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PI3-kinase and mitogen-activated protein kinase in cumulus cells mediate EGF-induced meiotic resumption of porcine oocyte

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Abstract

Previous studies have shown that epidermal growth factor (EGF) has the ability to promote *in vitro* cultured porcine oocyte maturation. However, little is known about the detailed downstream events in EGF-induced meiotic resumption. We designed this study to determine the relationship of EGF, EGFR, phosphatidylinositol 3-kinase (PI3-kinase), MAPK, and germinal vesicle breakdown (GVBD) during oocyte maturation. Our results showed that GVBD in cumulus-enclosed oocytes (CEOs) but not in denuded oocytes (DOs) was induced by EGF in a dose-dependent manner, which indicated that cumulus cells but not oocyte itself were the main target for EGF-induced meiotic resumption. Furthermore, we found that MAPK in cumulus cells rather than in oocyte was activated immediately after EGF administration. To explore whether EGF exerts its functions through MAPK pathway, the activities of EGF receptor (EGFR) and MAPK were inhibited by employing AG1478 and U0126, respectively. Inhibition of MAPK blocked EGF-induced GVBD, whereas inhibition of EGFR prevented MAPK activation. Both AG1478 and U0126 could lead to the failure of EGF-induced GVBD singly. Notably, we found that LY294002, a specific inhibitor of PI3-kinase, effectively inhibited EGF-induced MAPK activation as well as subsequent oocyte meiotic resumption and this inhibition could not be reversed by adding additional EGF. Thus, PI3-kinase-induced MAPK activation in cumulus cells mediated EGF-induced meiotic resumption in porcine CEOs. Together, this study provides evidences demonstrating a linear relationship of EGF/EGFR, PI3-kinase, MAPK and GVBD and presents a relatively definitive mechanism of EGF-induced meiotic resumption of porcine oocyte.

Keywords: Meiotic resumption; EGF/EGFR; MAPK; PI3-kinase; Cumulus cells

1. Introduction

Oocytes in most mammals are arrested at the diplotene stage of the first meiotic prophase, which is termed germinal vesicle (GV) stage. GV stage oocytes acquire the competence of resuming meiosis during the growth and development. *In vivo*, fully grown oocytes resume meiosis upon the stimulation of the LH surge, as indicated by germinal vesicle breakdown (GVBD), followed by entering metaphase of meiosis I (MI) and first polar body (PBI) emission. *In vitro*, oocytes can resume meiosis spontaneously when they are released from the inhibitory environment of follicles, as a result

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of breakdown in gap junction communication between different follicle compartments and interruption of the action of cAMP or other inhibitory molecules on the oocyte [1]. But the collected oocytes cannot achieve the same quality as those matured *in vivo*. Thus, it is important to search the factors participating in the process of oocyte maturation.

In current culture system, many different growth factors including epidermal growth factor (EGF) were added into the culture medium. It has been reported that administration of EGF can induce oocyte maturation in many species such as rat [2], mouse [3–5], goat [6–8], cow [9–11], pig [12–18] and human [19,20]. *In vivo*, EGF exists in follicular fluid [21,22] and EGF receptor (EGFR) exists not only in cumulus cells [9,23–30] but also in oocyte itself [23,27–31]. Although LH induces oocyte meiotic resumption, its role seems to be indirect because of the restricted expression of LH receptor in oocyte and the adjacent cumulus cells. A more recent study indicated that LH-stimulated meiotic resumption was mediated by EGF signal pathway [32]. All these findings indicate that EGF signal pathway may play an important role in *in vitro* and *in vivo* oocyte maturation. However, the exact molecular mechanism involving in the EGF cascade is still an enigma.

Mitogen-activated protein kinase (MAPK), also termed extracellular-regulated kinase (ERK), plays pivotal roles in oocyte maturation and fertilization [1,33,34]. The most widely studied MAPKs in oocyte are 44- and 42-kDa MAPK isoforms (ERK1 and ERK2, receptively), which can be phosphorylated by MEK (MAPK kinase), a molecule upstream of MAPK, on the serine and threonine residues to become fully activated [35]. Recent experimental evidences from our laboratory and others showed that MAPK in cumulus cells played key roles in oocyte meiotic resumption [34,36–43]. Intriguingly, a disputable understanding emerged considering these regulations and functions of MAPK pathway in meiosis. Meiotic resumption of *Xenopus* oocyte is induced by progesterone, which exerts its effect on oocyte directly and stimulates the MAPK pathway [44,45]. Additionally, in bovine oocytes, injection of mos mRNA elicited a rapid maximal activation of MAPK that resulted in accelerated resumption of meiosis, suggesting a positive effect of MAPK activation in oocyte itself on GVBD [46]. Another group found that just before GVBD, part of the activated MAPK translocated into the GV and exogenous MAPK injected into the GV induced GVBD in porcine oocytes [47,48]. Here, we first provide evidences to clarify the definite contribution of MAPK in cumulus cells and demonstrate its crosstalk with EGF/EGFR during meiotic resumption in porcine oocytes.

