Immature Oocyte-Specific Zap70 and Its Functional Analysis in Regulating Oocyte Maturation

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ABSTRACT : Previously, we obtained the list of genes differentially expressed between GV and MII oocytes. Out of the list, we focused on functional analysis of Zap70 in the present study, because it has been known to be expressed only in immune cells. This is the first report about the expression and its function of Zap70 in the oocytes. Synthetic 475 bp Zap70 dsRNA was microinjected into the GV oocytes, and the oocytes were cultured *in vitro*. In addition to maturation rates, meiotic spindle and chromosome rearrangements, and changes in expression levels of transcripts of three kinases, Erk1/2, JNK, and p38, were determined. Zap70 is highly expressed in immature GV oocytes, and gradually decreased as oocyte matured. When dsRNA of Zap70 was injected into the GV oocytes, Zap70 mRNA specifically and completely decreased by 2 hr and its protein expression also decreased significantly. Absence of Zap70 resulted in maturation inhibition at meiosis I (57%) with abnormalities in meiotic spindle formation and chromosome rearrangement. Concurrently, mRNA expression of Erk2, JNK, and p38, were affected by Zap70 RNAi. Therefore, we concluded that Zap70 is involved in MI-MII transition by affecting expression of MAP kinases.

Key words : Mouse oocyte, Zap70, dsRNA, GV, MII, Maturation.

INTRODUCTION

Mammalian oocytes accumulate RNAs and proteins during oogenesis for oocyte maturation, fertilization, and further embryonic development (DeJong, 2006). Previously, we have acquired list of the differentially expressed genes between germinal vesicle (GV) and metaphase II (MII) using an annealing control primer-PCR method to determine molecular mechanism of meiotic arrest and meiotic resumption (Yoon et al., 2005). In the list, we found that expression of zeta- chain-associated protein kinase (Zap70) mRNA is GV- specific but not expressed in MII oocytes.

Protein-tyrosine kinases (PTKs) play an integral role in T-cell activation. Stimulation of the T-cell antigen receptor results in tyrosine phosphorylation of a number of cellular substrates, and one of these substrates is the TCR-zeta chain. Zap70 is associated with the zeta chain and undergoes tyrosine phosphorylation following TCR stimulation (Chan et al., 1992).

It has been well known that the oocyte maturation is regulated by key kinases, such as MPF and MAPK (Hashimoto & Kishimoto 1988; Sobajima et al., 1993). Human dual specific phosphatase, VHR, has been reported as an activator of MPF in Xenopus oocytes (Aroca et al., 1995). It has also been reported that Zap70 phosphorylates VHR protein (Alonso et al., 2003). Thus, we could speculate that Zap70 may be involved in the regulation of oocyte maturation.

Zap70 and Syk are structurally and functionally homo-

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logous tyrosine kinases that function in the T- and B-cell signal transduction (Laurenti et al., 2005; Mortarino et al., 2009). Contrast to T-cell specific expression of Zap70, Syk is expressed in various tissues (Fluck et al., 1995; Tsuchida et al., 2000; Tsujimura et al., 2001; Yamada et al., 2001; Yanagi et al., 2001). In the $Zap70^{-/-}$ mice which are appeared healthy and fertile, it is thought that Syk compensates the absence of Zap70 (Negishi et al., 1995).

Our finding of Zap70 expression in the mouse oocyte is a significant and new discovery. Therefore, the objectives of the present study were 1) to characterize the expression of Zap70 together with its related genes, VHR and Syk, during oocyte maturation, and 2) to determine the role of Zap70 in oocytes with relation of oocyte maturation by RNA interference (RNAi).

MATERIALS AND METHODS

1. Animals

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

2. Isolation of Oocytes and Cells

To obtain GV stage oocytes, 3-wk-old female ICR mice were injected with 5 IU of PMSG (Sigma, St. Louis, MO). Forty six hr after injection, mice were sacrificed and isolated ovaries were placed in M2 medium (Sigma). Cumulus-Oocyte-Complexes (COCs) were obtained by puncturing the preovulatory follicles in M2 medium supplemented with 0.2 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma) to inhibit the germinal vesicle breakdown (GVBD). Cumulus cells (CCs) surrounding GV oocytes were removed mechanically. The CCs and granulosa cells (GCs) were collected and washed twice with PBS.

