Downstream Networking of *Zap70* in Meiotic Cell Cycle of the Mouse Oocytes

Hyun-Jung Kim, Hyun-Seo Lee, Eun-Young Kim and Kyung-Ah Lee†

Department of Biomedical Science, College of Life Science, CHA University
CHA Research Institute, Fertility Center, CHA General Hospital, Seoul 135-081, Korea

ABSTRACT : Previously, we found that *Zap70* (Zeta-chain-associated protein kinase) expressed in the mouse oocytes and played significant role in completion of meiosis specifically at MI-MII (metaphase I-II) transition. Microinjection of *Zap70* dsRNA into the cytoplasm of germinal vesicle oocyte resulted in MI arrest, and exhibited abnormalities in their spindles and chromosome configurations. The purpose of this study was to determine the mechanisms of action of *Zap70* in oocyte maturation by evaluating downstream signal networking after *Zap70* RNAi (RNA interference). The probe hybridization and data analysis were used by Affymetrix Gene Chip Mouse Genome 430 2.0 array and GenPlex 3.0 (ISTECH, Korea) software, respectively. Total 1,152 genes were up (n=366) and down (n=786) regulated after *Zap70* RNAi. Among those genes changed, we confirmed the expressional changes of the genes involved in the regulation of actin cytoskeleton and MAPK (mitogen-activated protein kinase) signaling pathway, since the phenotypes of *Zap70* RNAi in oocytes were found in the changes in the chromosome separation and spindle structures. We confirmed the changes in gene expression in the actin skeletal system as well as in the MAPK signaling pathway, and concluded that these changes are main cause of the aberrant chromosome arrangement and abnormal spindles after *Zap70* RNAi.

Key words : *Zap70*, RNAi, Microarray, Spindle, MAPK

INTRODUCTION

Zap70 is a 70 kD protein tyrosine kinase mainly found as surface member of normal T cells and natural killer cells. Because *Zap70* plays a critical role in T cell development and signaling, much effort has been focused on studying the regulation, structure, and function of *Zap70* T cells. Structurally, *Zap70* is composed of two SH2 domains and a carboxyl-terminal kinase domain (Wang et al., 2010).

Zap70 is also considered as involved in the regulation of the mouse oocyte maturation. We previously have shown that *Zap70* is highly expressed in the mouse oocytes, and *Zap70* RNAi caused abnormalities in the spindle formation and the chromosome segregation (Kim et al., 2009). MAPK has known as a critical regulatory factor in oocyte maturation (Sobajima et al., 1993), and in accordance the absence of *Zap70* and concurrent changes in MAPK components resulted in abnormal cytoskeletal systems during oocyte maturation (Kim et al., 2009).

This study was conducted to determine the molecular mechanism of *Zap70* function. To evaluate the downstream gene networking, we did *Zap70* RNAi at GV (germinal vesicle) stage, cultured oocytes 8 hours for obtaining complete knockdown of *Zap70*, and did microarray analysis. Microarray analysis showed that the multiple signaling pathways regulated by *Zap70* RNAi during oocyte maturation. Among those multiple pathways, the present study was focused on the evaluation of two major pathways, such as regulation of actin cytoskeleton and MAPK signaling pathway because we previously found that *Zap70* plays a role in spindle-chromosome configuration during MI-MII transition via MAPK pathway.

† Corresponding author: Kyung-Ah Lee, Dept. of Biomedical Science, College of Life Science, CHA University, 606-13 Yeoksam-1-dong, Gangnam-gu, Seoul 135-081, Korea. E-mail: leeka@ovary.co.kr

MATERIAL AND METHODS

1. Animals

ICR mice were obtained from Koatech (Pyengtaek, Korea) and maintained at the animal facility of the CHA Stem Cell Institute of CHA University. All procedures described 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. Isolation of Oocytes

For isolation of GV oocytes from preovulatory follicles, 3-week-old female ICR mice injected with 5 IU eCG and sacrificed 46 hours later. Cumulus-enclosed oocyte complexes (COCs) were recovered from ovaries by puncturing the preovulatory follicles with 27-gauge needles. M2 medium containing 0.2 mM IBMX (3-isobutyl-metyl-xanthine) was used to inhibit GVBD (germinal vesicle breakdown) while handling oocytes. Isolated oocytes were snap-frozen and stored at −70℃ prior for RNA preparation.