Phosphatidylinositol 3-kinase (PI3-kinase), which is an important enzyme in insulin and EGF [49–51] signal transduction, phosphorylates D-3 position of the inositol ring to produce phosphatidylinositol-3-phosphate and its analogues [52–54]. Existing evidence has demonstrated that PI3-kinase plays critical roles in meiotic resumption in *Xenopus* [55,56], mouse [57–59], rat [60], bovine [61,62] and porcine [63–66] oocytes. Further, some reports have implicated PI3-kinase as an important regulator during oocyte maturation through gap junctions between oocyte and cumulus cells [65] as well as during apoptosis [67,68]. More significant, Ras may contribute to progesterone-induced maturation by interacting with PI3-kinase since PI3-kinase -related enzyme is crucial for human Ras-induced MPF activation [69]. On the other hand, MOS/MEK/MAPK and PI3-kinase/Ras/Raf pathways have been thought to be two relative independent cascades during meiotic resumption [34,56], and there is no information about the definitive relationship between PI3-kinase and MAPK in cumulus cells of cumulus-enclosed oocytes (CEOs).

In this study, we investigated the mechanism of EGF-induced meiotic resumption in porcine CEOs. By acting on its receptor, EGF participates in the activation of MAPK in cumulus cells and the promotion of GVBD. As an essential intermediate molecule, PI3-kinase was also examined in this cascade and got its new position in this EGF-MAPK signal way.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO) except for those specifically mentioned. Monoclonal mouse anti-pERK1/2 antibody and polyclonal rabbit anti-ERK2 antibody were both purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Stock solutions of U0126 (10 mM, Calbiochem, La Jolla, CA), AG1478 (10 mM, Calbiochem), AG43 (10 mM, Calbiochem), LY294002 (100 mM, Upstate, Lake Placid, NY) were prepared in dimethyl sulfoxide (DMSO). Stock solutions of EGF (10 μg/ml, Calbiochem) were prepared in TCM-199 medium. All of the stock solutions were frozen and stored at −20 °C in the dark. The chemicals were diluted and added to the culture medium approximately 1 h before the oocytes culture.

2.2. Isolation and cultivation of porcine oocytes

Porcine ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory within 2 h in a thermos bottle containing warm (30–35 °C) saline (0.9% (w/v) NaCl supplemented with 40 IU/ml penicillin G and 50 μg/ml streptomycin sulfate). The contents of follicles measuring 2–6 mm in diameter were aspirated with an 18-gauge needle fixed to a 20-ml disposable syringe and pooled in 50 ml conical tubes (Falcon, Franklin Lakes, NJ). After sedimentation, the sediment was washed with 20 mM HEPES-buffered tissue culture medium-199 (H-TCM-199; Life Technologies, Inc., Grand Island, NY) supplemented with penicillin G (100 IU/ml), streptomycin sulfate (100 μg/ml), BSA (fraction V; 4 mg/ml; Calbiochem), and hypoxanthine (HX) (4 mM). H-TCM-199 was supplemented with the meiotic inhibitor HX to prevent premature meiotic progression during the oocytes collection, and subsequent removal of the inhibitor did not change the rate of maturation and development (data not shown).

Oocytes with at least four layers of intact, compact cumulus cells were recovered under a stereomicroscope and transferred to 35 mm petri dishes (Falcon) containing culture medium and subjected to three washes before the final culture. When denuded oocytes (DOs) were needed for culture, CEOs were mechanically denuded using a vortex instrument to remove all cumulus cells surrounding the oocytes in 0.5 ml H-TCM-199 containing 300 IU/ml hyaluronidase.

Culture of CEOs and DOs was carried out in 4-well dishes (Nunc, Roskilde, Denmark) at 39 °C in an atmosphere of 5% CO_2 in air and saturated humidity. The basic medium for culture of CEOs and DOs was TCM-199 medium supplemented with 0.23 mM sodium pyruvate, 2 mM glutamine, 3 mg/ml lyophilized crystallized BSA, 100 IU/ml penicillin G, and 50 μg/ml streptomycin sulfate. EGF, U0126, AG1478, AG43 and LY294002 were added to the medium approximately 1 h before the culture (detailed treated groups seen experimental design).

2.3. Nuclear status examination

Nuclear status of porcine oocytes was examined by the orcein staining method as described by us previously [70]. Briefly, denuded oocytes were mounted on slides, fixed in acetic acid/ethanol (1:3, v/v) for at least 48 h, stained with 1% orcein for 5 min, and then examined with a phase-contrast microscope (Nikon, Tokyo, Japan). Oocytes showing clear nuclear membrane were classified as GV stage oocytes and those that did not show nuclear membrane were classified as GVBD stage oocytes.

2.4. Electrophoresis and Western blot analysis

Cumulus cells for Western blot were isolated by removing the oocytes from CEOs by mechanically repeated pipetting with small-bore Pasteur pipettes. Oocytes for Western blot were denuded by vortexing in 300 IU/ml hyaluronidase to remove cumulus cells. MAPK activity in cumulus cells derived from 20 CEOs or in 150 oocytes was examined indirectly by detection of the phosphorylated (active) forms of MAPK [71] using Western blot analysis as described previously by us [72]. Briefly, the protein of cumulus cells was extracted with 2× Laemmli sample buffer, after denaturing by boiling for 4 min, the protein samples were separated by SDS-PAGE on 10% polyacrylamide gel and then transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking and incubating with the antibodies, enhanced chemiluminescence (ECL) was used for the detection. Phosphorylated MAPK was detected by using a monoclonal mouse pERK1/2 antibody (antibody:TBST = 1:1000). The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (antibody:TBST = 1:2000). To detect total ERK2, the membrane was washed in the washing buffer (100 mM β-mercaptoethanol, 20% SDS, and 62.5 mM Tris, PH 6.7) for 30 min at 50 °C. The total amount of ERK2 was assayed on the same membrane by using a polyclonal rabbit anti-ERK2 antibody (antibody:TBST = 1:1000) and HRP-conjugated goat antirabbit IgG (antibody:TBST = 1:2000).

