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Simultaneous Detection of Seven Phosphoproteins in a Single Lysate Sample during Oocyte Maturation Process

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난자성숙 과정의 단일 시료에서 일곱 가지 인산화 단백질의 동시 분석 방법

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목 적: 단백질 인산화는 세포신호전달에 매우 중요한 현상으로서, 수많은 조절인자들이 난자성숙에 관여하게 된다. 그러나 이들 중에서 어떤 단백질이 인산화되어 난자성숙을 조절하는지는 잘 알려져 있지 않다. 따라서 체세포의 신호전달과정에서 인산화를 통해 중요한 기능을 한다고 알려져 있는 일곱 가지 단백질들이 생쥐의 난자성숙과정에서 어떻게 인산화 되고 있는지 알아보고자 한 개 샘플에서 일곱 개의 변화를 한꺼번에 측정할 수 있는 bead-based multiplex phosphorylation assay를 이용하여 본 연구를 수행하였다.

연구방법: ICR 생쥐에 PMSG를 주사하고 46시간 후에 cumulus-oocyte complex (COCs) 형태로 미성숙 난자를 채취한 후 체외배양 하면서, 배양 2시간 후에 GVBD를, 배양 8시간 후에 MI을, 배양 16시간 후에 MII 단계의 난자를 얻었고 체내에서 배란한 MII 단계의 난자는 수란관에서 얻었다. 각 단계의 난자를 100개씩 모아서 mitogen-activated protein kinase (MAPK)에 속하는 세가지 단백질인 ERK1/2, JNK, p38 MAPK와 Akt, GSK-3α/β, IκBα, STAT3 등 총 일곱 단백질의 인산화를 Bio-Plex System을 이용하여 같은 시료에서 동시에 측정하였으며 세 번의 반복실험을 통하여 얻어진 결과를 통계적으로 분석하였다.

결 과: 생쥐의 난자성숙과정에서 측정된 일곱 가지 단백질 중에서 인산화가 현저히 증가하는 단백질로는 ERK1/2, JNK, p38 MAPK와 STAT3로서 미성숙 난자에 비해서 3배에서 20배까지 인산화되는 결과를 보였다. 반면에 GSK-3α/β, IκBα의 인산화의 변화는 미약하였으며, Akt의 경우에는 변화가 전혀 없었다. 난자성숙 과정에서 분석 대상 단백질들의 인산화는 GVBD 단계에서 활성화되기 시작하여 MI에서 현저히 높게 증가하며 MII까지 높게 유지되었다.

결 론: 본 연구는 난자성숙과정에서 일곱 가지 단백질의 인산화를 동시에 측정한 최초의 보고로서 이 방법은 난자와 같이 적은 양의 시료에서의 여러 개의 단백질 인산화를 동시에 분석하는데 유용할 것으로 생각된다. 본 연구 결과, 세 가지 MAPK 단백질인 ERK1/2, JNK, p38 MAPK 외에도 STAT3가 난자성숙에 있어서 매우 중요한 조절자로 생각되었다. 또한 Akt의 473번 serine기의 인산화는 난자성숙에 관여하지 않음을 알 수 있었다.

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중심단어: 난자성숙, 단백질 인산화, Mitogen-activated protein kinase (MAPK), Bio-Plex System

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Growing oocytes are arrested at the prophase I of the first meiotic division until they resume meiosis and progress to the metaphase of the second meiosis, where they are arrested again until they finally complete meiosis upon fertilization. It has been well known that this process, oocyte maturation, is controlled by several factors including maturation-promoting factor (MPF).[1] During the last decade, it has been determined that the mitogen-activated protein kinase (MAPK) cascade is a principal regulatory system that functions parallel to and interacts with MPF in driving the meiotic cell cycle of the oocyte.[2]

The family of MAPKs is involved in regulation of many signaling cascades in eukaryotic cells. Five groups of MAPK have so far been identified. The first group consists of extracellular signal-regulated kinase (ERKs) 1 and 2. The members of the second group are c-Jun N-terminal kinase (JNK) 1, 2 and 3. Isoforms α, β, γ and δ of kinase p38 comprise the third group. ERK3 and ERK4 are in the fourth group. The fifth group of MAPKs consists of large MAP kinase 1 (BMK1 or ERK5).[3~5] ERK1/2 plays an important role in cell proliferation and differentiation and in the area of oocyte maturation.[6] JNK was found in Xenopus oocytes,[7] and its involvement in mouse oocyte was reported in only one study.[8] JNK plays an active role in fragmentation of pig oocytes but p38 MAPK is not involved in the process.[9] Role of p38 MAPK on oocyte maturation has been reported in seastar,[10] Xenopus,[11] porcine,[2] and mouse.[12] In addition to MPF and MAPKs, many other signaling pathways may be involved in regulating oocyte maturation process.