3. RNAi for *Zap70*

To determine the possible role of *Zap70* in oocyte maturation, production of *Zap70* dsRNA (double-stranded RNA) and RNAi by microinjection was performed as described previously (Kim et al., 2009). We prepared dsRNA for *Zap70* (475 bp) using the MEGA script RNAi Kit (Ambion, Austin, TX, USA). GV oocytes were microinjected with *Zap70* dsRNA in M2 medium containing 0.2 mM IBMX. *Zap70* dsRNA-treated oocytes were cultured in M16 medium containing 0.2 mM IBMX for 8 hours, followed by culture in the plain M16 for 8 hours in 5% CO_2 at 37℃. Control oocytes were also cultured in M16 medium containing 0.2 mM IBMX for 8 hours, followed by culture in the plain M16 for 8 hours in 5% CO_2 at 37℃.

4. Messenger RNA Isolation

Oocytes' mRNA was isolated using the Dynabeads mRNA DIRECT Kit (Invitrogen Dynal AS, Oslo, Norway) according to the manufacturer's instructions. Briefly, oocytes were resuspended with lysis/binding buffer (100 mM Tris-HCl, 500 mM LiCl, 10 mM EDTA, 1% LiDS, and 5 mM DTT) and mixed with prewashed Dynabeads oligo dT_{25}. After RNA binding, the beads were washed with buffer twice, followed by buffer B, and RNA was eluted with Tris-HCl (10 mM Tris-HCl, pH 7.5) by incubation at 70℃. cDNA (Complementary DNA) was synthesized from mRNA using 0.5 ㎍ oligo (dT) primer, according to the Super Script Preamplification System protocol (Gibco-BRL, Grand Island, NY).

5. Real-time RT-PCR

Quantitative real-time RT-PCR analysis was performed using the iCycler (Bio-Rad, Hercules, CA). 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. The reaction mixture contained cDNA, 20 pmol forward and reverse primers and SYBR Green Supermix 2 (100 mM KCl, 40 mM Tris-HCl [pH 8.4], 0.4 mM each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein and stabilizers). Template was amplified with 40 cycles of denaturation at 95℃ for 40 sec, annealing at 60℃ for 40 sec, and extension at 72℃ for 40 sec. Upon completion of PCR, fluorescence was monitored continuously while slowly heating the samples from 55℃ to 95℃ at 0.5℃ intervals. The melting curves were used to identify any nonspecific amplification products. 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 RT-PCR reaction conditions and primer sequences for each gene are listed in Table 1.

Table 1. Sequence of oligonucleotide primers used in this study, their annealing temperature (AT), and expected PCR product sizes