2.5. Statistical analysis

All percentages from three repeated experiments are expressed as mean ± S.E.M. All frequencies were subjected to arcsine transformation. The transformed data were statistically compared by ANOVA using SPSS 10.0 software (SPSS Inc., Chicago, IL) followed by the Student–Newman–Keuls test. Differences of $P < 0.05$ were considered to be statistically significant.

2.6. Experimental design

In this study, we defined "TCM-199 culture medium" as "TCM-199 medium supplemented with 0.23 mM sodium pyruvate, 2 mM glutamine, 3 mg/ml lyophilized

crystallized BSA, 100 IU/ml penicillin G, and 50 μg/ml streptomycin sulfate". Different groups were described according to the differently treated groups in the results 3.1–3.7:

1 (result 3.1) (A) CEOs were cultured in TCM-199 culture medium supplemented with 0, 0.01, 0.1, 1 or 10 ng/ml EGF. (B) CEOs or DOs were cultured in TCM-199 culture medium supplemented with or without 10 ng/ml EGF.
2 (result 3.2) CEOs were cultured for 24 h in TCM-199 culture medium containing 10 ng/ml EGF supplemented with 0.01, 0.1, 1 or 10 μM AG1478 or AG43.
3 (result 3.3) (A) CEOs were cultured in TCM-199 culture medium supplemented with or without 10 ng/ml EGF. (B) CEOs or DOs were incubated in TCM-199 culture medium supplemented with 10 ng/ml EGF.
4 (result 3.4) CEOs were cultured in TCM-199 culture medium supplemented with 10 ng/ml EGF, either in the presence of 10 μM U0126 or 1/1000 (v/v) DMSO.
5 (result 3.5) CEOs were cultured in TCM-199 culture medium containing 10 ng/ml EGF supplemented with 10 μM AG1478 or 10 μM AG43.
6 (result 3.6) (A) CEOs were incubated in TCM-199 culture medium containing 10 ng/ml EGF, supplemented with 0, 0.1, 1, 10, or 50 μM LY294002. (B) CEOs or DOs were incubated in TCM-199 culture medium containing 10 ng/ml EGF, supplemented with or without 50 μM LY294002. Control group contained neither EGF nor LY294002.
7 (result 3.7) (A) CEOs were cultured in TCM-199 culture medium containing 10 ng/ml EGF, supplemented with or without 50 μM LY294002. (B) CEOs were cultured in TCM-199 culture medium containing 10 or 30 ng/ml EGF, supplemented with or without 50 μM LY294002.

3. Results

3.1. Cumulus-enclosed oocytes but not denuded oocytes can be induced to undergo GVBD by EGF in a dose-dependent manner

To explore the induction effect of EGF on porcine oocyte meiotic resumption, different doses of EGF were supplemented to the culture media. As shown in Fig. 1A, when 0.01 and 0.1 ng/ml of EGF were employed, the rates of GVBD were 13.3 and 15.1%, respectively, which have no significant difference compared to the control group (19.2%). This indicates that lower concentrations of EGF cannot promote GVBD. However, when the concentrations of EGF were increased to 1 and 10 ng/ml, the rates of GVBD reached 50.0 and 76.6%, respectively, which were significantly higher than the control group (Fig. 1A). These data show that higher doses (more than 1 ng/ml) of EGF can induce GVBD in porcine CEOs.

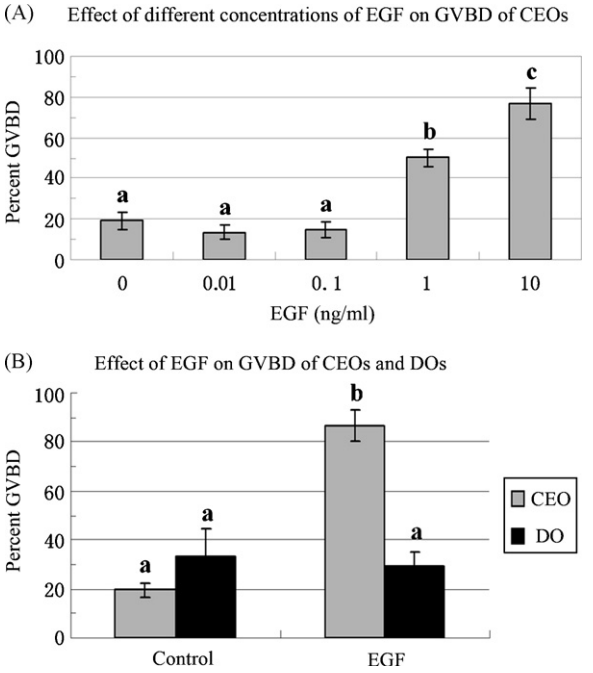

Fig. 1. CEOs but not DOs are induced to undergo GVBD by EGF in a dose-dependent manner. (A) Porcine CEOs were cultured in TCM-199 culture medium supplemented with 0, 0.01, 0.1, 1 or 10 ng/ml EGF. (B) CEOs or DOs were cultured in TCM-199 culture medium supplemented with or without 10 ng/ml EGF. Approximately, 50 oocytes were included in each group. The rate of GVBD was assessed by orcein staining at 24 h of the culture. Data are presented as the percentage of GVBD (mean ± S.E.M. of three independent experiments). Where no common letters are shown over the bars, the groups were significantly different ($P < 0.05$). Meiotic resumption could be induced by 1 or 10 ng/ml EGF in CEOs, but not in DOs.

