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Oocyte-dependent activation of mitogen-activated protein kinase (ERK1/2) in cumulus cells is required for the maturation of the mouse oocyte–cumulus cell complex

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Abstract

Luteinizing hormone (LH) induces maturational processes in oocyte–cumulus cell complexes (OCC) of preovulatory follicles that include both resumption of meiosis in the oocyte and expansion (mucification) of the cumulus oophorus. Both processes require activation of mitogen-activated protein kinase (MAPK) in granulosa cells. Here, it is reported that inhibition of MAPK activation prevented gonadotropin-stimulated resumption of meiosis as well as the rise in expression of two genes whose products are necessary for normal cumulus expansion, *Has2* and *Ptgs2*. However, inhibition of MAPK did not block gonadotropin-induced elevation of granulosa cell cAMP, indicating that the activation of MAPK required for inducing GVB and cumulus expansion is downstream of cAMP. Moreover, activation of MAPK in cumulus cells requires one or more paracrine factors from the oocyte to induce GVB and cumulus expansion; MAPK activation alone is not sufficient to initiate these maturational processes. This study demonstrates a remarkable interaction between the oocyte and cumulus cells that is essential for gonadotropin-induced maturational processes in OCC. By enabling gonadotropin-dependent MAPK activation in granulosa cells, oocytes promote the generation of a return signal from these cells that induces the resumption of meiosis. It also appears that an oocyte-dependent pathway downstream from oocyte-enabled activation of MAPK, and distinct from that promoting the resumption of meiosis, governs cumulus expansion.

Introduction

In each estrous cycle of a female mammal, after the surge of luteinizing hormone (LH), a series of well-coordinated physiological events must occur in a preovulatory follicle to enable the production of a fertilizable egg and ovulation. These processes take place in both the germ cell and somatic cell compartments of the follicle. In the oocyte, meiotic maturation is a crucial process in which meiosis is reinitiated and progresses to metaphase II, usually before ovulation. The most obvious morphological manifestation of the resumption of meiosis is the disappearance of the oocyte nucleus, or germinal vesicle (GV), a process known as germinal vesicle breakdown (GVB). Concurrent with meiotic maturation in vivo, a dramatic maturational change also occurs in the somatic cell compartment, in the cumulus oophorus, the specialized subpopulation of granulosa cells intimately associated with the oocyte. This change is cumulus expansion, or mucification, which requires gonadotropin-dependent increased expression of several key genes in cumulus cells, including *Has2*, *Ptgs2* (also known as *Cox2*), *Ptx3*, and *Tnfaip6* (Elvin et al., 1999; Fulop et al., 2003; Joyce et al., 2001; Lim et al., 1997; Ochsner et al., 2003; Varani et al., 2002). Cumulus expansion is required for normal ovulation rates (Chen et al., 1993; Hess et al., 1999).

LH initiates the maturation of the oocyte–cumulus cell complex in vivo and in cultured isolated intact ovarian follicles. The details of the signal transduction pathways connecting LH stimulation and the maturational processes are not yet resolved but, various studies suggest that LH promotes an increase in cyclic adenosine monophosphate

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(cAMP) levels within the granulosa cell compartment and a decrease in cAMP in the oocyte, thus inducing the resumption of meiosis as well as cumulus expansion (Downs and Hunzicker-Dunn, 1995; Tsafriri et al., 1996). Differences in expression and regulation of phosphodiesterase isoforms expressed in the two follicular compartments are likely key to the differential effects of LH on cAMP levels and to understanding the mechanisms governing the resumption of meiosis (Conti, 2002; Richard et al., 2001).