Gene	Accession no.	Primer sequence	AT (℃)	Size (bp)
*Zap70-A***	NM_009530	F-CACCCATCCACATTCACTCA R-TCTTTCCCAGCAGGAACTTG	60	475
*Zap70-B***	NM_009530	F-ACAGAGAAGGCCGACAAAGA R-TCGCTGATCTTGGCATAGTG	60	314
Nras (Ras)	NM_010937.2	F-TGACTTGCCAACAAGGACAG R-TAAAAGGCATCCTCCACACC	60	117
Cdc42	NM_001243769.1	F-ACTGCAGGGCAAGAGGATTA R-CCCAACAAGCAAGAAAGGAG	60	171
Rac	NM_133223.4	F-AAGCTGGCACCCATAACCTA R-CTGGCTTTTTCACTGGAGGT	60	160
Vav1	NM_011691.4	F-CACCACCTTGCAGTTTCCTT R-AGCCTTGCTGTCCCTTCTTA	60	185
NWASP	NM_028459	F-CCAGCTCCTTCAGGAAACAA R-TGAATGCCCTGTCGTATCTG	60	140
Pik3ca (PI3K)	NM_008839.2	F-AGCCTTGGACAAAACTGAGC R-CTGCTTGATGGTGTGGAAGA	60	115
Pik3cb (PI3K)	NM_029094.3	F-CTGAGCTCACATCGGTCAAA R-GCCTCGTCAAACTTCTGCTT	60	106
Pik3r1 (PI3K)	NM_001024955.1	F-CAAAGCGGAGAACCTATTGC R-CCGGTGGCAGTCTTGTTAAT	60	138
WAVE1(WASf1)	NM_031877.3	F-TAATGCCTCCATCTCCTCCA R-GCTCGTGTTTTGCTTCCTGT	60	171
WAVE2 (WASf2)	NM_153423.6	F-CAAGTCTTCATTGCCTGCTG R-TTCCGAGTCGCTGTACTCAA	60	178
APC1	NM_008569	F-TCTGAGTTCCTACCTGTGGTGA R-CAGAGTGGTAGACAAGGAAGCA	60	157
F-actin	NM_007393	F-TGTTACCAACTGGGACGACA R-CTTTTCACGGTTGGCCTTAG	60	130
Raf1	NM_029780	F-ATGACAACCCGTTCAGCTTC R-GACAGGATCTGGGGAAACAA	60	250
MEK2	NM_023138	F-TGTAGGTCATGGGATGGACA R-GTGGTTCATCAGCAGCTTCA	60	184
MEK1	NM_008927	F-TTGGGAGATACCCCATTCCT R-TTTGGAGGAGGCTCATTGAC	60	199
ERK1	NM_011952	F-ATGAAGGCCCGAAACTACCT R-GCTCCATGTCGAAGGTGAAT	60	232
ERK2	NM_001038663	F-GGTTGTTCCCAAATGCTGAC R-GTCGTCCAACTCCATGTCAA	60	182
JNK	NM_009158.2	F-ACTGCAGCATCCGTACATCA R-ATGGTGTGCTCCCTTTCATC	60	102
Tiam2 (Tiam1)	NM_011878.2	F-ACTTCCACCACCCTCTGAAA R-CCTCTTGGTGCTGTTTGCTT	60	191
p38	NM_011951.3	F-CCAGATGCCGAAGATGAACT R-TCATCATCAGGGTCGTGGTA	60	162
H1foo	NM_138312	F-GGCACAGGCTTTCTTTGTCT R-TCCAACACAAGTACCCGACA	60	173

**Primer set-A was used for the preparation of dsRNA, whereas set -B was used to confirm the gene knockdown after RNAi.

6. Immunofluorescence Staining

For immunofluorescence staining denuded oocytes were placed in PBS containing 0.1% polyvinyl alcohol (PBS-PVA), 4% paraformaldehyde, and 0.2% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA), and fixed for 40 minutes at room temperature. Fixed oocytes were washed three times in 1% BSA-PBS-PVA. Oocytes were blocked with 3% BSA-PBS-PVA for 1 hour and incubated overnight at 4℃ with α-Tubulin (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. After washing, the secondary antibody was hybridized (1:100, FITC-anti- Mouse IgG, Sigma Aldrich) for 1 hour at room temperature. After three times washing with 1% BSA-PBS-PVA, the oocytes were stained with propidium iodide (PI; 1 ㎎/㎖ in PBS, Sigma). After washing 3 times, the oocytes were mounted on slides and observed with a confocal laser scanning microscope (Zeiss LSM 510 META, Germany).

7. Microarray Analysis

Due to the small amounts of initial total RNA from 300 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 hybridization data were analyzed by using a GeneChip Array scanner 3000 7G (Affymetrix) and GenPlex 3.0 (ISTECH, Korea) software.

RESULTS AND DISCUSSION

To identify molecular mechanism of *Zap70* function, we compared gene expression of MI oocytes arrested after *Zap70* RNAi to those of control MI oocytes. Top 15 functional pathways in which many genes were changed by *Zap70* RNAi are summarized in Table 2. Fifty seven genes in the metabolic pathways were affected by *Zap70* RNAi. Over ten genes were changed in pathways in cancer, spliceosome, cell cycle, endocytosis, ubiquitin mediated proteolysis, regulation of actin cytoskeleton, neurotrophin signaling pathway, insulin signaling pathway, small cell lung cancer, oocyte meiosis, MAPK signaling pathway, focal adhesion, adherens junction, and prostate cancer. Among these top 15 pathways, we focused in two pathways such as regulation of actin cytoskeleton and MAPK signaling pathway based on the fact that our previous results demonstrated that *Zap70* is involved in the regulation of MI-MII transition, especially in chromosome and spindle structures.