Although EGF can induce GVBD of porcine CEOs, it is not clear whether this induction is mediated by EGFR located in cumulus cells or oocyte itself. Therefore, CEOs and DOs were incubated separately in the same culture media to examine the effect of EGF on them. In CEOs groups, EGF at 10 ng/ml could induce 86.7% of the oocytes to undergo meiotic resumption, significantly higher than that of the control group (19.5%). However, in DOs groups, there was no statistic difference between the control and EGF groups (EGF-treated group: 29.2%; control group: 33.2%) (Fig. 1B), indicating that it was cumulus cells rather than oocyte itself mediated EGF-induced meiotic resumption of porcine CEOs.

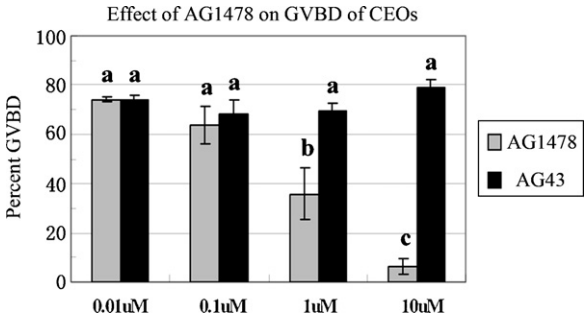

Fig. 2. Effects of AG1478 and AG43 on GVBD of porcine CEOs. Porcine CEOs were cultured for 24 h in TCM-199 culture medium containing 10 ng/ml EGF supplemented with 0.01, 0.1, 1 or 10 μM AG1478 or AG43. Approximately, 50 oocytes were included in each group. The rate of GVBD was assessed by orcein staining at 24 h of the culture. Data are presented as the percentage of GVBD (mean ± S.E.M. of three independent experiments). Where no common letters are shown over the bars, the groups were significantly different ($P < 0.05$). High concentrations (1 or 10 μM) of AG1478 but not AG43 could inhibit GVBD.

3.2. EGFR inhibitor AG1478 blocks GVBD of the CEOs in a dose-dependent manner

To determine whether EGFR participates in EGF-induced GVBD, the effect of AG1478, which can inhibit the activity of Tyr protein kinase of EGFR [73], was tested. As shown in Fig. 2, AG1478 could not inhibit EGF-induced GVBD when employed at 0.01 and 0.1 μM. However, when higher doses (1 and 10 μM) of AG1478 were used, a significant decrease of GVBD rate was observed. To rule out the possibility that the inhibition effect of higher dose of AG1478 was due to its chemical toxicity, AG43, an inactive analog of AG1478, was employed at the same time. The percentage of GVBD in AG43-treated group was significantly higher than that of AG1478-treated group (Fig. 2). These data reveal that activation of EGFR in cumulus cells plays a key role in EGF-induced GVBD in porcine CEOs.

3.3. EGF induces MAPK phosphorylation in cumulus cells derived from CEOs but not in oocytes of CEOs or DOs

In order to determine whether EGF-induced GVBD of CEOs is achieved via MAPK activation in cumulus cells, the active MAPK in cumulus cells was detected with Western blot after EGF administration. Our results showed that phosphorylated MAPK could be detected shortly after EGF treatment, whereas in the control group without EGF supplementation, phosphorylated MAPK could not be detected even after 20 min of cultivation,

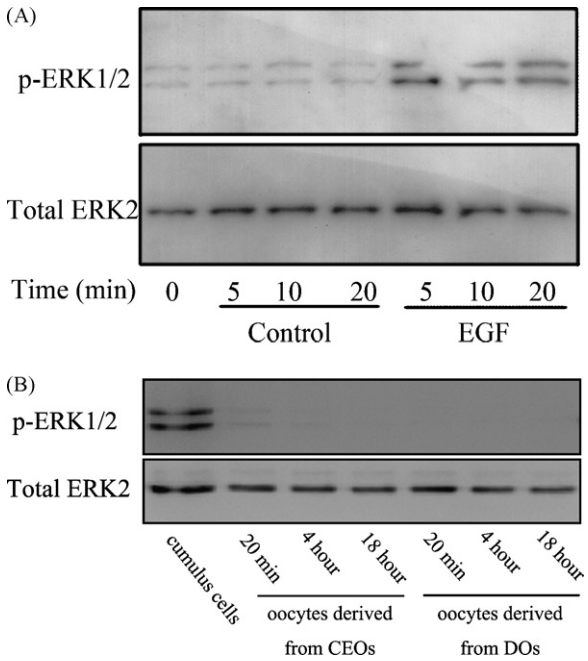

Fig. 3. EGF induces MAPK phosphorylation in cumulus cells derived from CEOs but not in oocytes of CEOs or DOs. (A) CEOs were cultured in TCM-199 culture medium supplemented with or without 10 ng/ml EGF. At each time point, cumulus cells derived from 20 CEOs were employed for Western blot with anti-p-ERK1/2 and anti-total-ERK2 antibodies. In the control group without EGF, MAPK in cumulus cells could not be activated even at 20 min, but evident activation of MAPK occurred at 5 min of the culture in EGF-supplemented group. (B) All the oocytes were incubated in TCM-199 culture medium supplemented with 10 ng/ml EGF. At each time point, oocytes without cumulus cells derived from 150 CEOs or DOs were employed for Western blot with anti-p-ERK1/2 and anti-total-ERK2 antibodies. The control group was cumulus cells derived from 20 CEOs. MAPK in oocyte could not be activated by EGF in either CEOs or DOs during meiotic resumption before GVBD.

which indicated that MAPK could be promptly activated by EGF in cumulus cells of porcine CEOs (Fig. 3A).

