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Changes in gene expression associated with oocyte meiosis after *Obox4* RNAi

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Objective: Previously, we found that oocyte specific homeobox (Obox) 4 plays significant role in completion of meiosis specifically at meiosis I-meiosis II (MI-MII) transition. The purpose of this study was to determine the mechanism of action of *Obox4* in oocyte maturation by evaluating downstream signal networking.
Methods: The *Obox4* dsRNA was prepared by *in vitro* transcription and microinjected into the cytoplasm of germinal vesicle oocytes followed by *in vitro* maturation in the presence or absence of 0.2 mM 3-isobutyl-1-metyl-xanthine. Total RNA was extracted from 200 oocytes of each group using a PicoPure RNA isolation kit then amplified two-rounds. The probe hybridization and data analysis were used by Affymetrix GeneChip® Mouse Genome 430 2.0 array and GenPlex 3.0 (ISTECH, Korea) software, respectively.
Results: Total 424 genes were up (n = 80) and down (n = 344) regulated after *Obox4* RNA interference (RNAi). Genes mainly related to metabolic pathways and mitogen-activated protein kinase (MAPK) signaling pathway was changed. Among the protein kinase C (PKC) isoforms, PKC-alpha, beta, gamma were down-regulated and especially the MAPK signaling pathway PKC-gamma was dramatically decreased by *Obox4* RNAi. In the cell cycle pathway, we evaluated the expression of genes involved in regulation of chromosome separation, and found that these genes were down-regulated. It may cause the aberrant chromosome segregation during MI-MII transition.
Conclusion: From the results of this study, it is concluded that *Obox4* is important upstream regulator of the PKC and anaphase-promoting complex action for maintaining intact germinal vesicle.

Keywords: Oocyte Maturation; *Obox4*, Mouse; RNA Interference; Microarray Analysis

Introduction

Oocyte maturation refers to the process that prophase I arrested germinal vesicle (GV) drives the progression of meiosis to metaphase II (MII) to have the capacity for fertilization and embryo development [1]. Spontaneous oocyte maturation *in vitro* is suppressed when oocytes are cultured with high levels of cAMP maintained by the addition of cAMP analogues or phosphodiesterase (PDE) inhibitors, such as 3-isobutyl-1-metyl-xanthine (IBMX), in the culture medium [2,3]. Thus, a high cAMP level in the oocyte is crucial for an environment that maintains the meiotic arrest of oocytes *in vitro* [2-7]. Active form of cAMP-dependent protein kinase A (PKA)-mediated cAMP action that inhibits the resumption of meiosis also prevents mitogen-activated protein kinase (MAPK) activation [8-11]. Also, protein kinase C (PKC) has been reported to plays an important role in inducing MAPK, maturation promoting factor (MPF) activation and oocyte maturation [12-15]. However, the role of PKC system in vertebrate oocytes is still not fully identified.

Oocyte specific homeobox (Obox) family proteins may play an im-

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portant role in follicle development and oogenesis, because their expression pattern is similar to that of growth differentiation factor-9 (GDF9) and bone morphogenetic protein-15 (BMP15), which are oocyte-specific and play important roles in follicle development and oogenesis [16]. In *Obox6* knockout mice, expressions of the *Obox1, 2, 3,* and *5* genes were increased during early embryo development, suggesting that these family members compensated for the loss of *Obox6* expression [17]. In addition, mice lacking the *Obox6* gene undergo normal morphological development with normal fertile [17].

In contrast, we found that the expression of the other *Obox* members such as 1, 2, 3, 5, and 6 genes not affected in the *Obox4* knocked down oocytes [18]. *Obox4* plays significant role in completion of meiosis specifically at meiosis I-meiosis II (MI-MII) transition with normal spindle-chromosome formation. Interestingly, *Obox4* RNA interference (RNAi) resulted in the MI-arrest regardless of the presence (77.7%) or absence (72.5%) of IBMX in the culture medium. Therefore, in that previous study, we concluded that *Obox4* is a key factor in cAMP-dependent GV-arrest in oocytes [18].