Table 2. Top 15 functional groups of genes differentially expressed in *Zap70* RNAi oocytes

KEGG pathway	Gene counts
Metabolic pathways	57
Pathways in cancer	27
Spliceosome	21
Cell cycle	21
Endocytosis	18
Ubiquitin mediated proteolysis	16
Regulation of actin cytoskeleton	15
Neurotrophin signaling pathway	13
Insulin signaling pathway	13
Small cell lung cancer	12
Oocyte meiosis	12
MAPK signaling pathway	12
Focal adhesion	12
Adherens junction	12
Prostate cancer	11

Previous research indicated that the *Zap70*, a Syk family tyrosine kinase, is expressed in oocytes (Kim et al., 2009) and in undifferentiated mESCs (mouse embryonic stem cells, Cha et al., 2010). In addition, *Zap70* RNAi resulted in MI arrest with chromosomal and spindle abnormalities

(Kim et al., 2009). Because oocytes failed cytokinesis without *Zap70*, we evaluated the meiotic spindle structure and chromosome alignment by confocal microscopy after immunofluoresence staining. Figure 1 depicts the normal spindle and chromosome in Control MI oocytes as well as the abnormal phenotype of spindle and chromosome structures in Zap70 RNAi treated MI oocytes. Control MI oocytes exhibited the clear barrel shape of normal characteristics of meiotic spindles. Moreover, control MI oocytes showed well-aligned chromosomes at the metaphase plate. In *Zap70* RNAi-treated oocytes, spindles shapes were more elongated than normal barrel shape and chromosomes were dotted and aggregated on the abnormally changed spindles.

To compare the gene expression between the control and *Zap70* RNAi-treated oocytes, the Affymetrix Mouse Genome 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 1,152 genes were significantly changed more than 2 folds in *Zap70* deficient oocytes. This analysis revealed that 366 genes were up-regulated and 786 genes were down-regulated by *Zap70* RNAi (Fig. 2).

Figures 3 and 5 depict the GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway of transcripts encoding genes related to the regulation of actin cytoskeleton and MAPK signaling pathway, respectively. While the real-time RT-PCR data for the selected genes are shown in Figures 4 and 6, respectively. Among changed genes by microarray analysis (red box), we selected genes with interesting functions (blue box) to confirm their expression by real-time RT- PCR after *Zap70* RNAi. These genes are related with spindle shape and chromosome configuration during oocyte meiosis. Up- or down-ward arrows beside the blue boxes indicate results of its expression in the microarray data.

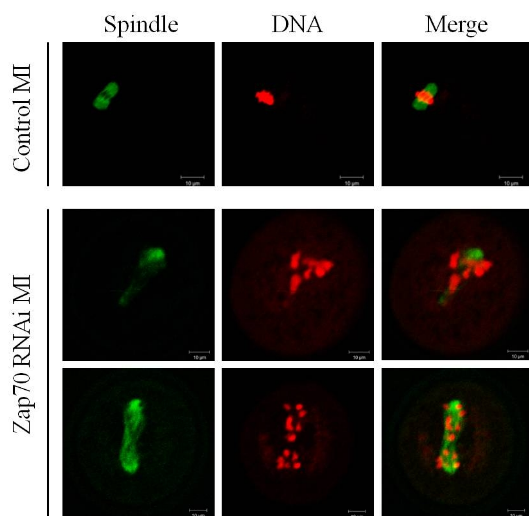

Fig. 1. Abnormal configuration of meiotic spindle and chromosome structures in *Zap70* RNAi-treated oocytes. Spindles were stained with α-Tubulin antibody (green) and chromosomes were counterstained with propidium iodide (red). (A) Control MI oocytes cultured for 8 hours exhibited the clear barrel shape of normal meiotic spindles with nicely arranged chromosomes at metaplate. (B, C) RNAi-mediated knockdown of *Zap70* arrested oocytes at the MI stage and the oocytes showed abnormally aggregated spindle and chromosomes. Left panel, spindle structure; middle panel, DNA; right panel, merged image. Bar = 10 ㎛

