immediately lysed in 350 μl of buffer, RNeasy lysis thioyanate (RLT), and homogenised by vortexing. After adding 1 volume of 70% ethanol, each lysate was applied to a RNeasy Mini spin column. The total RNA bound to the membrane was eluted in RNase-free water. Using this RNA, cDNA was synthesized and then amplified using the switch mechanism at the 5' end of RNA transcripts with (SMART™) PCR cDNA Synthesis Kit (Clontech/ Takara bio Inc., Shiga, Japan), according to the manufacturer's instructions. cDNA subtraction was performed using a PCR-select™ cDNA Subtraction Kit (Clontech) according to the manufacturer's instructions. The subtracted PCR products were subcloned into pGEM®-T Easy vector (Promega Co., WI) for screening. Next, the subtracted clones were differentially screened using PCR-Select Differential Screening Kit (Clontech). The inserted sequences of the subtracted clones were amplified by PCR using forward (TCGAGCGGCCGCCCGGGCAGGT) and reverse (AGCGTGGTCGCGGCCGAGGT) polylinker primers, and the PCR products were immobilized on nylon membranes in triplicate.

Hybridization probes were prepared by labeling the forward- and reverse-subtracted cDNA (20–90 μg) and forward- and reverse-unsubtracted cDNA (50–100 μg) libraries with digoxigenin (DIG; Roche Molecular Biochemicals, Basel, Switzerland). Subsequently, the PCR products immobilized on the nylon membranes were hybridized with 100 ng of each probe at 72°C for 60

min; the probes were prepared in ExpressHyb Hybridization Solution (Clontech). After rinsing, the hybridization signals of the membrane were read using a FujiFilm LAS-1000 Plus camera (Fujifilm Co., Tokyo, Japan), and the signal intensity was computed using FujiFilm ArrayGauge software (Fujifilm). The cDNA clones that hybridized with the forward-subtracted and forward-unsubtracted probes but not to the reverse-subtracted and reverse-unsubtracted probes were determined as specific to the MII oocytes and activated eggs. The results of the differential screening revealed that certain cDNA clones demonstrated a 2-fold increase in gene expression in the MII oocytes compared with the activated eggs. These genes were classified as MII oocyte-specific genes. The cDNA clones from the MII oocytes that exhibited respective signal intensities 2-fold weaker than those of the activated eggs were classified as activated egg-specific genes.

DNA sequencing analysis and BLAST search

DNA sequencing was performed using an automated ABI PRISM3130 Genetic Analyzer (ABI, CA). Sequencing reactions were carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI) using forward and reverse primers. The sequences obtained were compared against those in the Mouse Genome Informatics (MGI; http://www.informatics.jax.org/) database by using the online basic local alignment search tool (BLAST) analysis (www.ncbi.nlm.niho.gov/BLAST).

Quantitative gene expression analysis

cDNA was synthesized from ovulated MII oocytes and artificially activated eggs by using a Cells-to-cDNA™ II kit (Ambion, Inc., TX). Single blastocysts were placed in 13 μl of the lysis buffer and then treated with DNase. The cDNAs were synthesized in 20 μl of reaction solution by using a SuperScript RNase H-reverse transcriptase kit (Invitrogen, Co., CA). Further, this synthesized cDNA was employed for the quantitative analysis of gene expression using RT-PCR, which was carried out using a ready-to-use reaction mixture kit (LightCycler™ FirstStart DNA Master SYBR Green I; Roche Molecular Biochemicals). The primer sequences used for the PCR reaction, PCR conditions, and product sizes are listed in Table 1. The glyceraldehydes-3-phosphate dehydrogenase (*Gapdh*) gene was used as the loading control. The amplification protocol was as follows: DNA polymerase activation at 95°C for 10 min and 45 cycles of amplification with denaturation at 95°C for 15 s, annealing for 10 s at the optimum temperature for each gene (see Table 1), and extension at 72°C for 10 s. On completion of these amplification cycles, a melting curve analysis was performed to verify specific amplification. The relative expression levels of each gene tested were obtained from the standard curve that was generated using a pooled cDNA mixture extracted from E12.5 foetuses.

Statistical analysis

The gene expression levels were statistically analyzed using Student's *t*-test. Differences were considered significant at $p<0.05$ and greatly significant at $p<0.01$. In order to elucidate the functional network of the genes that were differentially expressed in the MII oocytes and those that were differentially expressed in the activated eggs at 8 h after oocyte activation. The gene lists were integrated into the Ingenuity Pathway Analysis (IPA) database (Ingenuity, CA).

Results and Discussion

Identification of differentially expressed genes

To identify genes that were differentially expressed following oocyte activation, we compared the cDNA libraries of freshly ovulated MII oocytes and activated eggs, obtained by interactive subtraction technology (Table 2). Total RNA was isolated from each of the approximately 3,000 MII oocytes and the pronuclear stage eggs at 8 h after activation, and the cDNA libraries were established for interactive subtraction analysis. cDNA subtraction was performed using the PCR-Select Subtraction Kit (Clontech), which combines the normalization and equalization of cDNA with the subtraction process. From the subtracted cDNA libraries, 2,304 cDNA clones were subcloned, and the forward and reverse sequences were subjected to probe hybridization in order to identify the differentially expressed genes (Table 2). We sequenced 635 subtracted clones that demonstrated approximately 2-fold or greater difference in the intensity of the hybridization signals between the subtracted forward and the reverse probes, and the identities of 515 of these genes were determined by online BLAST analysis. This set comprised 217 known genes (121 and 96 genes were strongly expressed in MII oocytes and activated eggs, respectively) and 181 novel or uncharacterized genes (29 and 152 genes strongly expressed in MII oocytes and activated eggs, respectively). The number of known genes represents the total number of genes remaining after the overlapping ones were excluded. The differentially expressed known genes, which were obtained by hybridization analysis