The present study was conducted to determine the molecular mechanism of *Obox4* function. We did *Obox4* RNAi at GV stage, cultured oocytes 4 hours for knockdown of *Obox4*, and did microarray analysis. We found the multiple signaling pathways regulated by *Obox4* RNAi during oocyte maturation. Among those multiple pathways, the current study was focused on cell cycle and MAPK pathway because we previously found that *Obox4* plays a role in spindle-chromosome configuration during MI-MII transition.

Table 1. Oligonucleotide primers, sequences, annealing temperatures (AT), and expected sizes of the real-time reverse transcription-polymerase chain reaction (RT-PCR) products

Genes	Gene title	Accession No.	Primer sequence	AT (°C)	Size (bp)
Obox4-1	Oocyte specific homeobox 4	AF461109	F - CCCTCATTGATCAACCCTTGG R - AGTTTTGGGTCATACTTGGAG	60	240
Obox4-2	Oocyte specific homeobox 4	AF461109	F - CCAACGTTCTTTGCTCACCT R - TGCACCGTGTTCTTCTCTGT	60	295
PKC-alpha	Protein kinase-alpha	NM_011101	F - CTACCCCAAATCCTTGTCCA R - CTCGCGTGAAGAACTTGTCA	60	230
PKC-beta	Protein kinase-beta	NM_008855	F - AATGTGGCGTATCCCAAGTC R - CTCCTTGCGTTCGAGTTTCT	60	163
PKC-delta	Protein kinase-delta	NM_011103	F - CCTGTACGAAATGCTCATCG R - GGGTGAATCCTGATGTTTCC	60	192
PKC-gamma	Protein kinase-gamma	NM_011102	F - TGATGGGGAAGATGAGGAAG R - GGTCTGAAAGGAGGTGCAAT	60	234
Erk1	Extracellular signal-regulated kinase 1	NM_011952	F- ATGAAGGCCCGAAACTACCT R- GCTCCATGTCGAAGGTGAAT	60	232
Erk2	Extracellular signal-regulated kinase 2	NM_001038663	F - GGTTGTTCCCAAATGCTGAC R - GTCGTCCAACTCCATGTCAA	60	182
Mek1	Mitogen-activated protein kinase kinase 1	NM_008927	F - TTGGGAGATACCCCATTCCT R - TTTGGAGGAGGCTCATTGAC	60	199
Mek2	Mitogen-activated protein kinase kinase 2	NM_023138	F - TGTAGGTCATGGGATGGACA R - GTGGTTCATCAGCAGCTTCA	60	184
Raf1	Threonin-protein kinase	NM_029780	F - ATGACAACCCGTTCAGCTTC R - GACAGGATCTGGGGAAACAA	60	250
Cdc20	Cell-division cycle 20	NM_023223	F - ATTTGGAACGTCTGCTCAGG R - CTTGGCCATGGTTGGATACT	60	156
APC1	Anaphase-promoting complex	NM_008569	F - TCTGAGTTCCTACCTGTGGTGA R - CAGAGTGGTAGACAAGGAAGCA	60	157
PTTG	Pituitary tumor-transforming gene 1	NM_001131054	F - AGTTTTGACCTGCCTGAGGA R - GGAGAGTACAGCGGATCAGATT	60	167
Espl1	Extra spindle poles-like 1	NM_001014976	F - GCTGGTATCGTGCTCAAGTACA R - TGGCTGGCATAGTAGAGAAAGG	60	158
Rad21	Cohesin	NM_009009	F - CAGCAGGTAGAGCAAATGGA R - CATCTGCTGAGTGCGTTTGT	60	216

F and R in the primer codes indicate forward and reverse.
The *Obox4*-1 primers were used for RT-PCR and synthesis of dsRNA.
The *Obox4*-2 primers were used to confirm the knockdown of *Obox4* after RNAi treatment.

Methods

1. Animals

ICR mice were obtained from Koatech (Pyeongtaek, Korea) and maintained at the animal facility of the CHA Stem Cell Institute of CHA University to obtain oocytes. All procedures described within were reviewed and approved by the University Institutional Animal Care and Use Committee (IACUC), and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

2. RNAi for *Obox4*

Production of *Obox4* double-stranded RNA (dsRNA) and RNAi by microinjection was performed as described previously [18]. We prepared dsRNA for *Obox4* (240 bp) using the MEGAscript RNAi Kit (Ambion, Austin, TX, USA). GV oocytes were microinjected with *Obox4* dsRNA in M2 medium containing 0.2 mM IBMX. *Obox4* dsRNA-injected oocytes were cultured in M16 medium containing 0.2 mM IBMX for 4 hours in 5% CO_2 at 37°C. Control oocytes cultured in M16 medium containing 0.2 mM IBMX for 4 hours in 5% CO_2 at 37°C.