It has been well recognized that the process of protein phosphorylation/dephosphorylation, which are mediated by protein kinases and protein phosphatases, play pivotal roles in the oocyte meiotic cell cycle.[6] However, measuring the amount of phosphorylation of one target protein by the classical method such as Western blot analysis requires many oocytes for one experiment. Recently, a new bead array phosphoprotein assay, Bio-Plex system (Bio-Rad, Hercules, CA) was introduced. This methodology allows the simultaneous analysis of the state of phosphorylation of several target proteins within a single 25 μl lysate sample.

The measurement of multiple proteins simultaneously in a single lysate is a powerful tool for proteomics and phosphoproteomics research, especially working with samples, such as oocytes, which are difficult to obtain in large quantities for biochemical studies. The present study was designed to evaluate the phosphorylation status of seven different proteins playing critical roles in different signal transduction systems by using the Bio-Plex system. We used seven types of beads coated with seven different phosphoprotein antibodies. Three MAPKs, namely ERK1/2, JNK, and p38 MAPK, and four other different signal transduction proteins such as Akt, GSK-3α/β, IκBα, and STAT3 were selected and detected during the mouse oocyte maturation.

MATERIALS AND METHODS

1. Mouse oocyte collection

Full-grown, GV-intact oocytes in a form of cumulus-oocyte complexes (COCs) were obtained from 4-week-old female ICR mice 46 h after intraperitoneal injection of 5 IU of pregnant mare's serum gonadotropin (PMSG; Folligon, Intervet). COCs were collected in the presence of 0.2 mM 3-isobutyl-1-methyl-xanthine (IBMX; Sigma, St. Louis, MO) in M2 medium in order to maintain the oocytes at the GV stage, then cultured in M16 medium containing 3 mg/ml bovine serum albumin (BSA; Sigma) for 2 h (GVBD), 8 h (MI), and 16 h (MII_vitro). After culture of COCs, cumulus cells were removed from the oocytes by repeated pipetting.

To obtain in vivo matured MII oocytes (MII_vivo), ICR female mice were injected with 5 IU of PMSG and human chorionic gonadotropin (hCG, Chorulon, Intervet) 46 h apart. Superovulated MII oocytes were obtained

Table 1. Phophorylated sites of the seven target proteins used in the present study

Name of phosphoprotein	Phosphorylation site
Akt	Ser^{473}
ERK1/2	Thr^{202}/Tyr^{204}
	Thr^{185}/Tyr^{187}
GSK	Ser^{21}/Ser^{9}
IκBα	Ser^{32}/Ser^{36}
JNK	Thr^{183}/Tyr^{185}
p38 MAPK	Thr^{180}/Tyr^{182}
STAT3	Tyr^{705}

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16 h after hCG injection. Cumulus cells surrounding MII oocytes were removed by treatment with hyaluronidase (300 U/ml; Sigma). Denuded oocytes were washed in phosphate-buffered saline (PBS) containing 0.1% polyvinyl alcohol (PVA; Sigma) for Bio-Plex analysis, Western blot analysis and kinase assay.

2. Bio-Plex analysis

Assays for changing in amount of phosphoproteins during oocyte maturation were performed by using the Bio-Plex 2200 system (Bio-Rad). In order to assess the relative level of phosphorylation in a given sample, 100 oocytes at each stage were used. Assays were carried out three times according to the manufacturer's protocol. On the day of assay, oocytes were treated with lysis buffer (40 μl of factor 1, 20 μl of factor 2, and 40 μl of 500 mM PMSF in 9.9 ml lysis buffer), while Bio-Plex filter plate was pre-wet with buffer prior to the sample addition. The coupled beads for seven different important protein targets (Akt, ERK1/2, GSK-3α/β, IκBα, JNK, p38 MAPK, and STAT3) were premixed for multiplex assays. Phosphorylation sites of the target proteins are summarized in Table 1. After loading bead solutions and samples, the plate was incubated with shaking at 4℃ overnight. After three times of washing the filter plate, detection antibodies were added for 30 min followed by addition of Streptavidin-PE for 10 min. At the end of incubation, plate was agitated to resuspend beads for reading the amount of phosphorylation by using Bio-Plex 2200. The fold change in phosphorylation state was calculated in comparison to that of GV oocyte as a control.