Since EGF could effectively activate MAPK in cumulus cells and trigger GVBD of CEOs but not DOs, we thus speculated that it was MAPK in cumulus cells but not in oocyte that mediated this event of meiotic resumption. To address this issue, CEOs and DOs were separately incubated in the culture media containing 10 ng/ml EGF. Oocytes derived from CEOs or DOs were then subjected to Western blot to examine the MAPK activity at different time points of culture. In agreement with our speculation, MAPK in oocytes from neither CEOs nor DOs could be activated by EGF at any time point of culture (Fig. 3B). These results suggested that, it was MAPK in cumulus cells but not in oocyte that regulated the EGF-induced resumption of meiosis.

3.4. EGF-induced activation of MAPK as well as GVBD can be inhibited by U0126

To explore whether MAPK activation in cumulus cells is essential for EGF-induced GVBD in porcine CEOs, we detected the effect of U0126, a specific inhibitor of MEK which is upstream of MAPK, on the phosphorylation of MAPK in cumulus cells and GVBD. The results demonstrated that in U0126-treated group, MAPK in cumulus cells could not be induced to activate by EGF during the culture. Conversely, EGF induced MAPK phosphorylation in the control group which was treated with DMSO, the U0126 carrier solution (Fig. 4A). Meanwhile, comparing to the control group, EGF-induced

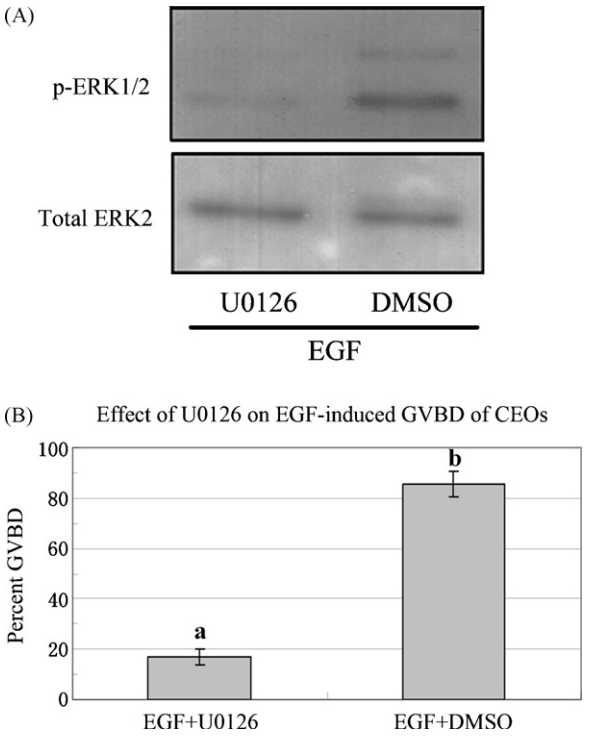

Fig. 4. EGF-induced activation of MAPK in cumulus cells and GVBD are inhibited by U0126. CEOs were cultured in TCM-199 culture medium supplemented with 10 ng/ml EGF, either in the presence of 10 μM U0126 or 1/1000 (v/v) DMSO. (A) At 5 min of the culture, cumulus cells derived from 20 CEOs were employed for Western blot analysis with anti-p-ERK1/2 and anti-total-ERK2 antibodies. The results of one representative out of three independent experiments are presented. In U0126-treated group, MAPK in cumulus cells could not be activated at 5 min, while MAPK activation occurred in the control group. (B) Approximately, 50 oocytes were included in each group. The rate of GVBD was assessed by orcein staining at 24 h of the culture. Data are presented as the percentage of GVBD (mean ± S.E.M. of three independent experiments). Where no common letters are shown over the bars, the groups were significantly different ($P < 0.05$). The rate of GVBD in U0126-treated group was significantly lower than that of the control group.

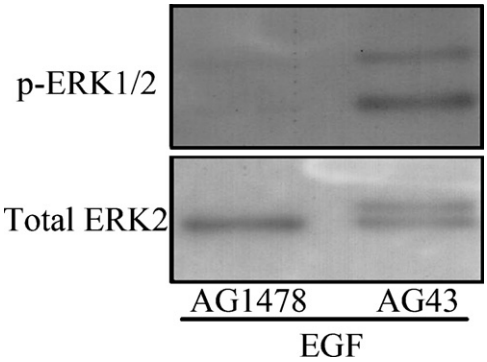

Fig. 5. EGF-induced activation of MAPK in cumulus cells is inhibited by AG1478. CEOs were cultured in TCM-199 culture medium containing 10 ng/ml EGF supplemented with 10 μM AG1478 or 10 μM AG43. After 5 min of the culture, cumulus cells derived from 20 CEOs were employed for Western blot analysis with anti-p-ERK1/2 and anti-total-ERK2 antibodies. The results of one representative out of three independent experiments are presented. In AG1478 group, MAPK in cumulus cells could not be activated, but MAPK activation could be detected in AG43 group at 5 min of the culture.

GVBD of CEOs was significantly prevented by U0126, which indicated that activation of MAPK in cumulus cells was necessary for EGF-induced GVBD (Fig. 4B).