In addition to cAMP, the mitogen-activated protein kinase (MAPK), specifically ERK1/2, pathway is also important for the maturation of the oocyte–cumulus cell complex. In oocytes, MAPK is activated indirectly by MOS (a MAPK kinase kinase, MAPKKK), whereas in granulosa cells, it is activated indirectly by RAS/RAF (another MAPKKK) signaling. MOS is exclusively expressed in germ cells. In both oocytes and granulosa cells, the activation of MAPK is directly mediated by MEK (a MAPK kinase, MAPKK) (Seger and Krebs, 1995). To study the role of MAPK in the regulation of maturational processes in both oocytes and cumulus cells, an in vitro model using isolated oocyte–cumulus cell complexes has generally been utilized. In this model, hypoxanthine, a purine found in follicular fluid (Downs et al., 1989; Eppig et al., 1985), is used to prevent spontaneous maturation, while the resumption of meiosis and cumulus expansion is induced by treatment with follicle-stimulating hormone (FSH) or 8Br-cAMP (Dekel and Kraicer, 1978; Downs et al., 1988; Downs and Hunzicker-Dunn, 1995; Eppig, 1979, 1989). Studies using this model system demonstrated that MAPK was activated in both cumulus cells and oocytes. Moreover, specific MEK inhibitors blocked oocyte GVB as well as cumulus expansion induced by either FSH or 8Br-cAMP (Leonardsen et al., 2000; Su et al., 2001, 2002). In sum, these studies suggested that the MAPK pathway is involved in gonadotropin-induced maturation of oocyte–cumulus cell complexes. However, MAPK activation in both cumulus cells and oocytes can be blocked by the same MEK inhibitors, making it difficult to distinguish in which cell type the activation of MAPK is actually required for gonadotropin-induced maturation of oocyte–cumulus cell complexes. To address this question, the role of MAPK in gonadotropin-induced oocyte GVB and cumulus expansion was studied by using Mos^{tmlEv}/Mos^{tmlEv} (*Mos*-null) mice. No activation of MAPK was detected in *Mos*-null oocytes induced to mature with gonadotropins either in vivo or in vitro, thus indicating that MAPK activation in oocytes is not necessary for gonadotropin-induced resumption of meiosis (Su et al., 2002). Moreover, FSH induced GVB and cumulus expansion in *Mos*-null oocytes as in the control wild type oocytes, but both GVB and cumulus expansion were blocked by the MEK inhibitor U0126 (Su et al., 2002). Since MAPK activation occurs only in the cumulus cells of *Mos*-null oocyte–cumulus cell complexes, and not in the oocyte, it was concluded that cumulus expansion as well as gonadotropin-induced GVB require MAPK activation in the cumulus cells (Su et al., 2002).

The experimental model using isolated oocyte–cumulus cell complexes cultured in medium containing hypoxanthine can mimic, at least in part, the effects of LH on maturation of both the oocyte and cumulus cell compartments in intact follicles. Nevertheless, this model has some disparities from LH-induced maturation in intact follicles. First, FSH, rather than LH, stimulation is required to induce GVB and cumulus expansion, since mouse cumulus cells do not express LH-receptors (Eppig et al., 1997). Second, the time course of GVB is delayed compared with that of LH-induced GVB in vivo. Therefore, the question of whether LH-induced oocyte GVB and cumulus expansion utilizes MAPK-dependent pathways was addressed in the present study in cultured intact antral follicles. Additionally, the potential requirement for MAPK-dependent transcription of two genes, *Has2* and *Ptgs2*, necessary for normal cumulus expansion was evaluated.

Since oocytes and granulosa cells communicate with each other via both paracrine signals and gap junctions (Eppig, 2001), whether MAPK activity in granulosa cells is influenced by the oocyte was also determined. A proposed oocyte–granulosa cell regulatory loop is driven by signals from oocytes and granulosa cells that affect the development and function of both cell types and are required to coordinate follicular development (see Eppig, 2001; Matzuk et al., 2002 for reviews). In mouse preovulatory follicles, cumulus expansion requires the participation of a cumulus expansion-enabling factor (CEEF) secreted by the oocyte (Buccione et al., 1990). An oocyte-specific member of the TGF-β superfamily, growth differentiation factor-9 (GDF-9) also promotes cumulus expansion, suggesting that GDF-9 is the CEEF (Elvin et al., 1999). However, recombinant GDF-9 exhibits some characteristics apparently different from native CEEF (Eppig, 2001; Su et al., 2002) and raises the question whether CEEF, GDF-9, or both, can regulate the activity of MAPK in cumulus cells. Since MAPK activation in cumulus cells is required for the maturation of both the oocyte and the cumulus oophorus, and if factors from the oocyte are required for activation of MAPK in the cumulus cells, we hypothesize that the oocyte plays a central role in both maturational processes, including control of its own meiotic maturation. We employed two model systems—isolated cumulus cell-enclosed oocytes and intact follicles—to test this hypothesis.