This instrument combines Lab-MAP multi-analyte profiling technology (Luminex, Austin, TX) with unique antigen-coated fluoromagnetic bead chemistry and versatile software. Briefly, dyed (fluorescent) bead sets, each of which is coated with a different, specific antibody, are mixed with sample. The principle of phosphoprotein assays using xMAP internally labeled beads is similar to that of a capture sandwich immunoassay (Bio-Rad). An antibody directed against the desired phosphoprotein target is covalently coupled to 5.6 μm fluorescent dye beads. The coupled beads are allowed to react with a sample containing an unknown amount of phosphoprotein target. After a series of washes to remove unbound protein, a biotinylated detection antibody specific to a different epitope on the target protein is added to the reaction. The result is the formation of a sandwich of antibodies around only phosphorylated target protein. The reaction mixture is detected by the addition of streptavidin-phycoerythrin, in which the streptavidin binds to the biotinylated detection antibodies.

3. Western blot analysis

Western blotting for ERK1/2 and JNK was conducted to confirm the results obtained from the Bio-Plex system. The phosphorylated ERK1/2 was detected by anti-phospho-ERK1/2 antibody in oocytes at 0, 2, 8, and 16 h after culture (GV, GVBD, MI, and MII, respectively), and super-ovulated MII (MII_vivo) oocytes. After denudation, groups of 50 oocytes were rinsed twice in PBS-PVA, dissolved in 15 μl of 2× sample buffer, boiled

for 5 min, and frozen at -20℃ before use. Samples were run on 10% SDS-polyacrylamide gels, and transferred to a hydrophobic polyvinylidene difluoride membrane (Immobilon; Millipore Co., Bedford, MA). The membrane was blocked with 5% non-fat dry milk in TBS-T (0.2 M NaCl, 0.1% Tween-20, 10 mM Tris pH 7.4) for 1 h.

For detection of phosphorylated ERK1/2, the membrane was incubated in 1% BSA in TBS-T overnight with rabbit monoclonal anti-phospho-ERK1/2 (1:1000; Cell Signaling, Beverly, MA) at 4℃. The total amount of ERK1/2 on the same membrane was assayed for loading control by using a rabbit polyclonal anti-ERK1/2 (1:1000; Cell Signaling) after stripping off the initial bound antibody. After washing in TBS-T, the membrane was treated with horseradish peroxidase (HRP)-conjugated anti-rabbit second antibody (1:2000; Cell Signaling) in blocking buffer for 1 h at room temperature. After three washes of 10 min each with TBS-T, peroxidase activity was visualized using the Western blotting luminal reagent system (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

4. Dual kinase activity assay

To examine the activities of MPF and ERK1/2 in mouse oocytes at the same time, the phosphorylation of myelin basic protein (MBP) and histone H1, substrates for ERK1/2 and MPF, respectively, was measured by dual kinase activity assay. After denudation and three washes in 0.1% PBS-PVA, the same number (1 or 3) of oocytes at each meiotic stage was transferred into an Eppendorf tube with 1 μl of PBS-PVA for the double kinase assay. Thereafter, 4 μl of ice-cold extraction buffer was added, and samples were kept at -70℃ until kinase assay. The extraction buffer was composed of 80 mM β-glycerophosphate, 25 mM HEPES (pH 7.2), 20 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM APMSF, 0.1 mM Na_3VO_4, 1 μg/ml leupeptin (Sigma), and 1 μg/ml aprotinin (Sigma).