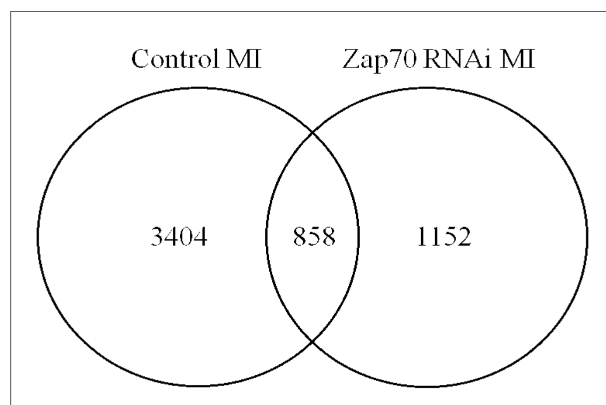

Fig. 2. Venn diagram showing the number of transcripts up- and down-regulated more than 2 folds in comparison between control and *Zap70* RNAi oocytes. Control MI oocytes, intact GV oocytes were cultured for 8 hours in IBMX supplemented medium, then transferred to IBMX-free M16 medium for 8 hours (non-treated), *Zap70* RNAi MI oocytes; *Zap70* dsRNA-treated GV oocytes were cultured for 8 hours in IBMX supplemented medium, and transferred to IBMX-free M16 medium for 8 hours (*Zap70* dsRNA-treated). Expression of transcripts in both MI oocytes were subtracted by control GV oocytes.

In Figure 3, we selected 1 up-regulated (*Ras*) and 7 down-regulated (*Pik3a, Pik3cd, Pik3rl, NWASP, WAVE1, APC, F-actin*) genes in the pathway related with regulation of actin cytoskeleton. A total of 13 genes selected including isomers of Pi3k, and separated *Vav1* and *Tiam* were selected for real-time RT-PCR and its names are listed with original gene name in Table 1. Names of selected genes in regulation of cytoskeleton were *Ras* (neuroblastoma ras oncogene), *Pik3ca* (phosphatidylinositol 3-kinase catalytic alpha polypeptide), *Pik3cd* (phosphatidylinositol 3-kinase catalytic beta polypeptide), *Pik3rl* (phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1), *Cdc42* (cell division cycle 42 homolog), *Rac* (RAS-related C3 botulinum substrate 3), *Vav1* (vav 1 oncogene), *Tiam2* (T cell lymphoma invasion and metastasis 2), *NWASP* (Wiskott-Aldrich syndrome protein), *WAVE1* (WAS protein family, member 1), *WAVE2* (WAS protein family, member 2), *APC1* (Anaphase- promoting complex), and *F-actin* (actin-beta). According to the real-time RT-PCR data after *Zap70* RNAi, expressions of 2 genes (*Vav* and *WAVE1*) were up-regulated, 2 genes (*Ras* and *WAVE2*) showed trends of up-regulation, while nine genes (*Pik3a, Pik3cd, Pik3rl, Tiam2, Rac, Cdc42, NWASP, APC,* and *F-actin*) were down-regulated (Fig. 4). The up-regulated *WAVE1* expression was not matched as in the microarray data that down-regulated in the microarray data (Fig. 3).

Zap70 leads to form a signaling complex with *Vav1*, activate *Cdc42* and *Rac*, and then promotes actin polymerization through multiple actin regulatory molecules like *WASP* and *WAVE2* (Burkhardt et al., 2008; Dombroski et al., 2005; Labno et al., 2003). The genes, *Cdc42* and *Rac*, are members of the *Rho* family and *Cdc42* activation