3. Preparation of dsRNA

Two sets of primers were designed according to Zap70 mRNA sequence (accession number NM_009539, Table 1). For preparing the double-stranded RNA (dsRNA), 475 bp long primers (Zap70-A) were used, and the other 314 bp primers (Zap70-B) were used for confirmation of mRNA knockdown. Using the primers and 3-wk-old female mice ovary cDNA as template, PCR reaction was performed and amplified 475 bp product was gel eluted. Then it was cloned into pGEM-T Easy (Promega, WI, USA) and linearized with SpeI. Single-stranded RNA of each orientation was transcribed with RNAi Kit (Ambion, Austin, TX) and T7 RNA polymerase. For annealing, each strand of RNA was mixed and incubated at 75℃, cooled to the room temperature, and followed by 30 min incubation with RNase A (Ambion). Formation of the dsRNA was confirmed with 1% agarose gel and the dsRNA was diluted at concentration 2.2 $\mu g/\mu \ell$.

4. Microinjection of dsRNA and *In Vitro* Culture

Synthesized dsRNA was microinjected into the cytoplasm of GV oocytes in M2 medium containing 0.2 mM IBMX. Injections were performed as previously described (Kim et al., 2008). Briefly, an injection pipette containing dsRNA solution was inserted into the cytoplasm of a GV oocyte and 10 pl dsRNA was microinjected using a constant flow system (Transjector; Eppendorf, Hamburg, Germany) and this injection of 10 pl corresponds ~4.3×10^7 molecules, respectively. To evaluate the injection damage, buffer alone was injected as a sham control group. The meiotic status of oocytes was measured after 16 hr culture in M16 medium containing 0.3% BSA in 5% CO_2 at 37℃.

5. Messenger RNA Isolation and RT-PCR

Messenger RNAs were isolated from oocytes, CCs, and GCs by using Dynabeads mRNA isolation kit (Dynal Asa, Oslo, Norway) according to the manufacturer's instructions. Cells were suspended with 300 $\mu \ell$ lysis/binding buffer for 5 min at room temperature and mixed with 20 $\mu \ell$ of pre-

Table 1. Primer sets used for PCRs

Gene	Gene Bank Accession No.	Primer sequence	AT (℃)	Size (bp)
Zap70-A	NM 009539	F : CACCCATCCACATTCACTCA R : TCTTTCCCAGCAGGAACTTG	60	475
Zap70-B	NM 009539	F : ACAGAGAAGGCCGACAAAGA R : TCGCTGATCTTGGCATAGTG	60	314
GFP	EU 056363	F : ATGGTGAGCAAGGGCGAG R : CTTGTACAGCTCGTCCAT	60	717
H1foo	NM 138311	F : GCGAAACCGAAAGAGGTCAGAA R : TGGAGGAGGTCTTGGGAAGTAA	60	378
Mos	J00372	F : TGGCTGTTCCTACTCATTTC R : CTTTATACACCGAGCCAAAC	60	297
Plat	NM 008872	F : CATGGGCAAGAGTTACACAG R : CAGAGAAGAATGGAGACGAT	60	650
JNK	NM 016700	F : TGCCACAAAATCCTCTTTCC R : TGATGTATGGGTGCTGGAGA	60	307
p38	NM 011951	F : CGGCACACTGATGATGAGAT R : TGGGCCAGAGACTGAATGTA	60	275
Erk1	NM 011952	F : ATGAAGGCCCGAAACTACCT R : GCTCCATGTCGAAGGTGAAT	60	232
Erk2	NM 011949	F : ATCTGTGACTTTGGCCTTGC R : CCTTATTTTTGTGCGGGAGA	60	325

F and R in the primer sequences indicated forward and reverse.
Zap70-A primer used for RT-PCR and preparation of dsRNA.
Zap70-B primer used for confirm knockdown after RNAi.

washed beads. After 5 min for annealing, beads were separated with Dynal MPC-S magnetic particle concentrator and washed with 500 $\mu\ell$ wash buffer A twice and 300 $\mu\ell$ wash buffer B. Poly(A) + RNAs were eluted with 10 $\mu\ell$ of Tris-HCl. To evaluate the recovery, mRNA of Green Fluorescent Protein (GFP) synthesized at the laboratory was added to the lysate.

Isolated mRNA was reverse transcribed with Promega M-MLV reverse transcriptase (Promega) using oligo dT as a primer. An amount of synthesized cDNA equivalent to a single oocyte was used as a template for PCR reaction.