After thawing on ice, samples were centrifuged, added to 5 μl of kinase buffer and 5 μl of substrate solution, and incubated for 20 min at 37℃. The kinase buffer was composed of 75 mM HEPES (pH 7.2), 75 mM β-glycerophosphate, 75 mM $MgCl_2$, 6 mM DTT, 10 mM EGTA, 60 μM ATP, 15 μM cAMP-dependent protein kinase inhibitor peptide (Sigma) and 0.3 μCi/μl [γ-^{32}P]ATP (250 μCi/25 μl, Amersham, Piscataway, NJ). The substrate solution for the double assay was a mixture of 4.5 μl of histone H1 (5 mg/ml; Roche, Indianapolis, IN) and 0.5 μl of MBP (5 mg/ml; Sigma). The reaction was terminated by the addition of 5 μl of 4× SDS sample buffer. Then samples were boiled for 5 min and loaded onto a 15% gel for separation of labeled histone H1 and MBP. After running, gels were dried and autoradiographed.

5. Protein kinase inhibitor treatment

To confirm the role of Akt, JNK, as well as ERK1/2 during oocyte maturation, we treated well-known inhibitors of those protein kinases during in vitro maturation, such as Akt inhibitor II (Calbiochem, La Jolla, CA), SP600125 (Calbiochem, San Diego, CA), and U0126 (Cell Signaling) for a specific inhibitor of Akt, JNK, and MEK1/2, respectively.

COCs were obtained according to the method explained previous session and cultured in the presence and absence of protein kinase inhibitors at concentration of 50 μM for U0126 and 100 μM for SP600125 and Akt inhibitor II during 16 h of culture period. These inhibitors were dissolved in dimethylsulphoxide (DMSO; Sigma), and DMSO was added at 0.2% (v/v) as a vehicle control group.

6. Data analysis

Statistical analysis was performed by using one-way analysis of variance (ANOVA) and log linear model.

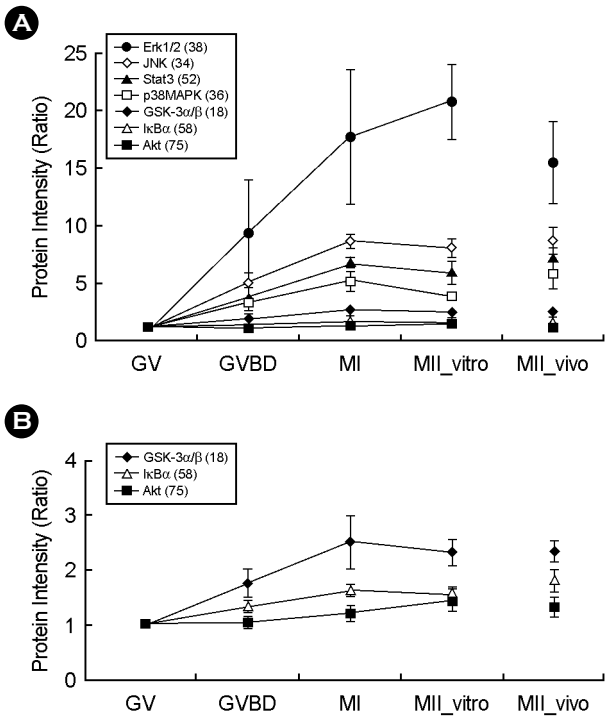

Figure 1. Changes in phosphorylation of seven different signal transduction proteins in various stages of oocytes according to the time-sequence of mouse in vitro oocyte maturation. GV, germinal vesicle (0 h); GVBD, germinal vesicle breakdown (2 h); MI, metaphase I (8 h); MII_vitro, in vitro cultured metaphase II (16 h); MII_vivo, in vivo developed metaphase II oocytes. The particular number of bead is indicated in each parenthesis. **(A)** Phosphorylation of seven proteins. **(B)** Phosphorylation of three proteins that showed minor changes less than 3 folds. Numbers in parentheses in the box indicates bead number of the Bio-Plex system.

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Data were shown as the mean ± SEM and a value of $p<0.05$ was considered to be statistically significant.

RESULTS

1. Analysis of phosphorylation by Bio-Plex

Phosphorylation of six proteins except Akt was detected from GVBD stage (2 h culture) and maximized and/or maintained at MI-MII. The phosphorylation of STAT3 and three MAPKs such as ERK1/2, JNK, and p38 MAPK was changed over 3 folds up to 20 folds (Figure 1A), while phosphorylation of the other three signal molecules such as Akt, GSK-3α/β, and IκBα was less than 3 folds (Figure 1B). Therefore, three MAPKs and STAT3 were grouped as major group, while the other three proteins were grouped as minor group. All of the changes except Akt were statistically significant ($p<0.05$). Among seven proteins, only the phosphorylation of Akt was not detected.