3. Microarray analysis

Due to the small amounts of initial total RNA from 200 oocytes, the process required an amplifying two-cycle target labeling assay step to obtain sufficient amounts of labeled cRNA target for analysis with microarrays. Total RNA was used to synthesize double-stranded cDNA with the MEGAscript kit (Ambion) with an oligo (dT) primer containing a T7 RNA polymerase promoter. The labeled cRNA was hybridized to the Affymetrix GeneChip Mouse Genome 430 2.0 array (Affymetrix, Santa Clara, CA, USA), which covers transcripts and variants from 34,000 well characterized mouse genes. Probe sets on this array are derived from sequences from GenBank and dbEST. The chips were analyzed by using a GeneChip array scanner 3000 7 G (Affymetrix) and GenPlex 3.0 (ISTECH, Goyang, Korea) software.

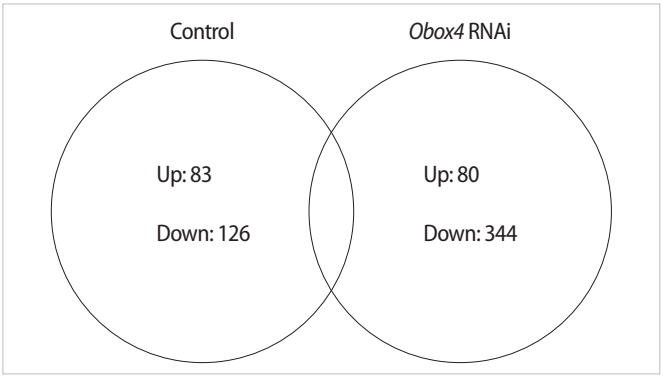

Figure 1. Venn diagram showing the number of transcripts up- and down-regulated more than 2-fold in comparisons between control and oocyte specific homeobox (Obox) 4 RNA interference, RNAi treated oocytes. Control group; germinal vesicles (GVs) cultured for 0 hour vs. GVs cultured for 4 hours in 3-isobutyl-1-metyl-xanthine (IBMX) supplemented medium (non-injected), *Obox4* RNAi group; GVs cultured for 0 hour vs. GVs cultured for 4 hours in IBMX supplemented medium (*Obox4* dsRNA-injected).

4. mRNA isolation and real-time RT-PCR

Messenger RNA was isolated from oocytes using the Dynabeads mRNA DIRECT kit (Invitrogen Dynal AS, Oslo, Norway), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from mRNA using oligo (dT) primer, according to the Super Script Preamplification System protocol (Gibco-BRL, Grand Island, NY, USA). Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed using the iCycler (Bio-Rad, Hercules, CA, USA). The iQ™ SYBR Green Supermix PCR reagents (Bio-Rad) were used for monitoring amplification and results were evaluated with the iCycler iQ real-time detection system software. Quantitation of gene amplification was performed by determining the cycle threshold (C_T), based on the fluorescence detected within the geometric region of the semi-log amplification plot. Expression of each mRNA species was normalized to that of H1foo mRNA. Relative quantitation of target gene expression was evaluated using the comparative C_T method and experiments were repeated at least three times. Real-time PCR reaction conditions and primer sequences for the encoding genes are listed in Table 1.

Results

To define and compare control and *Obox4*-deficient oocytes tran-

Table 2. Top 15 functional groups of genes differentially expressed in oocyte specific homeobox 4 RNAi oocytes

KEGG pathway	Gene counts
Metabolic pathways	23
MAPK signaling pathway	9
Chemokine signaling pathway	9
Pathways in cancer	9
Purine metabolism	6
Focal adhesion	6
Gap junction	6
Insulin signaling pathway	6
Pyrimidine metabolism	6
Ubiquitin mediated proteolysis	6
Adipocytokine signaling pathway	5
B cell receptor signaling pathway	5
Cell cycle	5
Regulation of actin cytoskeleton	5
Spliceosome	5

KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase.