6. Oocyte Dot Blot

Oocyte lysates were made as previously described (Kim et al., 2008) and loaded onto a Hybond-P PVDF membrane (Amersham Biosciences). The membrane was blocked for 3-4 hr in Tris-buffered saline-Tween (TBS-T; 0.2M NaCl, 0.1% Tween-20, and 10 mM Tris [pH 7.4]), containing 5% dry milk at room temperature. The blocked membranes then were incubated with goat polyclonal anti-Zap70 antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) or mouse monoclonal anti-α-tubulin antibody (1:1000, Santa Cruz Biotechnology) in TBS-T overnight. After incubation, membranes were washed several times with TBS-T, and then were incubated with HRP conjugated anti goat (1:1000, Santa Cruz Biotechnology) or anti mouse IgG (1:500, Santa Cruz Biotechnology) in TBS-T for 3-4 hr. After the washing with TBS-T, the blot was detected using an enhanced

chemiluminescence (ECL) detection system (Santa Cruz Biotechnology).

To quantify the amounts of Zap70 protein, we measured the intensity of the area for each dot using Bio 1D software (Vilber Lourmat). These values were normalized with α-tubulin dot, and expressed as a percentage in comparison to that of control oocytes.

7. Non-invasive Examination of Meiotic Spindle Structure

Meiotic spindle structure of oocytes was observed with the LC Pol-Scope optics and controller system, combined with a computerized image analysis system (Oosight™ Meta Imaging System, CRI Inc., MA).

8. Aceto-Orcein Staining

Oocytes were stained with aceto-Orcein for observation of chromosome arrangement. For staining, oocytes were fixed with aceto-methanol (Acetic acid : Methanol = 1:3) solution at 4℃. Fixed oocytes were transferred onto a slide and covered with a clean cover-glass. And then, aceto-Orcein solution was placed between the slide and the cover-glass and photographed.

9. Statistical Analysis

Each experiment was performed at least three times. The data in the text was analyzed with chi-square method and shown as the means ± SEM (standard error of mean) and a value of $P< 0.05$ was considered significant.

RESULTS

1. Expression of Zap70 mRNA

To evaluate Zap70 mRNA expression in oocytes, as well as VHR and Syk, RT-PCR was performed as oocytes matured (Fig. 1). Zap70 mRNA was detected in GV and decreased gradually through oocyte maturation, and disappeared in mature MII oocytes. But either VHR or Syk expression was not detected in oocytes at any stage. Thus,

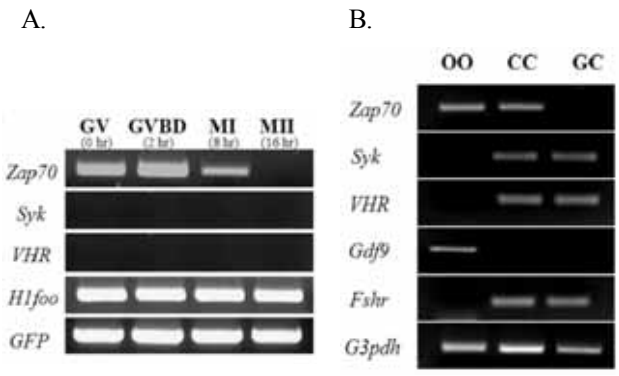

Fig. 1. Expression levels of Zap70, Syk and VHR mRNA in oocytes and follicular cells. A, Semi-quantitative RT-PCR analysis was performed on oocyte maturation. H1foo, GFP were used as internal control, external control, respectively. B, Determination of mRNA level in follicular components. OO, GV oocytes; CC, cumulus cells; GC, granulosa cells, Gdf9 mRNA was used as a marker for oocyte-specific expression, and Fshr mRNA used as marker for cumulus and granulosa cell. G3pdh was used as internal control.

we compared gene expression between GV oocytes, CCs, and GCs and confirmed that Zap70 was expressed in oocytes and CCs, VHR and Syk were expressed in CCs and GCs, but not in oocytes.