Table 1. Primers and conditions used for real-time PCR

Gene	Accession No.	Primer sequence (5'–3')	Mg2+ conc. (mM)	Annealing Tm (°C)	Amplified length (bp)
Lbr	AY148159	TTGGCCGAGAGTTAAACCCGCGACTTG CCAGAGGGCGTCCACCACATATAAGGG	3	62	200
Gdpd1	BC016541	TGCATGGAGTCACATCGGAA GTGACAATGCTGTCATGCCT	4	62	323
Ints9	BC055700	ATTGGGCAGTAGATGCACTT TTACCTCCCAGAACCACCTT	4	65	27
Il1rap	BC021159	CGGTGCATCCATTACCTTTC GAACTGGTTATTCCCTGCAA	4	60	286
Xpr	AK033421	GAGCCAGTTTGGTCTTTCTCC CCAGAGCATCTGAATACACGTT	3	60	185
Gdf9	BC052667	CCTTAGAGTGCCTGGGCAGAGA CTTGGTTTATGGCAACGACCAGTGAG	3	63	158
Lccr17	BC030317	AGTCTATGTGTTGCCTGACT TCTGTCAGCACCTTGATCTG	4	60	222
Slc7a1	AY766236	CAACACAGAGCTTAGCAAGT ACATTCTTCTGCAAGCATCA	5	60	144
Ggta1	BC006810	AGATAATGAAGCCAAAGGGA TGAGCCTGTAATATGTGAGA	4	60	256
Dkc1	BC099966	CTAGGGCCCTAGAAACTCTG GTACGCATAGTGTCCGAATG	4	60	186
Bub1b	BC031577	CTCTGCAGAGCTTCTTGGTG TGACTAATGACGGGGCAGAA	3	60	245
Slc23a2	BC050823	AACAAAGCTTACTAGTGGTT AAGTATTTGTGTGGTCAAGG	5	60	216
Dcp1a	BC064319	TAGGTGTGCCACGAGGACACAG ATGCCATTGTAACCCTGATAATGCAACA	3	62	184
Plat	BC011256	CTGAGTGCATCAACTGGAATAGCAGTGT TTTTCCCTTAGGGCAAGCTGGTGT	3	60	200
Spry4	BC057005	CTCCCAGGCTTCTTAGAAAGAG AAAGATAGAAGACAAACCCCTGC	4	55	175
Kpna2	U34229	ATGATGCTACTTCTCCGCTACAG GACTACAATCAGTTTTGCCCAAG	5	60	231
Exoc1	BC087543	GCAAGTCTCTATGAAATGGATGG CTTCAGTGACCTGAGGGAACAT	3	55	152
Gapdh	–	GTCGTGGAGTCTACTGGTGTC GAGCCCTTCCACAATGCCAAA	2	61	240

with forward subtracted and unsubtracted probes and with reverse subtracted and unsubtracted probes, were classified into 3 groups: Group A genes were detected by both subtracted and unsubtracted probes; Group B genes were detected by only subtracted probes; Group C genes were detected by only unsubtracted probes.

To understand the functions of the genes with dynamically changed expressions after oocyte activation, we performed a cluster analysis for Group A genes (21 and 27 genes from the MII oocytes [Table 3] and activated eggs [Table 4], respectively) by using the MGI database (http://www.informatics.jax.org/). The results revealed that the genes down-regulated after activation were mainly responsible for enzyme and receptor activities and also for DNA, chromatin, ATP, protein, and ion binding (Table 3). On the other hand,

Table 2. Summary of subtraction analysis using MII oocytes and activated eggs

Stage	Number of cDNA clones			Number of genes (Known gene)[1]		
	Analyzed	Differentially expressed	Sequenced and BLAST searched	Class A	Class B	Class C
MII oocytes	1,152	247	231	28 (21)	47 (38)	75 (62)
activated eggs	1,152	388	284	125 (27)	60 (38)	63 (31)
Total	2,304	635	515	153 (48)	107 (76)	138 (93)

[1]The numbers of the known genes do not contain overlaps.

Table 3. Genes specifically expressed in MII oocytes

Accession No.	Symbol name	Gene name	Gene ontology (GO) function
BC011256	*Plat*	plasminogen activator, tissue	hydrolase activity, peptidase activity
BC094587	*Glce*	glucuronyl C5-epimerase	isomerase activity, racemase and epimerase activity, acting on carbohydrates and derivatives
BC031577	*Bub1b*	budding uninhibited by benzimidazoles 1 homolog, beta (S. cerevisiae)	ATP binding, kinase activity
X60672	*Rdx*	radixin	actin binding, binding
BC016541	*Gdpd1*	glycerophosphodiester phosphodiesterase domain containing 1	glycerophosphodiester phosphodiesterase activity, hydrolase activity
AY148159	*Lbr*	lamin B receptor	DNA binding, nucleic acid binding
AY007195	*H1foo*	H1 histone family, member O, oocyte-specific	DNA binding
BC021159	*Il1rap*	interleukin 1 receptor accessory protein	interleukin-1 receptor activity, protein binding
BC052667	*Gdf9*	growth differentiation factor 9	cytokine activity, growth factor activity
AK033037	*Astl*	astacin-like metalloendopeptidase	astacin activity, hydrolase activity
BC055700	*Ints9*	integrator complex subunit 9	unknown
AK033421	*Xpr1*	xenotropic and polytropic retrovirus receptor	receptor activity
AB021132	*Rapgef4*	Rap guanine nucleotide exchange factor (GEF) 4	cAMP binding, cAMP-dependent protein kinase regulator activity
BC050823	*Slc23a2*	solute carrier family 23 (nucleobase transporters), member 2	L-ascorbate:sodium symporter activity, sodium ion binding
AY766236	*Slc7a11*	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	amino acid transmembrane transporter activity
BC030317	*Lrrc17*	leucine rich repeat containing 17	protein binding
AY280964.	*Kcnn2*	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	calcium-activated potassium channel activity, calmodulin binding
BC064319	*Dcp1a*	decapping enzyme	hydrolase activity, transcription factor binding
BX908741	*Depdc7*	DEP domain containing 7	unknown
BC099966	*Dkc1*	dyskeratosis congenita 1, dyskerin homolog (human)	isomerase activity, pseudouridylate synthase activity
BC056440	*Asb4*	ankyrin repeat and SOCS box-containing protein 4	unknown