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scriptomes, gene expressions of control and *Obox4* RNAi treated oocytes were analyzed with the Affymetrix Mouse Genome 430 2.0 arrays and GenPlex 3.0 (ISTECH) software. The Affymetrix 430 2.0 mouse microarray was used because it is the most comprehensive array system that allows expression inquiry for over 34,000 well substantiated mouse genes.

A total of 424 genes were significantly changed at least 2-fold in *Obox4* deficient oocytes. This analysis revealed that 80 genes were up-regulated and 344 genes were down-regulated by *Obox4* RNAi (Figure 1). Top 15 functional Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways changed after *Obox4* RNAi were summarized in Table 2. Among 424 genes, 306 genes were not included for this pathway analysis since functions of these genes are still unknown. Many genes were changed in the pathways related with metabolism, MAPK, and chemokine signaling.

Selected genes in the MAPK signaling pathway are presented in the box with bold characters (Figure 2) and we confirmed their expression by real-time RT-PCR (Figure 3). Four genes (PKC-beta, PKC-delta, PKC-gamma, mitogen-activated protein kinase kinase 2 [MEK2]) were down-regulated and only Threonin-protein kinase (*Raf1*) was up-regulated (Figure 3).

Selected 5 genes in the cell cycle pathway are presented in bold type (Figure 4) and we confirmed their expression by real-time RT-PCR (Figure 5). These genes are related to spindle and chromosome configuration during oocyte maturation, and we selected it based on our previous findings that *Obox4* may be involved in the chromosome segregation [18]. Two genes (*APC1*, *PTTG*) were down-regulated, while *Cdc20* was up-regulated (Figure 5).