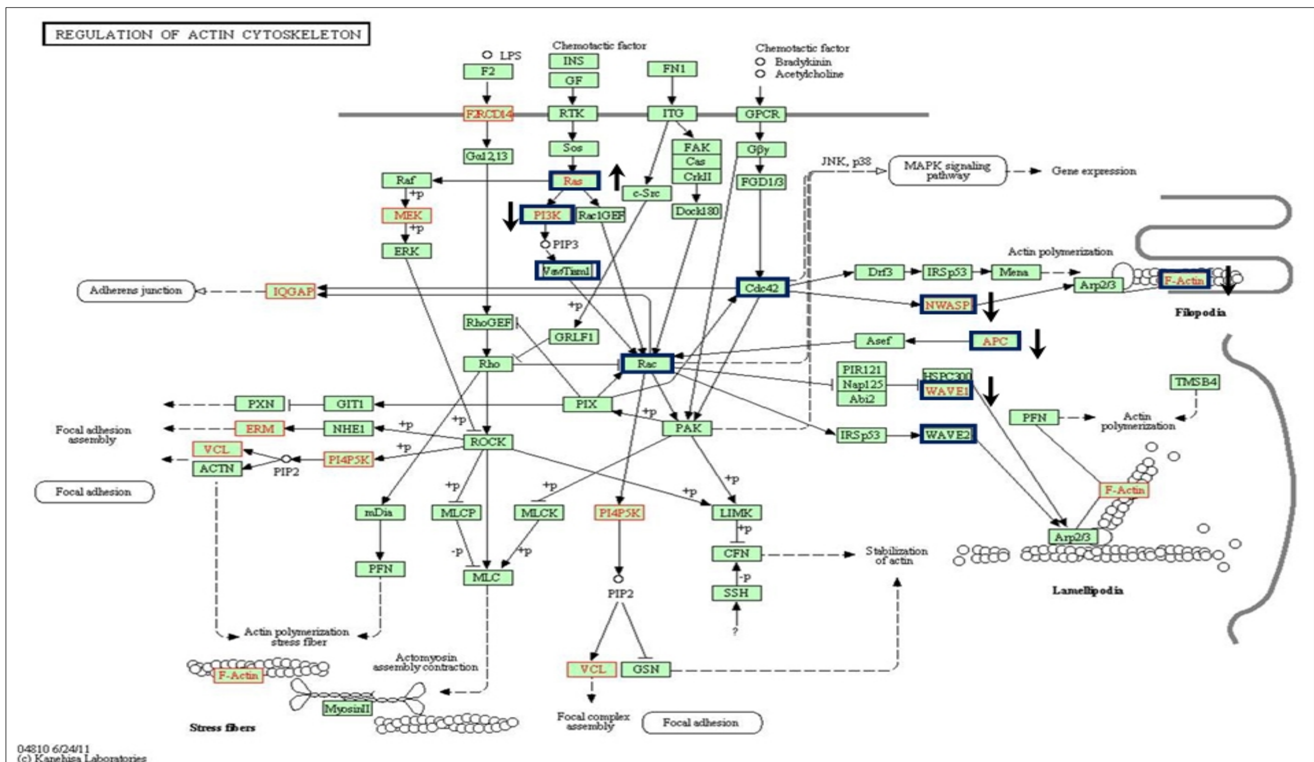

Fig. 3. GO and KEGG pathway display of transcripts encoding regulation of actin cytoskeleton pathway. The significantly up- and down-regulated transcripts by *Zap70* RNAi more than 2-fold change were marked in red. The genes confirmed by real-time RT-PCR are indicated with blue boxes. Arrows indicate the up-(↑) and down-regulated genes (↓) in microarray analysis after *Zap70* RNAi.

regulated during asymmetric cytokinesis (Kwon & Lim, 2011; Leblanc et al., 2011). When suppressed the activity of *Cdc42*, oocytes also cannot complete cytokinesis (Na & Zernicka-Goetz, 2006). Inhibition of Rac during oocyte maturation resulted in a cell cycle block at prometaphase I and spindle elongation (Halet & Carroll, 2007). Therefore, we concluded that these genes are involved in actin polymerization in oocyte meiosis.