2. Effects of Zap70 RNAi

1) Specific Knockdown of Zap70 mRNA

To determine the degradation speed of endogenous Zap70 mRNA after Zap70 RNAi, oocytes were collected at 30 min interval during *in vitro* culture after RNAi, and RT-PCR was performed. Zap70 mRNA was constitutively expressed for 2 hr in control oocytes, but with RNAi, Zap70 transcript disappeared by 2 hr (Fig. 2A). To identify the knockdown of Zap70 protein, modified Western blot, namely oocyte dot blot was performed (Fig. 2B). Zap70 protein was also decreased in Zap70 RNAi oocytes. The changes in amount of mRNA and protein were expressed numerically as in Fig. 2C; mRNA was decreased almost completely (94%) but protein was decreased 40%.

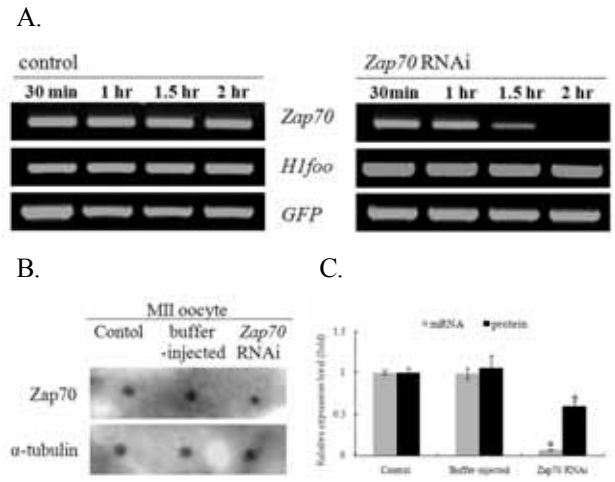

Fig. 2. Determination of mRNA and protein levels knockdown after Zap70 RNAi. A, Typical patterns of Zap70 mRNA degradation during *in vitro* oocyte maturation. B, Protein levels in oocytes were measured by dot blot analysis. Proteins were extracted from three MII oocyte for each dot. C, Zap70 mRNA and protein expression were calculated using Image-J software. * represents statistical significance at $p<0.05$

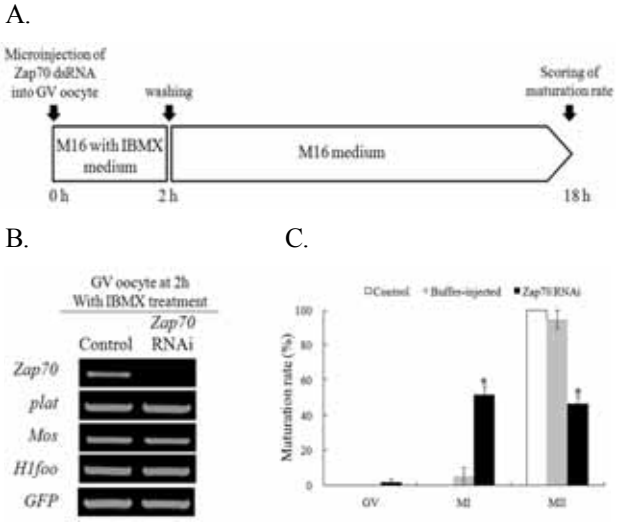

Fig. 3. Effect of Zap70 knockdown on oocyte maturation. A, B, After Zap70 RNAi, culture in M16 medium containing IBMX resulted in specific repression of Zap70 mRNA in GV oocyte. C, Maturation rates of oocytes after culture with IBMX for 2 hr followed by 16 hr of culture in plain M16 medium.

2) Oocyte Maturation

To assess the effect of Zap70 knockdown on oocyte maturation, oocytes were microinjected with Zap70 dsRNA, cultured in M16 medium, and maturation rates were scored. To give oocytes time for degradation of present Zap70 mRNA, oocytes were placed in IBMX supplemented medium for 2 hr prior to culture further 16 hr (Fig. 3A). Knockdown of Zap70 mRNA at the 2 hr point was confirmed by RT-PCR (Fig. 3B). Oocytes without either GV or polar body were regarded as MI. All control oocytes completed meiosis I, and 95% of buffer-injected sham control oocytes also underwent meiosis normally. But in the Zap70 RNAi group, only 47% oocytes extruded the first polar body that counted as metaphase II (MII) oocytes, and the rest of 52% oocytes were arrested at MI (Fig. 3C).