the genes that were up-regulated after activation were mainly responsible for enzyme activities, ion binding, and the maintenance of cell structure (Table 4).

From the subtraction analysis we established 2 cDNA libraries of genes specifically expressed in the MII oocytes and activated eggs. Since we used

Table 4. Genes up-regulated after oocyte activation

Accession No.	Symbol name	Gene name	Gene ontology (GO) function
AF033115	*Siva1*	SIVA1, apoptosis-inducing factor	CD27 receptor binding, metal ion binding
AJ250192	*unknown*	Mus musculus partial mRNA for muscle protein684	Unknown
AJ132889	*Kif9*	kinesin family member 9	ATP binding, microtubule motor activity
BC015259	*Eps15l1*	epidermal growth factor receptor pathway substrate 15-like 1	calcium ion binding, protein binding
AK153747	*mt-Nd4*	NADH dehydrogenase 4, mitochondrial	NADH dehydrogenase (ubiquinone) activity, oxidoreductase activity
BC059028	*Asxl1*	additional sex combs like 1 (Drosophila)	metal ion binding, protein binding
BC051943	*Elp2*	elongation protein 2 homolog (S. cerevisiae)	protein binding, protein kinase binding
BC014287	*Pigq*	phosphatidylinositol glycan, class Q	phosphatidylinositol N-acetylglucosaminyl-transferase activity, transferase activity
CT010241	*Nono*	non-POU-domain-containing, octamer binding protein	DNA binding, nucleic acid binding
BC057005	*Spry4*	sprouty homolog 4 (Drosophila)	protein binding
BC080732	*Tox*	thymocyte selection-associated HMG box gene	DNA binding
AL669931	*Stc2*	stanniocalcin 2	hormone activity
BC087543	*Exoc1*	exocyst complex component 1	unknown
BC085314	*Copz1*	coatomer protein complex, subunit zeta 1	protein binding, protein transporter activity
AL772227	*Atp10b*	ATPase, class V, type 10B	unknown
U34229	*Kpna2*	karyopherin (importin) alpha 2	binding, protein binding
U96760	*Vbp1*	von Hippel-Lindau binding protein 1	protein binding, unfolded protein binding
BC094904	*Clec12a*	C-type lectin domain family 12, member a	protein phosphatase binding, sugar binding
AY363102	*Fhit*	fragile histidine triad gene	bis (5'-adenosyl)-triphosphatase activity, catalytic activity
BX293563	*Echdc2*	enoyl Coenzyme A hydratase domain containing 2	catalytic activity, lyase activity
AL845502	*Cdk5rap2*	CDK5 regulatory subunit associated protein 2	unknown
AL606908	*Ncdn*	neurochondrin	unknown
AL627349	*Dock7*	dedicator of cytokinesis 7	GTP binding, GTPase binding
BC056487	*Hexdc*	hexosaminidase (glycosyl hydrolase family 20, catalytic domain) containing	hydrolase activity
AL844204	*Mal*	myelin and lymphocyte protein, T-cell differentiation protein	unknown
AC136515	*Dock1*	dedicator of cytokinesis 1	guanyl-nucleotide exchange factor activity, protein binding.
AL732450	*Phka2*	phosphorylase kinase alpha 2	calmodulin binding, kinase activity

parthenogenetically activated eggs, all cDNA clones represented maternal genome-derived transcripts. The cDNA library for MII oocytes comprised well-known genes that are expressed at high levels in MII oocytes: linker histone (*H1oo*) [4, 26–29], growth differentiation factor 9 (*Gdf9*) [4, 29], and *Plat* [4, 9]. Similarly, the cDNA library for activated eggs comprised genes that are known to be activated after fertilization: sprouty 4 (*Spry4*) [9, 29, 30] and karyopherin-alpha2 (*Kpna2*) [10]. We believe that the present interactive subtraction analysis successfully detected a substantial number of genes that are differentially expressed at the above-mentioned stages.