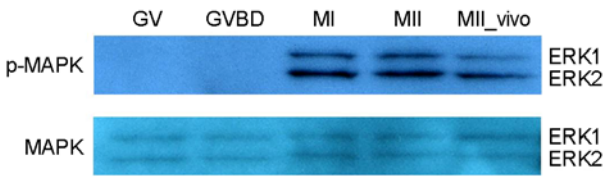

Figure 2. Expression of the ERK1/2 MAPK in mouse oocytes. The whole lysate of 50 oocytes at each meiotic stage was subjected to Western blot analysis with anti-phospho ERK1/2 antibody (p-MAPK, upper). The same membrane was re-probed to detect total ERK1/2 (MAPK, lower) for loading control. GV, germinal vesicle (0 h); GVBD, germinal vesicle breakdown (2 h); MI, metaphase I (8 h); MII, in vitro cultured metaphase II (16 h); MII_vivo, in vivo developed metaphase II oocytes.

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2. Expression and activation of ERK1/2 and JNK during oocyte maturation

Western blot analysis showed a good correlation with results obtained by Bio-Plex with no detection of phosphorylation in GV and highly increased phosphorylation in MI and MII stages in each of 50 oocytes (Figure 2). Membranes were re-probed with anti-ERK1/2 antibody for loading control. Both bands of non-phosphorylated total ERK1/2 (44 and 42 kDa) were detected at similar levels in every meiotic stage. However, we could not detect neither total nor phospho-JNK bands even in the lysate from 100 oocytes (data not shown). This result implies that the amount of total and phospho-JNK proteins, compared to those of ERK1/2, might too small to be detected by Western blot analysis.

3. Dual kinase activity assay

We also evaluated the activation pattern of MPF and ERK1/2 during oocyte maturation according to its ability to phosphorylate their substrates histone H1 and MBP, respectively by using dual kinase activity assay. We observed the highest level of phosphorylated MBP as well as histone H1 at MI and MII stages (Figure 3).

4. Effect of protein kinase inhibitors

The effects of inhibitors for Akt, JNK, and ERK1/2 (MEK inhibitor) on the in vitro mouse oocyte maturation were shown in the Figure 4. As expected, Akt inhibitor treatment showed no effect on the maturation rates, and the maturation rates were similar in control, vehicle control, and Akt inhibitor treatment groups.

However, treatment of U0126 (ERK1/2 inhibitor) resulted in the inhibition of oocyte maturation. Majority of oocytes were stopped at MI (80.7%) stage, and the

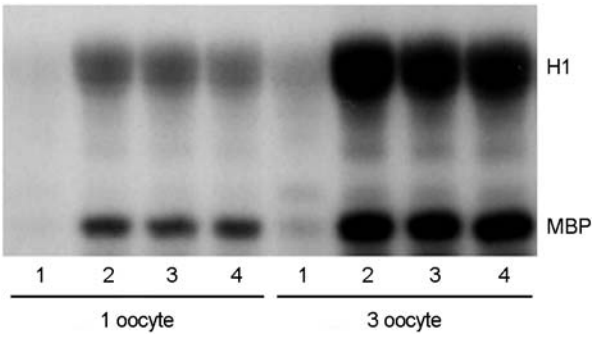

Figure 3. Dual kinase activity assay to assess the activities of MPF and ERK1/2, respectively. One or three oocytes were loaded per lane. Lane 1: GV, germinal vesicle (0 h); lane 2: MI, metaphase I (8 h); lane 3: MII_vitro, in vitro cultured metaphase II (16 h); lane 4: MII_vivo, in vivo developed metaphase II oocytes.