3) Abnormalities in Spindle and Chromosome Structures

Because the oocytes failed to complete the meiosis I, we

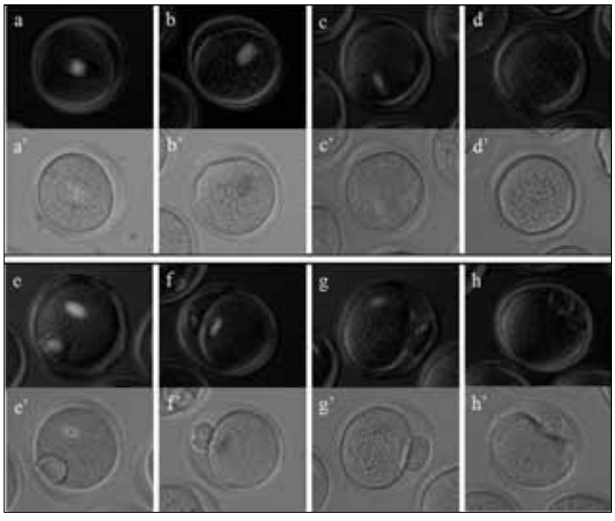

Fig. 4. Observation of the spindle structures in oocytes after Zap70 RNAi. The Pol-Scope images show the same oocytes under bright (upper column) and dark field (lower column). Microphotographs of a-d, MI and e-h, MII oocytes cultured *in vitro*. a and e Control oocytes; b and f, buffer- injected oocytes; c and g, Zap70 RNAi oocytes weakened spindle; d and h, Zap70 RNAi oocytes without any spindle.

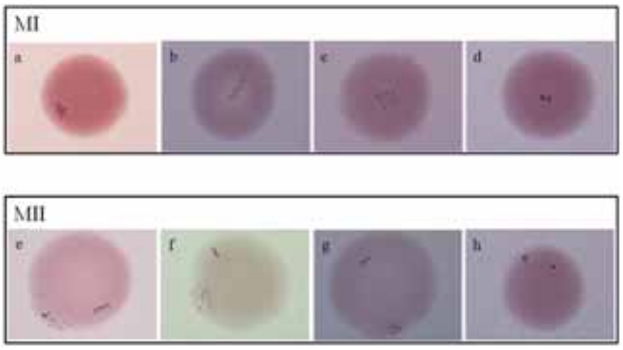

Fig. 5. Configuration of chromosomes following Zap70 RNAi. a and e, Control oocyte, b and f, buffer-injected oocyte, c and g, Zap70 RNAi oocyte and spindle existence, d and h, Zap70 RNAi oocyte and spindle absence.

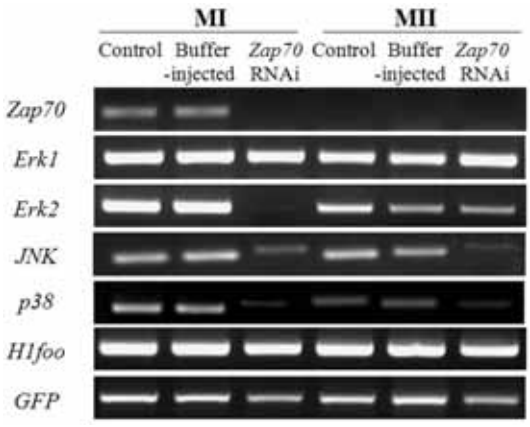

Fig. 6. Changes in mRNA expression levels of components of MAP kinase. RT-PCR analysis was used to determine mRNA level of components of MAP kinases (Erk1, Erk2, JNK, p38) in MI and MII oocytes after Zap70 RNAi.

evaluated the changes in the meiotic spindle and chromosome arrangements of oocytes without Zap70. Meiotic spindle was observed non-invasively using Pol-scope (Fig. 4). After Zap70 RNAi, 37.0% of MI oocytes and 45.7% of MII oocytes showed weakened spindle structure, but the remaining 63.0% of MI, 54.3% of MII oocytes did not show any spindle. No spindle structure resulted in severe chromosome abnormalities (Fig. 5).

4) Changes in the Expression of mRNA of MAP Kinases

To assess whether the expression of the MAP kinases (ERK1/2, JNK, p38) was influenced by the absence of Zap70, mRNA levels of the components of the well known three kinases were determined by RT-PCR (Fig. 6). Expression levels of Erk2, JNK and p38 were significantly changed but not that of Erk1 including internal house keeping gene, H1foo. Expression levels of JNK and p38 mRNA were decreased in both MI and MII oocytes after Zap70 RNAi, but Erk2 expression level was decreased only in arrested-MI oocyte.