3.5. EGF-induced activation of MAPK in cumulus cells is inhibited by EGFR inhibitor AG1478

Although MAPK phosphorylation in cumulus cells can be induced by EGF, it is still not clear whether EGFR mediates EGF-induced MAPK activation in cumulus cells and the subsequent meiotic resumption in oocytes. Therefore, the consequence of EGFR activation and MAPK activation was investigated by employing AG1478, a specific inhibitor of EGFR. The results showed that when CEOs were cultured in the medium supplemented with AG1478, EGF did not induce MAPK activation in cumulus cells. Conversely, phosphorylated MAPK was detected in the control group treated with EGF combined with AG43, indicating that EGF induced-activation of MAPK was mediated by EGFR (Fig. 5).

3.6. PI3-Kinase inhibitor LY294002 can inhibit EGF-induced GVBD of CEOs in a dose-dependent manner, but has no effect on the GVBD of DOs

To investigate whether PI3-kinase involves in the regulation of EGF-induced meiotic resumption in CEOs, its specific inhibitor LY294002 was employed to examine the relationship between the EGF pathway and PI3-kinase. As shown in Fig. 6A, the rates of GVBD in the groups with lower concentrations (0.1 and 1 μM) of

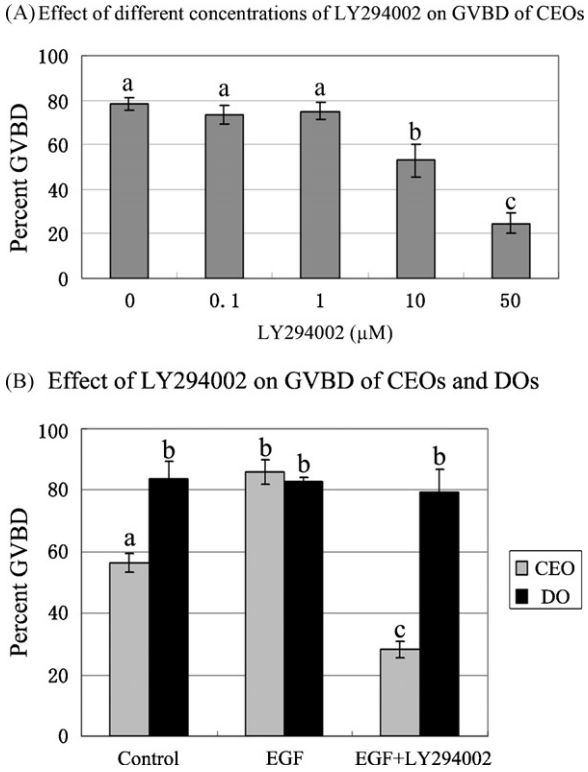

Fig. 6. LY294002 can inhibit EGF-induced GVBD of CEOs in a dose-dependent manner, but has no effect on the GVBD of DOs. (A) Porcine CEOs were incubated in TCM-199 culture medium containing 10 ng/ml EGF, supplemented with 0, 0.1, 1, 10, or 50 μM LY294002. (B) CEOs or DOs were incubated in TCM-199 culture medium containing 10 ng/ml EGF, supplemented with or without 50 μM LY294002. Medium with neither EGF nor LY294002 was employed as a control. Approximately, 50 oocytes were included in each group. The rate of GVBD was assessed by orcein staining at 24 h (28 h for B) of the culture. Data are presented as the percentage of GVBD (mean ± S.E.M. of three independent experiments). Where no common letters are shown over the bars, the groups were significantly different ($P < 0.05$). Meiotic resumption could be blocked by 10 μM or higher concentration of LY294002 in CEOs, but not in DOs.

LY294002 were not significantly different compared to that of the control group which contained DMSO. When higher concentrations of LY294002 were employed, the rates of GVBD decreased, which were significantly lower than that of the control group. These data suggested that LY294002-sensitivated PI3-kinase participated in EGF-induced GVBD in porcine CEOs.

Although LY294002 can inhibit GVBD of porcine CEOs, it is not clear whether this block is due to the inhibition of PI3-kinase in cumulus cells or intra-oocyte. Therefore, CEOs and DOs were incubated separately in the same culture media to examine the effect of LY294002 on them. It is worth mentioning that, because DOs display a 3–4 h delay of GVBD during their maturation compared with CEOs in this culture model, to investigate the effect of LY294002 on GVBD of CEOs and DOs, in this part, all the oocytes were incubated for 28 h (different from the other parts with 24 h) followed by GVBD examination. Furthermore, control groups without either EGF or LY294002 were also employed to exclude the possibility that the higher GVBD rate of DOs in these parts was due to the long-time exposure to EGF. Fig. 6B showed that, in CEOs groups, LY294002 at 50 μM could effectively inhibit GVBD of CEOs, the rate of which was significantly lower than that of the EGF group. However, in DOs groups, there was no statistic difference between LY294002-treated group and the other groups (LY294002-treated group: 79.17%, EGF-treated group: 82.6%, control group: 83.6%), confirming that it was the PI3-kinase in cumulus cells rather than in oocyte itself that mediated EGF-induced meiotic resumption of porcine oocyte.