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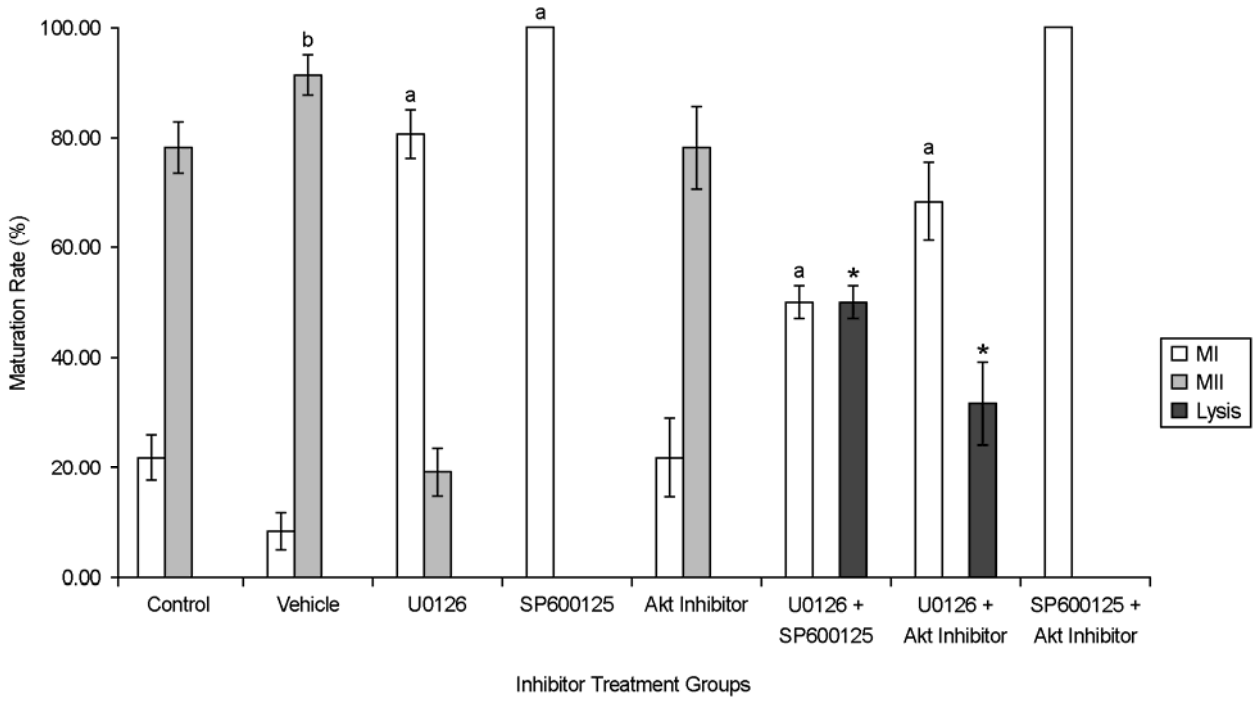

Figure 4. Effects of U0126 (ERK1/2 inhibitor), SP600125 (JNK inhibitor) and Akt inhibitor II on the mouse oocyte maturation in vitro. The solution of 0.2% DMSO was added for vehicle control. U0126 and SP600125 treatment inhibited meiotic resumption of oocytes and arrested the majority of oocytes at metaphase I. Akt inhibitor II showed no effects for the oocyte maturation.

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percentages of oocytes reaching MII was reduced significantly (19.3%). The treatment of SP600125 (JNK inhibitor) showed the same pattern, and all oocytes was arrested at MI stage (100%).

Treatment of dual inhibitors in combination confirmed their inhibitory effects. When inhibitors for ERK1/2 and JNK combined, 50% oocytes were arrested at MI and 50% oocytes were dead by lysis. When inhibitors for JNK and Akt combined, 68.3% oocytes were arrested at MI and 31.7% oocytes were dead by lysis. However, Akt inhibitor did not show additive effect of JNK on inhibition of oocyte maturation.

DISCUSSION

By applying a new technology of a multiplex assay of phosphoproteins, namely the Bio-Plex system, we succeeded in measuring the changes in the phosphorylation of seven different important proteins simultaneously in a single tube of lysate samples from various stages of oocytes during maturation process. We found that: 1) phosphorylation of all three MAPKs, ERK1/2, JNK, and p38 MAPK are involved in mouse oocyte maturation, 2) substantial phosphorylation of STAT3 also occurs during oocyte maturation, and 3) phosphorylation of Akt is not altered during the gonadotropin-independent spontaneous oocyte maturation of the mouse.