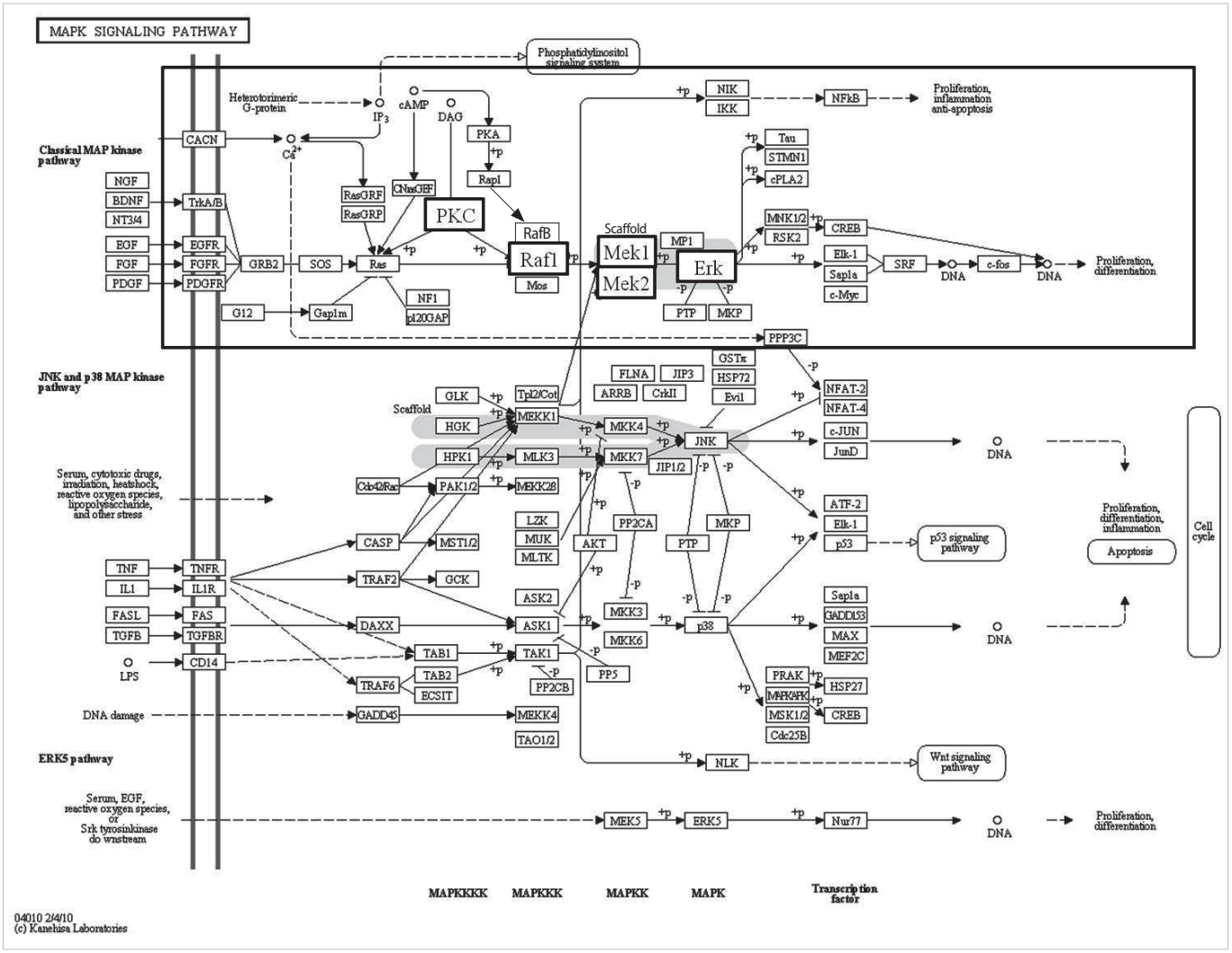

Figure 2. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway displaying transcripts in mitogen-activated protein kinase (MAPK) signaling pathway. Selected genes are presented with bold characters in a small box and their expression was confirmed by real-time reverse transcription-polymerase chain reaction as summarized in Figure 3 [34]. PKC, protein kinase; Raf1, threonin-protein kinase; MEK, mitogen-activated protein kinase kinase; Erk, extracellular signal-regulated kinase.

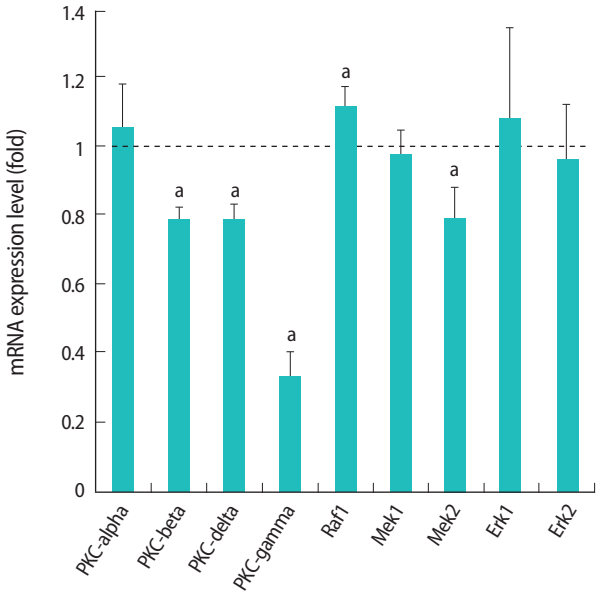

Figure 3. Relative expression of genes related to mitogen-activated protein kinase pathway in germinal vesicle (GV) oocytes after oocyte specific homeobox 4 RNA interference. Expression of mRNA was confirmed by real-time reverse transcription-polymerase chain reaction analysis. The expression levels were calculated cycle threshold values, and then mRNA expression ratio was determined relative to that of control GV oocytes. Experiments were repeated least three times, and data was expressed as mean ± SE. *PKC*, protein kinase; *Raf1*, threonin-protein kinase; *MEK*, mitogen-activated protein kinase kinase; *Erk*, extracellular signal-regulated kinase.[a]Statistical significance at $p < 0.05$.