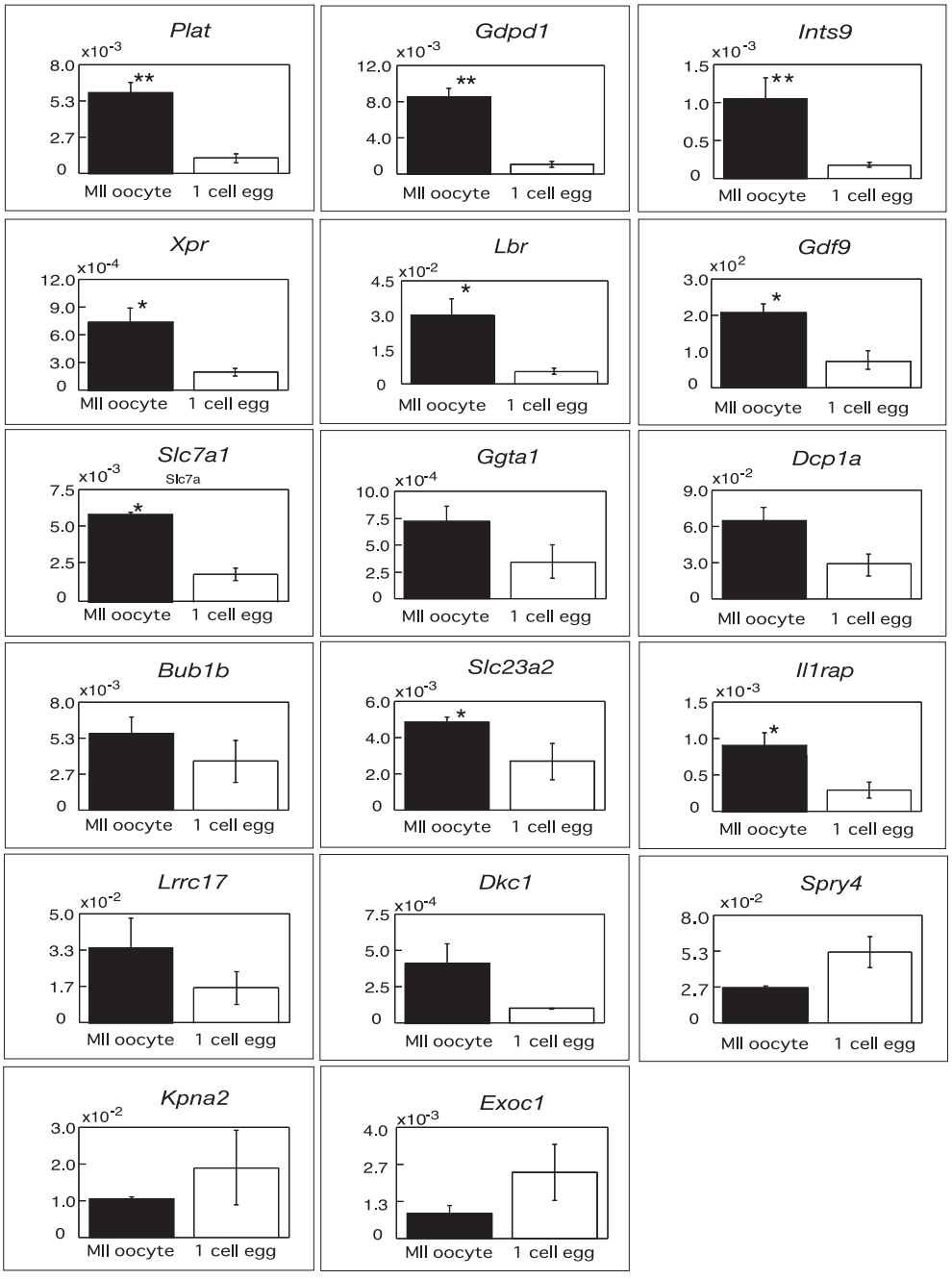


Fig. 1. Quantitative analysis of the gene expression in MII oocytes and activated eggs. The genes were selected from the list of genes differentially expressed in MII oocytes and activated eggs, as determined by cDNA subtraction analysis. The relative mRNA expression levels were calculated by determining the intensity of gene expression using *Gapdh* as internal control. The ordinate shows the mean of the relatively expressed levels. The values denote the mean ± S.E. obtained from 3 independent experiments. Asterisks denote significant difference between the oocytes and activated eggs (*p<0.05, **p<0.01).

Validation of subtraction data by Q-PCR

We validated our results of gene expression levels by using real-time quantitative PCR. From Group A, we examined 14 and 3 genes that were identified as having been specifically expressed in the MII oocytes and activated eggs, respectively (Fig. 1). The quantitative

PCR analysis revealed that out of the 14 genes tested, 3 MII oocytes-specific genes, including *Plat*, glycerophohodiester phosphodiesterase domaincontaining 1 (*Gdpd1*) and integrator complex subunit 9 (*Ints9*), were expressed at levels 5-fold higher than in the activated eggs. The differences in the expression levels were greatly significant at $p<0.01$ levels. Moreover, 6 genes, lamin B-receptor (*Lbr*), interleukin 1 receptor accessory protein (*Il1rap*), xenotropic and polytropic retrovirus receptor 1 (*Xpr1*), Gdf9, solute carrier family 7 member 1 (*Slc7a1*) and solute carrier family 23 member 2 (*Slc23a2*), were expressed at significantly ($p<0.05$) higher levels in the activated eggs. In contrast, 3 genes whose expressions were elevated after oocyte activation, including sprouty homolog 4 (*Spry4*), karyopherin (importin) alpha 2 (*Kpna2*) and exocyst complex component 1 (*Exoc1*) were expressed at levels of 2-fold or more in the activated eggs, however, significant differences were not seen. *Gapdh* gene, which was tested as the internal control, demonstrated almost equal expression levels in both the MII oocytes and activated eggs.

The present data is representative of genes expressed specifically in the MII oocytes and activated eggs, because the results of real-time PCR correlated well with those of the subtraction analysis. Accordingly, we believe that the present interactive subtraction analysis successfully detected a substantial number of differentially expressed genes, and identified a small number of candidate genes that may significantly contribute to future studies into the molecular mechanisms in oocytes.

Networks

In order to obtain further insight into the function of the genes classified into Group A, gene networks were constructed using the IPA database. The first 5 functional networks in each case are shown in Fig. 2a, b and Tables 5 and 6.