The MAPK superfamily consists of ERK1/2, JNK, and p38 MAPK, and all of these are serine-threonine protein kinases that have multiple functions in various biological processes.[3~5] The role of the MOS/MEK1/ERK1/2 system on the oocyte maturation in many species including the mouse has been intensively studied and many reviews have been published.[6,13,14] However, results have been conflicting and vary according to the species and culture conditions, and role of MAPK during oocyte meiotic resumption remain unclear. Relatively little attention has been paid to the role of JNK and p38 MAPK compared to that of ERK1/2 in mouse oocyte maturation. In the present study, we succeeded in finding that three MAPKs are involved in the mouse oocyte maturation by revealing their phosphorylation patterns according to maturational stage by using Bio-Plex system. Phosphorylation of ERK1/2 showed the biggest change (around 20 folds), while that of JNK and p38 MAPK increased up to 10 folds and 5 folds, respectively (Figure 1).

The confirmation of the involvement of MAPKs during oocyte maturation was conducted by parallel experiments using Western blot analysis, kinase activity assay, and kinase inhibitor treatment. Western blot analysis showed confirmatory results in comparison with the Bio-Plex system in the case of ERK1/2 (Figure 2). However, JNK protein, total or the phosphorylated form, was not detected using up to 100 oocytes, the twice number of ERK1/2 (data not shown). This implies that the amount of total and phosphorylated JNK is too low to be detected by Western blot analysis. These data suggest that the detection of changes in various different proteins simultaneously, even at very low expression levels by using the Bio-Plex system is a powerful technique to apply when working with small quantities of sample.

We also confirmed the activation of ERK1/2 during oocyte maturation according to its ability to phosphorylate MBP simultaneously together with the activation of MPF to phosphorylated Histone H1 using dual kinase activity assay in a single mouse oocyte. We observed the highest level of phosphorylated MBP as well as histone H1 at MI and MII stages (Figure 3). It is very powerful tool with reproducible results as reported in our published studies of the effect of RNA interference (RNAi) in a single oocyte during the oocyte meiotic process.[15,16] This dual kinase analysis is applicable with different substrate combinations such as activated transcription factor 2 (ATF-2) and MBP as the substrate of p38 MAPK and ERK1/2, respectively.[2] Histone H1 and histone H3 were

also used for the substrates of Cdc2 kinase and histone H3 kinase, respectively.[17]

The role of ERK1/2 in the events of spontaneous oocyte maturation has been studied extensively, and known that it activated after GVBD and MPF activation in mouse, and rat.[18~20] However, ERK1/2 is activated before GVBD in porcine and bovine spontaneous oocyte maturation.[21,22] As the case of ERK1/2 in the mouse, JNK also appears to be involved more to the MI-MII transition rather than the GVBD (Figure 4). Although JNK protein was not detected by Western blot analysis, its involvement in oocyte maturation was confirmed by measuring maturation rates after treatment with a JNK inhibitor during in vitro maturation. The JNK inhibitor, SP600125, at 100 μM inhibited normal oocyte maturation with arrest occurring at the MI stage (Figure 4). However, further examinations such as the study of phosphorylation status after the treatment of various inhibitors using Bio-Plex, Western blotting or kinase assay are required. Two possibilities can be given to explain the phenomenon of MI arrest after treatment of various MAPK inhibitors from the start point of in vitro culture of GV oocytes. First, there are likely to be many signal transduction pathways involved for germinal vesicle breakdown rather than just one MAPK exclusively involved. The other possibility is the involvement of other MAPK-independent mechanisms, such as the well-known MPF, and other signal transduction pathways. There is also likely to be substantial cross-talk among MAPKs or between MAPK and the other signal transduction pathways. The simultaneous treatment of ERK1/2 inhibitor, U0126 with SP600125 or Akt inhibitor resulted in high rates of cell lysis (Figure 4). However, there was no lysis phenomenon in the treatment of SP600125 with Akt inhibitor. It is possible that ERK1/2 inhibitor induced synergistic effect of apoptotic cell death with concomitant JNK or Akt inhibitor treatment.

Phosphorylation of Akt increased slightly (up to 1.5 folds) in MII stage, and was not statistically significant. Treatment of an Akt inhibitor during in vitro maturation confirmed that the signal transduction pathway including Akt protein is not involved in mouse oocyte maturation. Conflicting results regarding involvement of the Akt system in oocyte meiotic maturation may have resulted from the use of different source of oocytes in different species and with different culture conditions, such as in the presence of cumulus cells and hormones in the medium.[23~25] Phosphorylation of Akt occurs at two residues, Thr^{308} and Ser^{473}.[26] It has been reported that phosphatidylinisitol 3-kinase (PI3K) and its downstream target Akt participated in the gonadotropin-induced meiotic maturation of mouse oocytes, and phosphorylation of Akt has been involved separately according to the position of phosphorylation.[23] Unfortunately, these previous data used a gonadotropin-induced maturation system and measured Akt activation after stimulating its upstream PI3K. In contrast, we evaluated changes of phosphorylation in a spontaneous maturation system and the antibody we used for Bio-Plex system in this study only measured Ser^{473} phosphorylation. Therefore, involvement of Akt in the regulation of the mouse oocyte maturation needs to be scrutinized further.