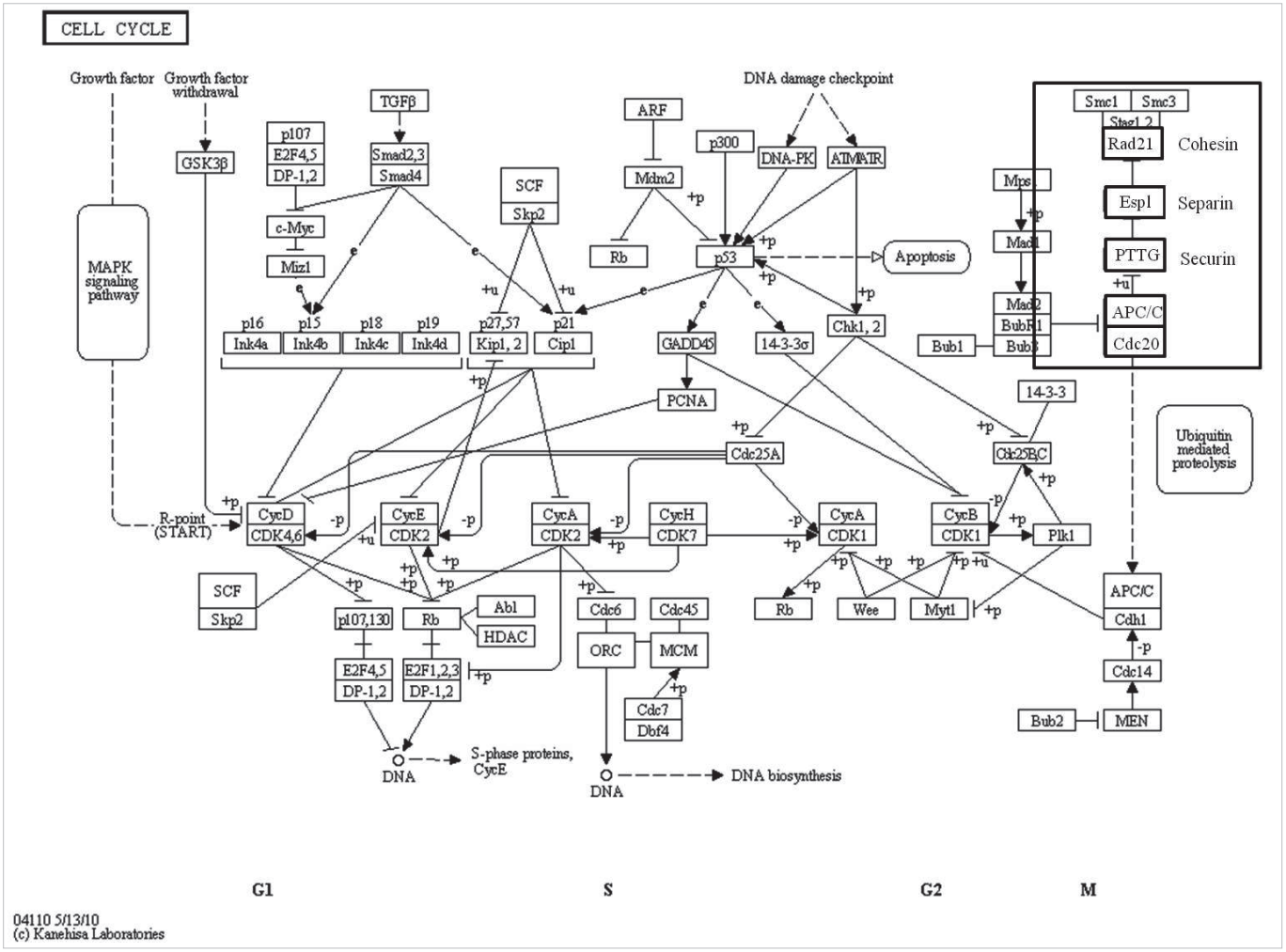

Figure 4. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway display transcripts in cell cycle pathway. Selected genes are presented in bold characters in a small box and their expression was confirmed by real-time reverse transcription-polymerase chain reaction. *Rad21* encodes cohesin, *esp1* encodes separin, and *PTTG* encodes securin [35]. Cdc20, cell-division cycle 20; APC1, anaphase-promoting complex; PTTG, pituitary tumor-transforming gene 1; Espl, extra spindle poles-like; Rad21, cohesin.