DISCUSSION

Zap70 is cytoplasmic nonreceptor tyrosine kinase that associated with TCR signaling in activated T-cells (Chan et al., 1992). This protein is a member of the Syk family (Chan et al., 1991) and expressed only in T cells, NK cells, and thymocytes, and no transcript was detected in other tissues (Chan et al., 1992). Previously, we found the Zap70 expression in mouse immature GV oocytes (Yoon et al., 2005) and in the present study, we characterized its expression and analyzed the role of Zap70 on oocyte maturation.

During the oocyte maturation, Zap70 transcript decreased gradually and disappeared in mature MII oocytes. Without normal Zap70 expression, GVBD has occurred, suggesting Zap70 may not involved in initial event of the resumption of oocyte maturation. However, 52% of oocytes could not complete the first meiosis, but the rest of oocytes developed to MII (47%). We suggested the reason for this phenomenon as a threshold effect (Yoon et al., 2006). Each oocyte has various amount of Zap70 transcript and it may affect efficiency of Zap70 RNAi, so oocytes with less Zap70 transcript might have more rapidly knockdowned Zap70 and could not complete meiosis with abnormalities in the spindle and chromosome structures. Therefore Zap70 may be necessary for MI-MII transition, but not for GVBD.

Meiotic maturation in oocytes is a complex process that involves rearrangement of microtubules and actin filaments (Roth & Hansen 2005). After GVBD, the meiotic spindle forms and migrates to the cortex before polar body extrusion (Verlhac et al., 2000). GVBD and meiotic spindle formation are not regulated by microfilaments, but polarized movement of the chromosomes depends on a microfilament-mediated process in maturating oocytes (Sun & Schatten 2006). Microfilaments are needed for microtubule functions, and the segregation of chromosomes requires interaction between microtubules and microfilaments (Sun & Schatten 2006).

Zap70 is required for actin remodeling in T-cells; Zap70 leads to form a signaling complex including Vav1, activate Cdc42 and Rac1, and then promotes actin polymerization via multiple actin regulatory molecules like WASp (Wiskott-Aldrich syndrome protein) and WAVE2 (WAS protein family, member 2) (Burkhardt et al., 2008; Dombroski et al., 2005; Labno et al., 2003). Therefore, we suggest that knockdown of Zap70 may affect oocyte maturation by interrupting the actin polymerization. In Zap70 knockdowned oocytes, GVBD occurs normally and division couldn't occured because the asymmetric division was affected by disrupted actin network but GVBD was not.

In $Zap70^{-/-}$ mice, Syk is thought that compensated the absence of Zap70 (Negishi et al., 1995), and in the oocytes with Zap70 RNAi, Syk could also compensate the function. But we couldn't find out the expression of Syk in oocytes, cannot confirm that. Although Syk was not in oocytes, expressed in CCs and GCs, thus Syk protein was synthesized in CCs and GCs and might be transferred to the oocytes and functions at oocytes.

It has been reported that dual specific phosphatase VHR acts as a repressor of ERK activation, but not other MAPKs, JNK and p38 (Todd et al., 1999). Also, VHR protein participates in the process of meiotic maturation by activating MPF in Xenopus oocytes (Aroca et al., 1995). We previously found that all three MAPKs, Erk1/2, JNK, and p38, are expressed in mouse oocytes and its phosphorylation are occurred during oocyte maturation *in vitro* (unpublished). Therefore, we confirmed differences in the expression of 3 MAPKs in the present study that occurred after Zap70 RNAi in mouse oocytes. We found interesting results that Zap70 RNAi affected expression of Erk2, p38 and JNK, but not Erk1 mRNA expression.

MAPK has been known as a critical regulatory factor in oocyte maturation (Sobajima et al., 1993). MAPK activity is maintained high level from GVBD until 6-8 hr after fertilization. The MAPK pathway is activated after GVBD and is involved in meiotic resumption and regulation of microtubule organization (Fan & Sun 2004). Therefore, we concluded that the absence of Zap70 and following changes of MAP kinases resulted in abnormal spindle and chromosome arrangement in oocytes.

In conclusion, Zap70 is necessary for MI-MII transition and further investigation of the action mechanism of Zap70 in relation with MAPKs and cytoskeletal systems in oocyte maturation is valuable.

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