Materials and methods

Follicle, cumulus cell, and oocyte isolation and culture

Immature 22-to 24-day-old (C57BL/6J × SJL/J) F_1 mice were raised in the research colony of the investigators at The Jackson Laboratory. Follicle development was stimulated by ip injection with 5 IU (equine chorionic gonado-

tropin (eCG; National Hormone and Peptide Program, NIDDK) 44–48 h before harvesting follicles. The culture medium used for all experiments was bicarbonate-buffered Minimum Essential Medium α (Life Technologies, Inc., Grand Island, NY) with Earle's salts, supplemented with 75 mg/liter penicillin G, 50 mg/liter streptomycin sulfate, 0.23 mM pyruvate, and 3 mg/ml crystallized lyophilized bovine serum albumin. All media components were purchased from Sigma (St. Louis, MO).

Large antral follicles were isolated in medium supplemented with 10% fetal bovine serum as described previously (Dekel and Sherizly, 1985; Tsafriri et al., 1972). After three washes in medium, the follicles were cultured on collagen-coated membranes as described previously (Eppig and O'Brien, 1996). Each culture well contained approximately 1.5 ml of medium beneath the membrane insert and only a thin film of medium covering the follicles. Follicle culture was carried out at 37°C in a modular incubation chamber (Billups Rothenberg, Del Mar, CA) infused with 5% CO_2 and 95% air. During culture, follicles were treated with 10 μg/ml of ovine LH (National Hormone and Pituitary Program, NIDDK) or LH plus various doses of the highly specific MEK1/2 inhibitor U0126 or its inactive analog U0124 (Favata et al., 1998) (Calbiochem, La Jolla, CA).

To isolate cumulus cell-enclosed oocytes, large antral follicles on the surface of ovaries were punctured with 26-gauge needles. Cumulus cells and denuded oocytes were obtained by drawing the oocyte–cumulus cell complexes (OCCs) into and out of a small-bore pipette, gently shearing off the cumulus cells. Culture of OCCs, cumulus cells, or cumulus cells cocultured with denuded oocytes was carried out in 30 μl drops of medium under washed paraffin oil at 37°C in a modular incubation chamber infused with 5% O_2, 5% CO_2, and 90% N_2. During culture, the cells were treated with FSH (National Hormone and Pituitary Program, NIDDK), recombinant GDF-9 (generously provided by Dr. Martin M. Matzuk at Baylor College of Medicine, Houston, TX), the MEK inhibitor U0126, or its inactive analog U0124.

Activation of MAPK

MAPK activity in cumulus cells was determined indirectly by detection of phosphorylated (active) forms of MAPK using Western blot analysis as described previously (Su et al., 2002). Phosphorylated MAPK was detected by using a monoclonal anti-diphosphorylated ERK1 and-2 antibody (Sigma). The second antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The total amount of MAPK (phosphorylated and unphosphorylated forms) on the same membrane was assayed by using a polyclonal anti-MAPK antibody (Sigma) after stripping off the initial bound antibodies. In this reaction, horseradish peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) was used as the secondary antibody.