Phosphorylation of the other two proteins, GSK-3α/β, and IκBα, were slightly changed (~3 folds), but may be sufficient to induce certain physiological processes. Results of immunoblot analysis of GSK-3α and β in fully-grown GV, MI, and MII mouse oocytes have been reported,[27] but these data assessed total protein, not the phosphorylated form. In addition, it is well known that activities of GSK-3α/β are negatively regulated by serine phosphorylation but positively controlled by tyrosine phosphorylation.[27] Taken together, the increase in GSK-3α/β phosphorylation during oocyte maturation measured in the present study is thought to play an important role in maturation, but in an opposite direction compared to the other signaling pathways in terms of inducing

meiotic resumption. More study will be necessary in this area.

Changes in the phosphorylation of STAT3 were substantial compared to that of p38 MAPK. The presence of STAT3 in mouse MII oocytes and its phosphorylation by leptin has been reported by Matsuoka and coworkers. They suggested a possible role of leptin during oocyte maturation through the STAT3 signal transduction pathway.[28] The present study is the first demonstration of changing phosphorylation of STAT3 in oocytes according to the changes in maturational status. STAT proteins become activated by means of a variety of soluble factors, such as cytokines, growth factors, and hormones.[26] In addition to leptin, other stimulatory signals for STAT3 and its downstream signal transduction pathways would be other areas of investigation.

In conclusion, by using a new, simple, and relatively easy technique, namely Bio-Plex multiplex assay system, we obtained several significant new findings. The Bio-Plex system is a powerful and useful tool to study phosphoproteomics in the area where dealing with small amount of sample such as mammalian oocytes and preimplantation embryos. Results of the present study will contribute to uncover the molecular mechanisms involved in mammalian oocyte maturation.

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= **Abstract** =

Objective: Phosphorylation and dephosphorylation of proteins are important in regulating cellular signaling pathways. Bead-based multiplex phosphorylation assay was conducted to detect the phosphorylation of seven proteins to maximize the information obtained from a single lysate of stage-specific mouse oocytes at a time.

Methods: Cumulus-oocyte complexes (COCs) were cultured for 2 h, 8 h, and 16 h, respectively to address phosphorylation status of seven target proteins during oocyte maturation process. We analyzed the changes in phosphorylation at germinal vesicle (GV, 0 h), germinal vesicle breakdown (GVBD, 2 h), metaphase I (MI, 8 h), and metaphase II (MII, 16 h in vitro or in vivo) mouse oocytes by using Bio-Plex phosphoprotein assay system. We chose seven target proteins, namely, three mitogen-activated protein kinases (MAPKs), ERK1/2, JNK, and p38 MAPK, and other 4 well known signaling molecules, Akt, GSK-3α/β, IκBα, and STAT3 to measure their phosphorylation status. Western blot analysis and kinase inhibitor treatment for ERK1/2, JNK, and Akt during in vitro maturation of oocytes were conducted for the confirmation.

Results: Phosphorylation of ERK1/2, JNK, p38 MAPK and STAT3 was increased over 3 folds up to 20 folds, while phosphorylation of the other three signal molecules, Akt, GSK-3α/β, and IκBα was less than 3 folds. All of these results except for Akt were statistically significant ($p<0.05$).

Conclusion: This is the first report on the new and valuable method measuring many phosphoproteins simultaneously in one minute sample such as oocyte lysates. All of the three MAPKs, ERK1/2, JNK, and p38 MAPK are involved in the process of mouse oocyte maturation. In addition, STAT3 might be important regulator of oocyte maturation, while Akt phosphorylation at Serine 473 may not be involved in the regulation of oocyte maturation.

Key Words: Oocyte maturation, Protein phosphorylation, Mitogen-activated protein kinase (MAPK), Bio-Plex system