In the next experiment, we selected 3 up-regulated (*Ras, MEK2,* and *JNK*) and 4 interesting most well known genes (*Raf1, MEK1, ERK,* and *p38*) in the pathway (Fig. 5). Names of these selected genes in MAPK signaling pathway were *Raf1* (Threonin-protein kinase), *MEK1* (Mitogen-activated protein kinase kinase 1), *MEK2* (Mitogen-activated protein kinase kinase 2), *ERK1* (Extracellular signal-regulated kinase 1), *ERK2* (Extracellular signal- regulated kinase 2), *JNK*

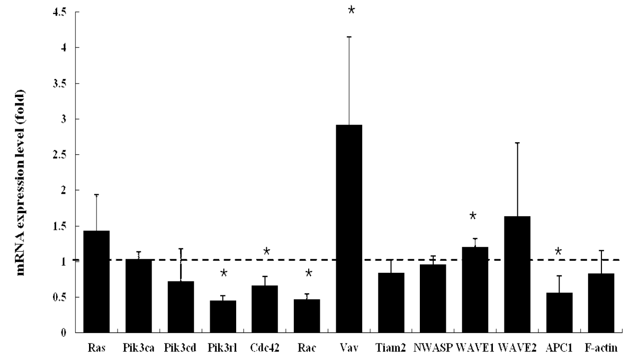

Fig. 4. Relative expression of 13 genes involved in the regulation of actin cytoskeleton systems in MI-arrested oocytes after *Zap70* RNAi. Expression of transcript was confirmed by quantitative real-time RT-PCR analysis. The X-axis represents the genes and the Y-axis shows the relative expression. Dotted horizontal line designates the normal expression level of each gene. The expression levels were calculated C_T values, and then mRNA expression ratio was determined relative to that of control MI oocytes. The number of biological replicates is three, and data were expressed as the mean±SEM. Asterisks represent statistical significance at p<0.05.