Measurement of cAMP levels

The levels of cAMP in OCCs were measured by using the cAMP-^{125}I-Direct Biotrak Assay by Amersham Sciences (Piscataway, NJ). OCCs were incubated in control medium or in medium with FSH (100 IU/liter) or FSH plus U0126 or U0124 (10 μM). All groups were cultured in medium supplemented with 50 μM isobutyl methylxanthine. Complexes were cultured for 3 h before cells were lysed and cAMP assayed. The experiment was conducted three times with the samples in each group analyzed in triplicate.

Evaluation of oocyte GVB and cumulus expansion

Oocyte GVB were scored at 4 h after intact follicle culture. OCCs were released by puncturing the follicles with a 26-gauge needle, and GVB was evaluated under a stereomicroscope after the cumulus cells were stripped off with a small-bore glass pipette. Cumulus expansion was evaluated after 12 h of intact follicle culture by histological assessment. Follicles were fixed in Bouin's fixing solution overnight, then washed, dehydrated, and embedded in paraffin wax. Sections were cut at 4-μm thickness and stained with periodic acid/Schiff reagent and hematoxylin.

Comparison of Has2 and Ptgs2 mRNA steady state levels

The steady-state expression by cumulus cells of *Has2* and *Ptgs2* mRNA was determined by RNase protection assay as described previously (Eppig et al., 1998; Joyce et al., 2001). The *Has2* RNA probe was generated from a mouse *Has2* cDNA provided by Dr. Martin M. Matzuk. The *Ptgs2* RNA probe was generated from a mouse *Ptgs2* cDNA provided by Dr. S.K. Dey (Vanderbilt University School of Medicine). Antisense *Has2* and *Ptgs2* probes were labeled with [γ-^{32}P]CTP (NEN Life Science Products, Boston, MA), using MAXI script kits (Ambion, Inc., Austin, TX). A [γ-^{32}P]CTP-labeled antisense *Rpl19* RNA probe was included in each sample to allow differences in the quantity of mRNA between samples to be corrected mathematically (Eppig et al., 1998). After electrophoresis, band intensity was quantified by using a phosphor imaging system (Fuji Photo Film Co., Ltd., Stamford, CT) and standardized to account for variable mRNA levels per sample.

Statistical analysis

Experiments were repeated independently a minimum of three times. The number of experimental replicates is indicated in the figure legends. Data were statistically compared by ANOVA using StatView software (SAS Institute, Inc., Cary, NC). When a significant F ratio was defined by

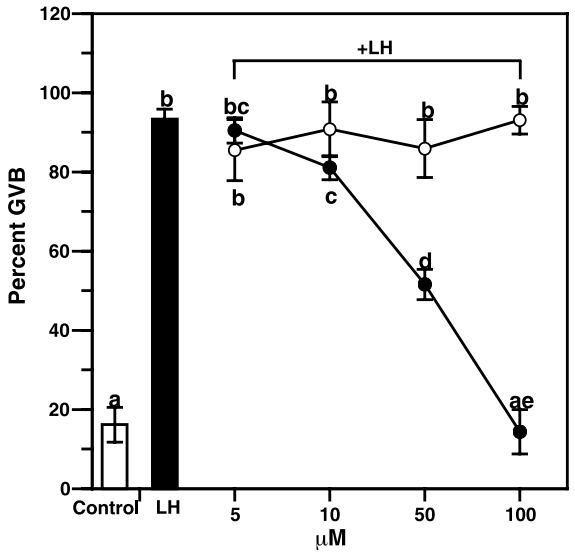

Fig. 1. MEK inhibitor dose-dependently blocks LH-induced GVB in intact isolated follicles. Large antral follicles isolated from eCG-primed 22-to 24-day-old mouse ovaries were cultured in vitro using approximately 30–35 follicles per well. Follicles were initially treated with 5–100 μM U0126 (solid circles) or U0124 (open circles) for 1 h; LH was then added to the culture medium to a final concentration of 10 μg/ml. The incidence of oocyte GVB was scored after 4 h of culture. Results are presented as the mean and standard errors of the mean of five independent experiments. Where there are no common letters over the points, the groups were significantly different ($P < 0.05$). Control indicates the group with no treatment. LH indicates the group treated only with 10 μg/ml LH without MEK inhibitor.