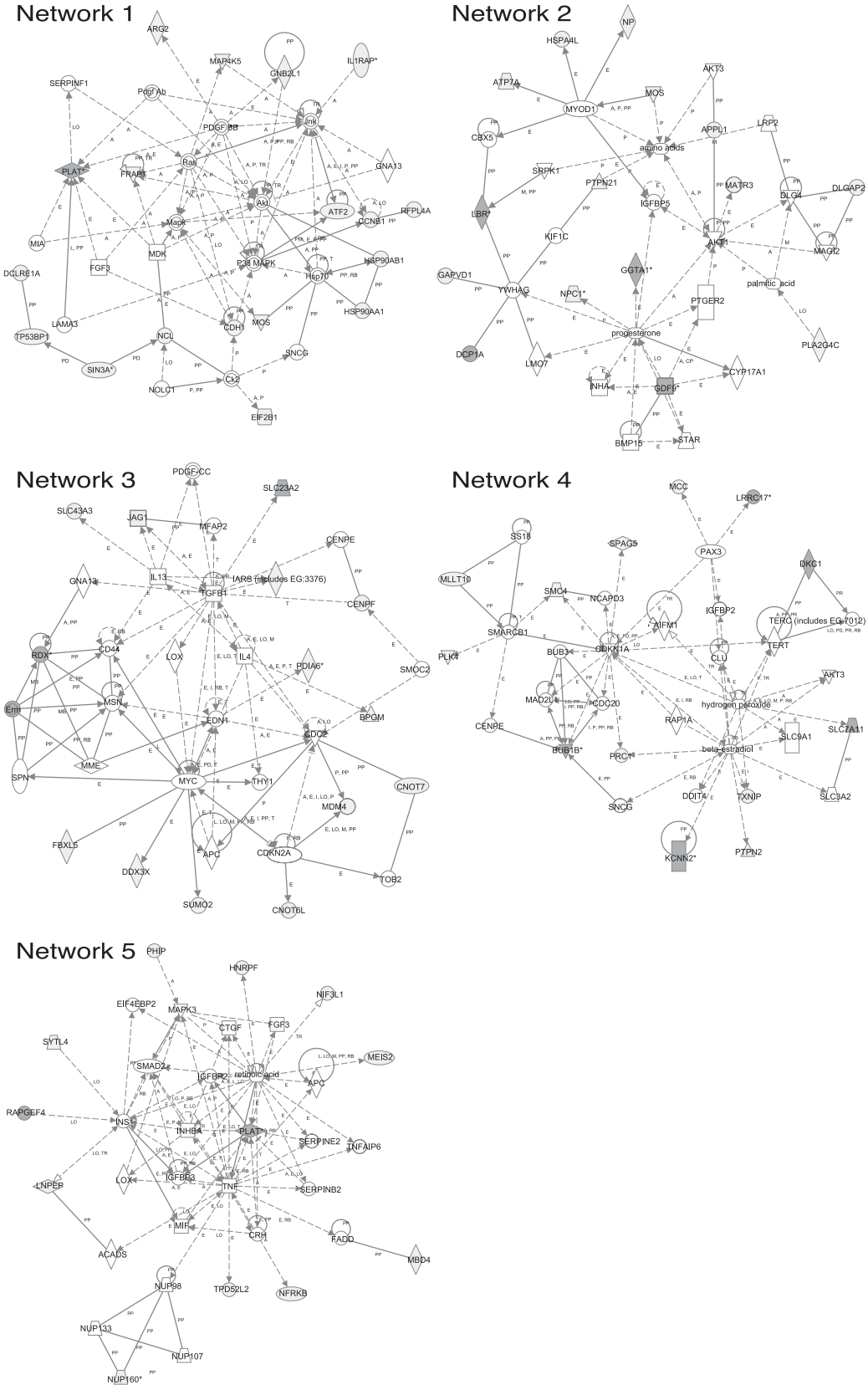
Gene networks constructed using genes expressed specifically in the MII oocytes were as follows (Fig. 2a, Table 5). Network 1 (*Plat*): cellular movement, cardiovascular system development and function, and organismal development. Network 2 (*Gdf9*): DNA replication, DNA recombination, and DNA repair, cell cycle regulation, and cellular assembly and organization. Network 3 (solute carriers family 23 member 1 [*Slc23a*]): lipid metabolism, small molecule biochemistry, and cell death. Network 4 (*Slc7a1*, dyskeratosis congenital 1 [*Dkc1*]): DNA replication, DNA recombination, DNA repair; cell cycle regulation, and connective tissue development and function. Network 5 (*Plat*): cellular assembly and organization, anticancer effect, and cell cycle regulation.

The networks constructed using genes with rapidly enhanced expressions after oocyte activation were as follows (Fig. 2b, Table 6). Network 1 (*Kpna2*): cellular movement, cardiovascular system development and function, and organismal development. Network 2 (*Exoc1*): DNA replication, DNA recombination, DNA repair, cell cycle regulation, and cellular assembly and organization. Network 3 (additional sexcombs-like 1 protein [*Asxl1*], non-POU-domain-containing, octomer binding protein [*Nono*]): lipid metabolism, small molecule biochemistry, and cell death. Network 4 (aldo-keto reductase family 1, member B1 [*Akrb1*]): DNA replication, DNA recombination, DNA repair, cell cycle regulation, and connective tissue development and function. Network 5 (epidermal growth factor receptor pathway substrate 15-like 1 [*Eps15l1*], stanniocalcin 2 [*Sct2*]): cellular assembly and organization, anticancer effect, and cell cycle regulation.

Genes expressed specifically in the MII oocytes were primarily involved in the cell cycle and cancer. Furthermore, the networks generated from these genes also suggest that the genes highly expressed in MII oocytes are involved in the cell cycle and are responsible for cell proliferation. In addition to the cell cycle and cancer, the genes with significantly enhanced expressions after activation were involved in cell proliferation and cell adhesion. This result is fairly understandable because it is expected that the genes responsible for the cell cycle and development would be activated following the resumption of meiosis and subsequent development.