3.7. EGF-induced activation of MAPK in cumulus cells can be inhibited by LY294002 and this inhibition cannot be reversed by adding additional EGF

Although both U0126 and LY294002 can block EGF-induced GVBD, the relationship between MAPK and PI3-kinase is not clear. To identify the role of PI3-kinase in EGF-induced MAPK activation in cumulus cells derived from CEOs, PI3-kinase activity was inhibited by LY294002. Then the phosphorylated MAPK in cumulus cells was detected. Our results showed that, with EGF administration, MAPK in cumulus cells derived from CEOs treated by LY294002 was barely activated even at 20 min, whereas a significant phosphorylation of MAPK was observed at 5 min during the cultivation in the control group lack of LY294002. It is indicated that EGF-induced activation of MAPK in cumulus cells can be inhibited by LY294002, which means that PI3-kinase activity is required for EGF-induced activation of MAPK in porcine cumulus cells (Fig. 7A).

To provide a further evidence to determine the relationship of EGF/EGFR, PI3-kinase, and MAPK, additional EGF was added to the media to examine its effect on the phosphorylation of MAPK. As shown in Fig. 7B, 30 ng/ml EGF induced activation of MAPK in cumulus cells much more conspicuously than that of 10 ng/ml EGF. However, the extra EGF could not reactivate the MAPK inhibited by LY294002, which further revealed that PI3-kinase lay between EGF/EGFR and MAPK in the MAPK cascade.

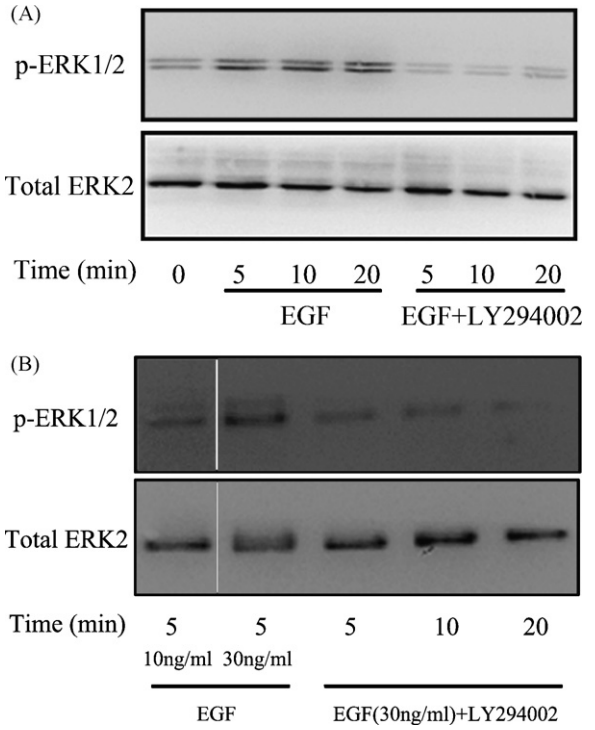

Fig. 7. EGF-induced activation of MAPK in cumulus cells derived from CEOs can be inhibited by LY294002 and the inhibition cannot be reversed by adding additional EGF. CEOs were cultured in TCM-199 culture medium containing EGF, supplemented with or without 50 μM LY294002. At each time point, cumulus cells derived from 20 CEOs were employed for Western blot analysis with anti-p-ERK1/2 and anti-total-ERK2 antibodies. The results of one representative out of three independent experiments are presented. (A) In the control group containing 10 ng/ml EGF without LY294002, MAPK activation could occur at 5 min of the culture, while MAPK activation in cumulus cells could not be detected even at 20 min in LY294002-supplemented group. (B) Higher concentration (30 ng/ml) of EGF phosphorylated MAPK much more effectively than that of control group (10 ng/ml), but was unable to activate MAPK in LY294002-treated groups.

4. Discussion

In this study, we have provided evidences showing that EGF, by acting on EGF receptor, stimulates MAPK activation in cumulus cells, and thus oocyte meiotic resumption. PI3-kinase in cumulus cells is a critical intermediate molecule in this cascade.

Various reports have shown that EGF plays a pivotal role in porcine oocyte meiotic maturation [15,74–77], however, the signal pathway of this process is not well understood. In our study, we used cumulus-enclosed oocyte as an *in vitro* model to investigate the molecular mechanism of EGF-induced meiotic resumption. Although GVBD of CEOs can be induced by EGF, it is not clear whether EGF receptor plays the functional role, since EGF receptor is expressed in both cumulus cells and oocyte itself. It was claimed that EGF could directly act on DOs and promote their maturation [9], but our present study showed that EGF induced GVBD only through cumulus cells of CEOs, indicating that EGFR expressed in cumulus cells played a dominant role in meiotic resumption, which was supported by the previous studies, demonstrating that EGFR in cumulus cells made a major contribution to meiotic resumption [6,32,78]. Moreover, it has been shown that, in goats, only the EGFR in cumulus cells is able to be phosphorylated after transient EGF stimulation [6], although the receptor is expressed in both cumulus cells and oocytes. The absence of EGFR activation in oocytes is possible due to the immature receptor because EGF strongly stimulated expansion of porcine oocyte cumulus complexes (OCCs) isolated from large follicles (>6 mm) but it could not promote expansion of OCCs from small (3–4 mm) follicles [16]. The differences between EGFR in cumulus cells and oocytes as well as their responses to EGF need to be further studied.