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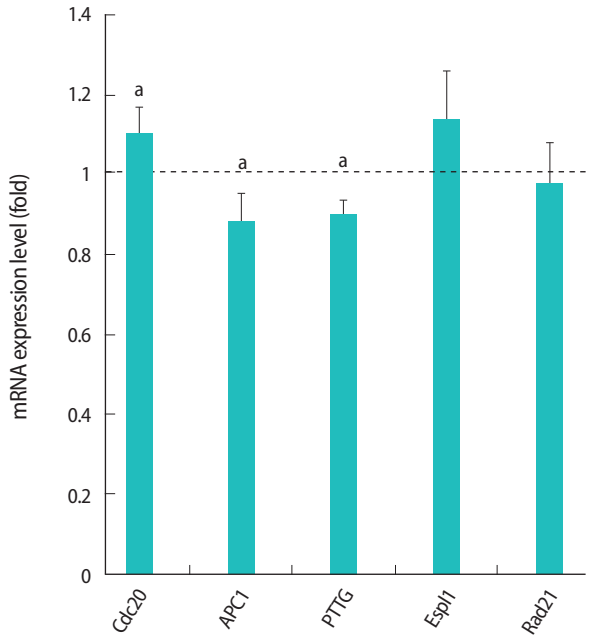

Figure 5. Relative expression of genes related to cell cycle pathway in germinal vesicle (GV) oocytes after oocyte specific homeobox 4 RNAi. Expression of mRNA was confirmed by real-time reverse transcription-polymerase chain reaction analysis. The expression levels were calculated cycle threshold values, and then mRNA expression ratio was determined relative to that of control GV oocytes. Experiments were repeated least three times, and data was expressed as mean ± SE. *Cdc20*, cell-division cycle 20; *APC1*, anaphase-promoting complex; *PTTG*, pituitary tumor-transforming gene 1; *Espl*, extra spindle poles-like; *Rad21*, cohesin. [a]Statistical significance at $p < 0.05$.

Discussion

MAPK plays crucial roles in maturation of oocytes in all animals [19]. Activation of MAPKs requires the dual-specific kinase MEK (MAPKK), which phosphorylates MAPK on critical threonine and tyrosine residues [20-22]. MAPK super-family can be divided in to three major subgroups: extracellular signal-regulated kinases (ERK), *c-jun*-N-terminal kinase, and p38 as depicted in Figure 2 [23]. The first characterized subfamily of MAP kinase, Raf/MEK/ERK signaling pathway [24]. Raf proteins have been shown to phosphorylate and activate MAP kinase kinases (MAPKKs) called MEKs (MAPK or ERK kinases) which in turn phosphorylate and activate ERKs (Raf→MAPKK→MEK cascade) [25,26]. This cascade is inhibited by PKA [5].

We observed that PKC affected by *Obox4* RNAi among the MAPK signaling pathway in microarray analysis. The PKC is another major kinase for oocyte maturation in MAPK signaling pathway [27-30]. It has been identified that PKC superfamily has at least 12 isoforms [31]. We focused on the expression of conventional (alpha, beta, gamma) and novel (delta) PKC. These isoforms have been reported in human or mouse oocytes [27-30]. Especially, among 4 isoforms of PKC, 3 isoforms were down-regulated and the expression of PKC-gamma was dramatically decreased after *Obox4* RNAi. PKC activity suppressed by PKC antagonists have been shown to induce germinal vesicle breakdown (GVBD) in denuded mouse oocytes in a medium contained hypoxanthine [30]. The authors demonstrated that the meiotic inhibitors such as hypoxanthine might activate PKC in the oocytes [30].

The function of each PKC isoform in oocytes is still largely unknown. Relationship between PKC and *Obox4* in regulation of GVBD requires further study. The APC complex is composed of at least 11 APC components and APC1 is the largest of these subunits. The APC/C-CDC20 is known to be suppressed in the metaphase arrest by the spindle assembly checkpoint [32]. CDC20 accumulates during the GV stage but does not activate APC until the resumption of GVBD, because the phosphorylation of APC subunits by the MPF is required for the binding of CDC20 to APC [33]. The APC/C-CDC20 mediated degradation of securin and the degradation of securin activate separase [32]. Separase then cleaves the cohesion, allowing chromosome segregation [32].

We suggest that up-regulation of *Cdc20* (Figure 5) may be involved in inducing the meiotic resumption in the *Obox4*-deficient oocytes in the IBMX medium. In addition, deficiency of spindle formation and aberrant chromosome configuration may be affected by abnormal expression of *PTTG*, of which protein product is securin.

Taken together, we suggest that *Obox4* is important upstream regulator of the PKC and APC action for maintaining intact GV. Further elucidation of the molecular mechanism of the *Obox4* would lead us to understand how to maintain the GV arrest.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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