Although cDNA microarray analysis is a robust technique for the identification of genes that are specifically expressed in particular cells and tissues, it is complementary to subtractive hybridization analysis [31]. Recently, a microarray study presented several novel findings regarding gene expression profiles in early-stage embryos [32]. For example, fertilization leads to extensive changes in the transcript profiles, which are much greater than previously recognized, and genome activation during the 2-cell stage may be selective on a qualitative scale with genes involved in transcription and RNA processing. Cui *et al.* (2007) [8] reported a comparison of gene expression profiles of germinal vesicle (GV)- and MII-stage oocytes. Their results indicate that the genes specifically up-regulated in MII oocytes are more likely to be involved in DNA replication, amino acid metabolism, and the expression

a


Network 1
ARG2
MAP4K5
GNB2L1
IL1RAP*
SERPINF1
Pdgf Ab
PDGF BB
Jnk
Rac
GNA13
PLAT*
FRAP1
Abl
ATF2
CCNB1
RPPL4A
Mapk
MIA
MDK
FGF3
P38 MAPK
HSP90AB1
Hsp70
DDR1
LAMA3
CDH1
MOS
HSP90AA1
TP53BP1
NCL
SNCG
SIN3A*
Ck2
NOLC1
EIF2B1
Network 2
NP
HSPA4L
AKT3
ATP7A
MOS
MYOD1
CBX5
APPL1
LRP2
amino acids
SRPK1
PTPN21
MATR3
DLG4
DLGAP2
IGFBP5
LBR*
KIF1C
AKT1
MAGI2
GAPVD1
GGTA1*
palmitic acid
YWHAG
NPC1*
PTGER2
progesterone
PLA2G4C
DCP1A
LMO7
CYP17A1
NHA
GDF9*
BMP15
STAR
Network 3
PDGF-CC
SLC23A2
SLC43A3
JAG1
MFAP2
CENPE
GNA13
IL13
IARS (includes EG:3376)
TGFB1
CENPF
RDX*
CD44
LOX
IL4
PDIA6*
SMOC2
Erm
MSN
EDN1
BPGM
CDC2
SPN
MME
MYC
THY1
CNOT7
MDM4
FBXL5
APC
CDKN2A
DDX3X
TOB2
SUMO2
CNOT6L
Network 4
MCC
LRRC17*
SS18
SPAG5
PAX3
DKC1
MLLT10
SMC4
NCAPD3
AIFM1
IGFBP2
TERC (includes EG:7012)
SMARCB1
CDKN1A
TERT
PLK4
BUB3
CLU
AKT3
MAD2L1
PCDC20
hydrogen peroxide
CENPE
RAP1A
SLC9A11
SLC7A11
BUB1B*
PRC1
beta-estradiol
DDIT4
TXNIP
SLC3A2
SNCG
KCNN2*
PTPN2
Network 5
PHIP
HNRPF
NIF3L1
EIF4EBP2
MAPK3
CTGF
FGF3
SYTL4
MEIS2
SMAD2
IGFBP2
retinoic acid
APC
RAPGEF4
PNS1
INHBA
PLAT*
SERPINE2
TNFAIP6
LNPEP
LOX
IGFBP3
TNF
SERPINB2
MIF
CRH
FADD
ACADS
MBD4
NUP98
TPD52L2
NFRKB
NUP133
NUP107
NUP160*