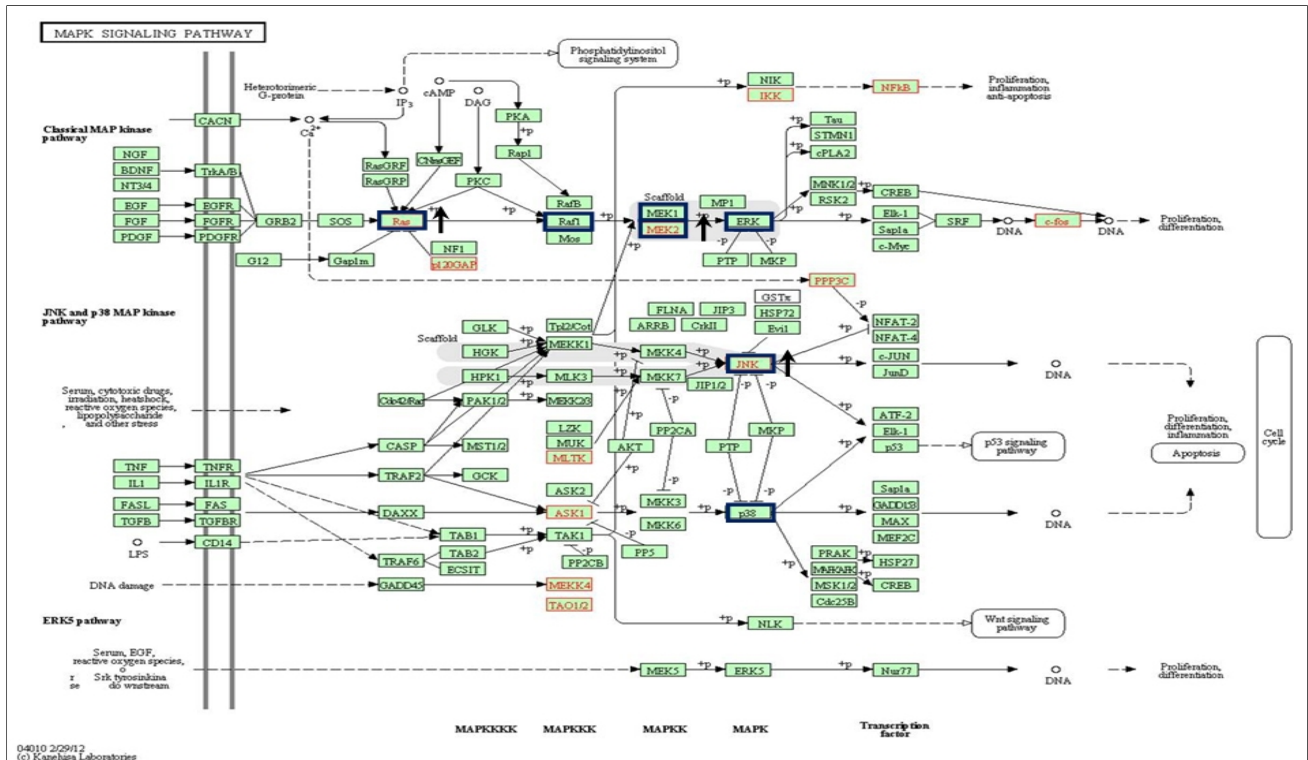

Fig. 5. GO and KEGG pathway display of transcripts encoding MAPK signaling pathway. The significantly up- and down-regulated transcripts by *Zap70* RNAi were defined by more than 2-fold change, and were shown in boxes that were marked in red. The genes confirmed by quantitative real-time RT-PCR are indicated in red and blue box. Black arrows showed up-(↑) and down-regulated genes (↓) by *Zap70* RNAi in microarray analysis.

(mitogen-activated protein kinase 10), and *p38* (mitogen-activated protein kinase 14). Its real-time RT-PCR data displayed that 2 genes (*MEK2* and *JNK*) were up-regulated and 5 genes (*Raf1, MEK1, ERK, ERK1* and *ERK2,* and *p38*) were down-regulated (Fig. 6). *Ras* was selected in both pathways and its real-time RT-PCR data was shown in Figure 4.

Previously, we found that *Zap70* RNAi affected expression of *ERK2, p38* and *JNK*, but not *ERK1* mRNA expression (Kim et al., 2009). MAPK has been known as a critical regulatory key factor during oocyte maturation in mammals (Sobajima et al, 1993; Abrieu et al., 2001; Zhang et al., 2010). Activation of MAPKs requires the dual-specific kinase MEK (MAPKK), which phosphorylates MAPK on critical threonine and tyrosine residues (Sun et al., 2002; Fan & Sun, 2004; Liang et al., 2007). MAPK family consists of three major subgroups: *ERK, JNK*, and *p38* (Davis et al., 1994). The firstly characterized subfamily of MAPK is Raf-MEK-ERK pathway (Schmitt & Nebreda, 2002). The Raf proteins have been shown to phosphorylate and activate MAPKKs called MEKs (MAPK or ERK kinases) which in turn phosphorylates and activates *ERK*s (Howe et al., 1992; Kyriakis et al., 1992). Therefore, we confirmed that the absence of Zap70 and following changes of MAPK components and its regulators resulted in abnormal spindle formation and aberrant chromosome configuration in mouse oocytes. In conclusion, we propose that *Zap70* is a critical key regulator of meiotic normal spindle formation and chromosome arrangement and it may act through regulation of many genes in the pathway of regulation of actin cytoskeleton and MAPK signaling in mouse oocytes.

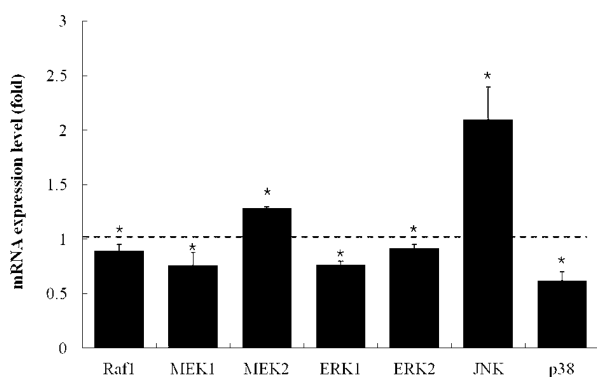

Fig. 6. Relative expression of 7 genes involved in MAPK signaling pathway in MI-arrested oocytes after *Zap70* RNAi. Expression of transcript was confirmed by quantitative real-time RT-PCR analysis. The X-axis represents the genes and the Y-axis shows the relative expression. Dotted horizontal line designates the normal expression level of each gene. The expression levels were calculated C_T values, and then mRNA expression ratio was determined relative to that of control MI oocytes. The number of biological replicates is three, and data were expressed as the mean±SEM. Asterisks represent statistical significance at $p<0.05$.

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