Previous studies have shown that EGF, EGFR and the associating Tyr protein kinases may participate in cumulus expansion during porcine oocyte maturation [16,79,80]. In bovine oocytes, EGF can induce polar body emission in the oocytes from small follicles through MPF and MAPK pathways [11]. The MAPK pathway cannot only induce cumulus expansion [43], but also mediate the interruption of gap junctions between oocytes and cumulus cells [40], as well as oocyte maturation induced by FSH [38]. Therefore, we speculate that the functions of EGF and its receptor may be achieved through the MAPK pathway in cumulus cells. Here, we showed that EGFR specific inhibitor AG1478 had the ability to block GVBD of CEOs as well as phosphorylation of MAPK in cumulus cells. Moreover, MEK specific inhibitor U0126 could completely block EGF-induced meiotic resumption and MAPK activation. These findings suggested that, activated MAPK in cumulus cells rather than in oocyte itself, was one of the downstream events of EGF/EGFR and essential for EGF-induced GVBD. Nevertheless, results reported by Inoue et al. [47] showed that, before GVBD, a slight amount of activated MAPK immigrated into GV and led to GVBD. These data enlightened us to investigate the delicate signal way of MAPK in oocyte. However, in our current culture model, we did not detect phosphorylated MAPK in the oocyte, confirming that, in the EGF-induced meiotic cell cycle, MAPK activation in oocyte might not directly regulate GVBD in porcine oocyte. Herein, we still emphasized the crucial contributions of activated MAPK in cumulus cells to meiotic reinitiation. Although the definite mechanism that how MAPK in cumulus

cells triggers GVBD is not clear, accumulative evidences have established some possible links between MAPK and GVBD: activated MAPK in cumulus cells breaks gap junctions between the somatic cells and oocyte through phosphorylating gap junctional proteins Connexins [40,81], which are the most important proteins of gap junctions and play key roles in oocyte meiotic maturation [81,82], so that most of the inhibitors in cumulus cells which hold oocyte arrested such as cAMP cannot flow into oocyte. Another potential mechanism is the MAPK-induced production of meiosis stimulating substance(s) in cumulus cells. Various reports have demonstrated that meiosis activating sterol (MAS) which is secreted by cumulus cells [83,84] can promote GVBD in mouse and porcine oocyte [85–87] and its synthesis is dependent on MAPK [34], which indicates that MAS serves as one of the important intermediate molecules between MAPK and GVBD and gives a positive stimulation to meiotic resumption.

Studies have suggested that PI3-kinase participates in meiotic resumption in various species [58], however, disputations still exist [56,88,89] and there is no report about the regulatory mechanism of this kinase in cumulus cells for meiotic resumption. A recent study reported that gap junctional communications between cumulus cells and oocytes might be closed by phosphorylation of connexin-43 through PI3-kinase activation in cumulus cells, leading to the activation of MAPK in porcine oocytes [65], but our previously published work suggested that activation of MAPK in cumulus cells but not in oocytes was necessary for gonadotropin-induced meiotic resumption [1,34,38]. In this study, we noted that PI3-kinase was not only involved, but also served as the intermediate molecule between EGF/EGFR and MAPK, because the inhibition of EGF-induced meiotic resumption by LY294002 could not be reversed by adding additional EGF into the culture medium though this extra EGF could strongly activate the MAPK in control group without LY294002, which indicated that PI3-kinase was downstream of EGF/EGFR in this pathway. To further explore the relationship between MAPK and PI3-kinase, MAPK activation in cumulus cells was detected in EGF-induced meiotic resumption after the inhibition of PI3-kinase activity. The effective inhibition of LY294002 on MAPK activation indicated that, in EGF-induced meiotic resumption, PI3-kinase was one of the molecules upstream of MAPK in cumulus cells. Eventually, we propose a linear relationship of EGF, EGFR, PI3-kinase, MAPK and GVBD because the inhibition of either PI3-kinase or MAPK could abrogate the EGF effect on meiotic resumption as described in the results. A recent review summarized regular mechanisms and roles of MAPK during meiotic resumption in lower vertebrates and mammals [34], meanwhile, it raised an unidentified pathway from EGF to MAPK in cumulus cells. Thus, the present study possibly gives a supplement to the MAPK network in mammalian oocyte maturation.

Notably, androgens (especially testosterone) were evidenced as the novel and important candidates for activating MAPK and promoting the release of meiotic inhibition in mammals [90,91]. Furthermore, a recent study suggested that steroid production served as one of many integrated signals triggered by EGFR signaling to promote oocyte maturation [5]. Our studies therefore provide an insight into this issue. In summary, EGFR on the membrane of cumulus cells in CEOs is activated by EGF, and then PI3-kinase is stimulated, which subsequently phosphorylates MAPK in cumulus cells, the active MAPK in turn causes a series of events (possibly through disrupting cumulus cell-oocyte gap junctions [92,93] and attenuating G protein-mediated signals which hold oocyte in meiotic arrest [94,95]) to promote GVBD of oocytes. Because steroidogenesis in mammals occurs in granulosa cells (consist of mural granulosa cells and cumulus granulosa cells) and theca cells of ovary [96–98], cumulus cells, as the closest somatic cells surrounding oocyte, are considered to play pivotal roles in communicating with the germ cell and conveying signals to oocyte to regulate meiotic events. Furthermore, a report which focused on sertoli cells (somatic cells surrounding sperm in male) demonstrated that androgens activated MAPK via EGF receptor [99]. These results implied that there might be some similar or conserved signals in cumulus cells and this EGF-triggered pathway of meiotic reinitiation might involve in the androgen-mediated meiotic resumption. Anyway, during mammalian oocyte meiotic cycle, paracrine signals in the ovary, especially in the cumulus cells and the surrounded oocyte, play dominant roles in regulating oocyte maturation and the detailed mechanisms of the signal transductions need to be further explored.

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