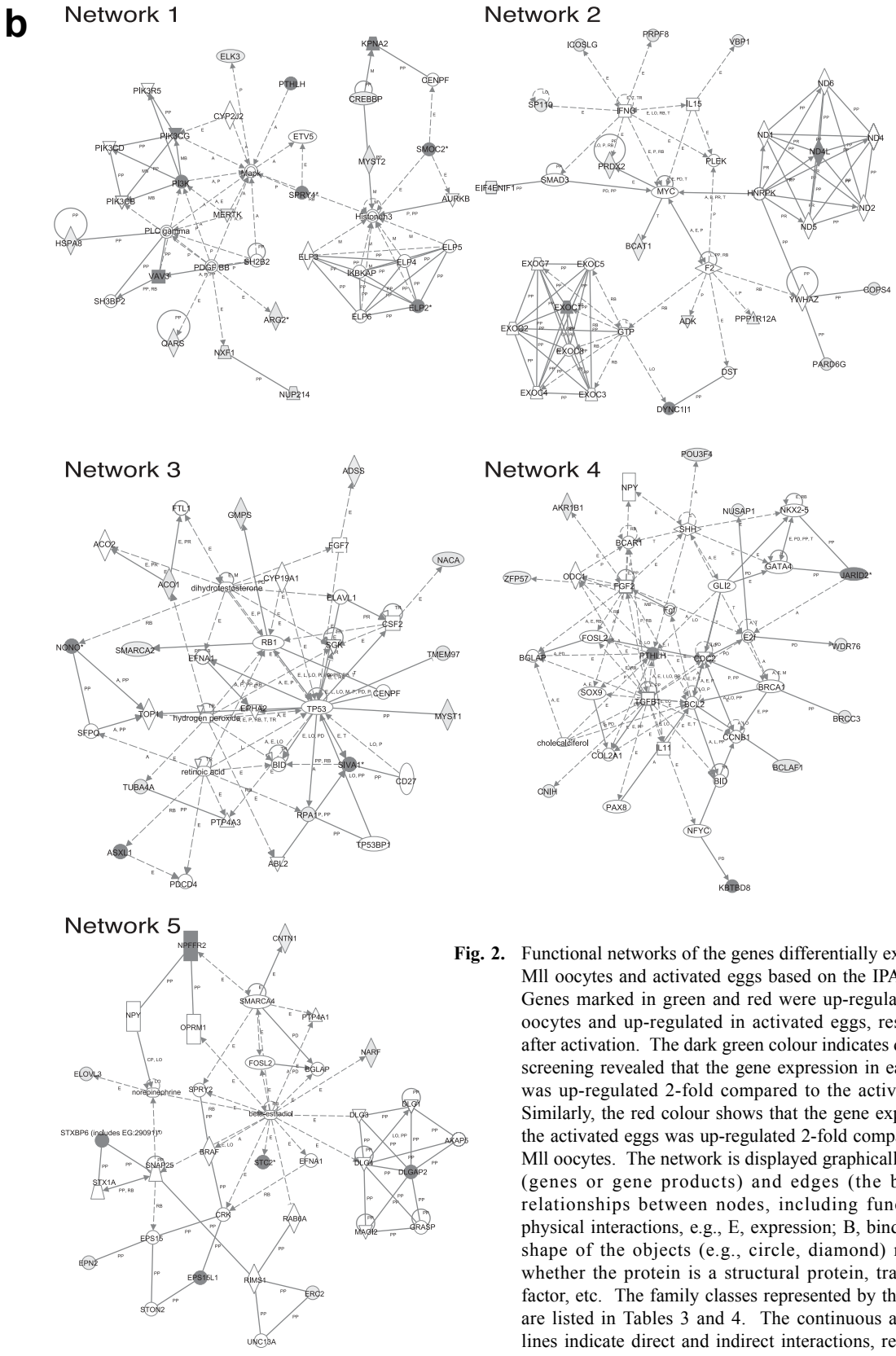


Fig. 2. Functional networks of the genes differentially expressed in Mll oocytes and activated eggs based on the IPA database. Genes marked in green and red were up-regulated in Mll oocytes and up-regulated in activated eggs, respectively, after activation. The dark green colour indicates differential screening revealed that the gene expression in each oocyte was up-regulated 2-fold compared to the activated eggs. Similarly, the red colour shows that the gene expression in the activated eggs was up-regulated 2-fold compared to the Mll oocytes. The network is displayed graphically as nodes (genes or gene products) and edges (the biological relationships between nodes, including functional or physical interactions, e.g., E, expression; B, binding). The shape of the objects (e.g., circle, diamond) represents whether the protein is a structural protein, transcription factor, etc. The family classes represented by these shapes are listed in Tables 3 and 4. The continuous and dashed lines indicate direct and indirect interactions, respectively, between the gene products.

Table 5. Top 5 networks generated from the IPA for genes specifically expressed in Mll oocytes

Network ID	Genes in network	Categories of top functions
1	*Akt*, ***ATF2***, ***CCNB1***, ***CDH1***, *Ck2*, ***DCLRE1A***, ***EIF2B1***, *FGF3*, ***FRAP1***, *GNA13*, ***GNB2L1***, *Hsp70*, ***HSP90AA1***, ***HSP90AB1***, ***IL1RAP***, *Jnk*, *LAMA3*, *MAP4K5*, *Mapk*, *MDK*, *MIA*, ***MOS***, ***NCL***, *NOLC1*, *MAPK14*, *Pdgf Ab*, *PDGF BB*, ***PLAT***, *Ras*, ***RFPL4A***, *SERPINF1*, ***SIN3A***, *SNCG*, ***TP53BP1***	Cellular Movement, Cardiovascular System Development and Function, Organismal Development
2	*AKT1*, ***AKT3***, *amino acids*, ***APPL1***, ***ATP7A***, *BMP15*, *CBX5*, *CYP17A1*, ***DCP1A***, *DLG4*, *DLGAP2*, ***GAPVD1***, ***GDF9***, ***GGTA1***, ***HSPA4L***, *IGFBP5*, *INHA*, *KIF1C*, ***LBR***, *LMO7*, *LRP2*, *MAGI2*, ***MATR3***, ***MOS***, *MYOD1*, *NP*, *NPC1*, *palmitic acid*, ***PLA2G4C***, *progesterone*, *PTGER2*, ***PTPN21***, *SRPK1*, *STAR*, *YWHAG*	Cancer, Lipid Metabolism, Molecular Transport
3	*APC*, *BPGM*, *CD44*, *CDC2*, *CDKN2A*, *CENPE*, ***CENPF***, ***CNOT7***, *CNOT6L*, ***DDX3X***, *EDN1*, *Erm*, ***FBXL5***, *GNA13*, ***IARS*** *(includes EG:3376)*, *IL4*, *IL13*, ***JAG1***, *LOX*, ***MDM4***, *MFAP2*, *MME*, *MSN*, *MYC*, *PDGF-CC*, ***PDIA6***, ***RDX***, ***SLC23A2***, *SLC43A3*, *SMOC2*, *SPN*, ***SUMO2***, *TGFB1*, *THY1*, *TOB2*	Cancer, Cellular Growth and Proliferation, Tumor Morphology
4	*AIFM1*, ***AKT3***, *beta-estradiol*, *BUB3*, ***BUB1B***, *CDC20*, *CDKN1A*, *CENPE*, *CLU*, *DDIT4*, ***DKC1***, *hydrogen peroxide*, *IGFBP2*, ***KCNN2***, ***LRRC17***, *MAD2L1*, ***MCC***, ***MLLT10***, *NCAPD3*, *PAX3*, ***PLK4***, ***PRC1***, ***PTPN2***, *RAP1A*, *SLC3A2*, ***SLC7A11***, *SLC9A1*, *SMARCB1*, *SMC4*, *SNCG*, ***SPAG5***, *SS18*, *TERC (includes EG:7012)*, *TERT*, ***TXNIP***	Cancer, Cellular Growth and Proliferation, Cell Death
5	*ACADS*, *APC*, *CRH*, *CTGF*, *EIF4EBP2*, *FADD*, *FGF3*, ***HNRPF***, *IGFBP2*, *IGFBP3*, *INHBA*, *INS1*, ***LNPEP***, *LOX*, *MAPK3*, ***MBD4***, ***MEIS2***, *MIF*, ***NFRKB***, *NIF3L1*, *NUP98*, *NUP107*, *NUP133*, ***NUP160***, ***PHIP***, ***PLAT***, ***RAPGEF4***, *retinoic acid*, *SERPINB2*, *SERPINE2*, *SMAD2*, ***SYTL4***, *TNF*, *TNFAIP6*, ***TPD52L2***	Cancer, Cellular Growth and Proliferation, Cell Cycle

The genes with bold letters were in the cDNA library obtained by the subtraction analysis.

Table 6. Top 5 networks generated from IPA for genes upregulated after oocyte activation

Network ID	Genes in network	Categories of top functions
1	*AURKB*, *CENPF*, ***CREBBP***, *CYP2J2*, *ELK3*, *ELP2*, *ELP3*, *ELP4*, *ELP5*, *ELP6*, *ETV5*, *Histone h3*, ***HSPA8***, *IKBKAP*, ***KPNA2***, *Mapk*, ***MERTK***, ***MYST2***, ***NUP214***, ***NXF1***, *PDGF BB*, *PI3K*, *PIK3CB*, *PIK3CD*, *PIK3CG*, ***PIK3R5***, *PLC gamma*, *PTHLH*, ***QARS***, *SH2B2*, *SH3BP2*, *SMOC2*, ***SPRY4***, *VAV3*	Immune Response, Cell Signaling, Cell-To-Cell Signaling and Interaction
2	***ADK***, ***BCAT1***, ***COPS4***, *DST*, *DYNC1I1*, ***EIF4ENIF1***, ***EXOC1***, *EXOC2*, *EXOC3*, *EXOC4*, *EXOC5*, *EXOC7*, *EXOC8*, *F2*, *GTP*, *HNRPK*, *ICOSLG*, *IFNG*, *IL15*, *MYC*, *ND1*, *ND2*, ***ND4***, *ND5*, *ND6*, *ND4L*, ***PARD6G***, *PLEK*, ***PPP1R12A***, ***PRDX2***, ***PRPF8***, *SMAD3*, ***SP110***, ***VBP1***, *YWHAZ*	Vitamin and Mineral Metabolism, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function
3	*ABL2*, ***ACO1***, *ACO2*, ***ADSS***, ***ASXL1***, *BID*, *CD27*, *CENPF*, *CSF2*, *CYP19A1*, *dihydrotestosterone*, *EFNA1*, *ELAVL1*, *EPHA2*, *FGF7*, *FTL1*, ***GMPS***, *hydrogen peroxide*, ***MYST1***, *NACA*, ***NONO***, *PDCD4*, *PTP4A3*, *RB1*, *retinoic acid*, ***RPA1***, *SFPQ*, *SGK*, ***SIVA1***, ***SMARCA2***, ***TMEM97***, *TOP1*, *TP53*, *TP53BP1*, ***TUBA4A***	Cell Cycle, Cancer, Cellular Growth and Proliferation
4	***AKR1B1***, *BCAR1*, *BCL2*, ***BCLAF1***, *BGLAP*, *BID*, *BRCA1*, ***BRCC3***, *CCNB1*, *CDC2*, *cholecalciferol*, ***CNIH***, *COL2A1*, *E2f*, *Fgf*, *FGF2*, *FOSL2*, *GATA4*, *GLI2*, *IL11*, *JARID2*, *KBTBD8*, *NFYC*, *NKX2-5*, *NPY*, ***NUSAP1***, *ODC1*, *PAX8*, *POU3F4*, *PTHLH*, *SHH*, *SOX9*, *TGFB1*, ***WDR76***, ***ZFP57***	Embryonic Development, Tissue Development, Cancer
5	*AKAP5*, *beta-estradiol*, *BGLAP*, *BRAF*, *CNTN1*, *CRK*, *DLG1*, *DLG3*, *DLG4*, *DLGAP2*, *EFNA1*, ***ELOVL3***, ***EPN2***, *EPS15*, ***EPS15L1***, *ERC2*, *FOSL2*, *GRASP*, *MAGI2*, ***NARF***, *norepinephrine*, *NPFFR2*, *NPY*, *OPRM1*, *PTP4A1*, *RAB6A*, *RIMS1*, *SMARCA4*, *SNAP25*, *SPRY2*, ***STC2***, *STON2*, *STX1A*, *STXBP6 (includes EG:29091)*, *UNC13A*	Cancer, Endocrine System Disorders, Molecular Transport

The genes with bold letters were included in the cDNA library obtained by the subtraction analysis.

of G protein-coupled receptors and signalling molecules. It has also been reported that the transcripts encoding components of signalling pathways essential for maintaining the unique characteristics of the MII-arrested oocyte, such as those involved in protein kinase pathways, are the most prominent among the stable transcripts [33].

The present results also provide a comprehensive profile of the dynamism in gene expression following oocyte activation in mice. Further understanding of the biological role of these genes would increase our knowledge regarding the meiotic cell cycle, maternal genome activation, chromatin remodeling, pluripotency, early cleavage, and epigenetic reprogramming. We believe that the approach we have described in which differential gene expression can be identified will lead to a better understanding of the molecular mechanisms underlying the unique functions of these genes in oocytes, including their long-term effects on oocyte development.

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