ANOVA, groups were compared by using Fisher's protected least significant difference post hoc test. $P < 0.05$ was considered significant. In the oocyte maturation experiments, 30–35 follicles or OCCs were utilized in each experiment. The data are reported as the mean percentage of GVB ± SEM. All frequencies were subjected to arcsin transformation before analysis.

Results

Effect of MEK inhibitor on LH-induced oocyte GVB and cumulus expansion in intact large antral follicles in vitro

The hypothesis that a MAPK-dependent pathway participates in LH-induced oocyte GVB and cumulus expansion in intact follicles was tested. Large antral follicles were initially treated with various doses of U0126 [a specific MEK1/2 inhibitor (Favata et al., 1998)] or U0124 (an inactive analog of U0126) for 1 h, LH was then added to the medium at a final concentration of 10 μg/ml. After 4 h of culture, the majority of the oocytes isolated from the large antral follicles in the control group without LH or MEK inhibitor treatments were maintained at the GV stage, with a GVB rate of less than 20% (Fig. 1). In contrast, when follicles were treated with 10 μg/ml of LH alone, almost all the oocytes in these follicles underwent GVB. The stimulatory effect of LH on GVB in follicle-enclosed oocytes was, however, dose-dependently inhibited by U0126; 10 μM U0126 was effective to inhibit LH-induced GVB, and 100 μM U0126 almost totally blocked the effect of LH on GVB rate. At the same concentration, U0124, the inactive analog of the MEK inhibitor U0126, had no inhibitory effect on LH-induced GVB. Although there was a significant inhibition of LH-induced GVB at a concentration of 50 μM U0126, a high concentration (100 μM), which could produce nonspecific effects, was required to produce maximum inhibition. However, it is impossible to know the concentration of inhibitor deep within the cultured intact follicles. Indeed, a high concentration of 50 μM U0126 was found to be efficient to inactivate MAPK and induce mouse egg parthenogenetic activation (Phillips et al., 2002). These results with LH-treated intact follicles, strongly support the hypothesis that gonadotropin-induced resumption of meiosis requires somatic cell activation of MAPK. In addition, this helps to validate the physiological significance of FSH-induced MAPK activation and GVB in the isolated cumulus cell-enclosed model system.

Cumulus expansion was evaluated in intact follicles cultured for 12 h. No expansion was observed in the control group follicles; the intact cumulus layers remained in close contact with GV-stage oocytes (Fig. 2A). Conversely, in the group treated with 10 μg/ml LH, cumulus cells underwent full expansion and their tight association with the oocyte was lost. Both the mature oocyte and cumulus cells became separated from the follicle wall (Fig. 2B). LH-induced cumulus expansion was completely blocked by 100 μM U0126 (Fig. 2C), but not by the same dose of U0124 (Fig. 2D).

In summary, in follicles treated with U0126 plus LH, the oocyte was maintained at GV-stage, and the intact cumulus layers remained tightly associated with both the oocyte and the follicle wall. By contrast, in follicles treated with the inactive analog plus LH, the oocyte underwent GVB and the cumulus underwent full expansion, exactly as in the LH-treated follicles. Thus, LH-induced activation of follicular MAPK was required for the maturation of both the oocyte and cumulus oophorus.

Effect of MEK inhibitor on steady-state levels of gonadotropin-induced Has2 and Ptgs2 mRNA expression in cumulus cells

The observation that the MAPK-dependent pathway is essential for both LH-induced cumulus expansion within intact follicles (above) and FSH-induced cumulus expansion of OCCs in vitro (Su et al., 2002) suggest that MAPK participates in gonadotropin-induced expression of the genes involved in cumulus expansion. Both *Has2* and *Ptgs2* are expressed in the cumulus cells, and their expression is essential for cumulus expansion. Therefore, it was determined whether MAPK activity is required for gonadotropin-

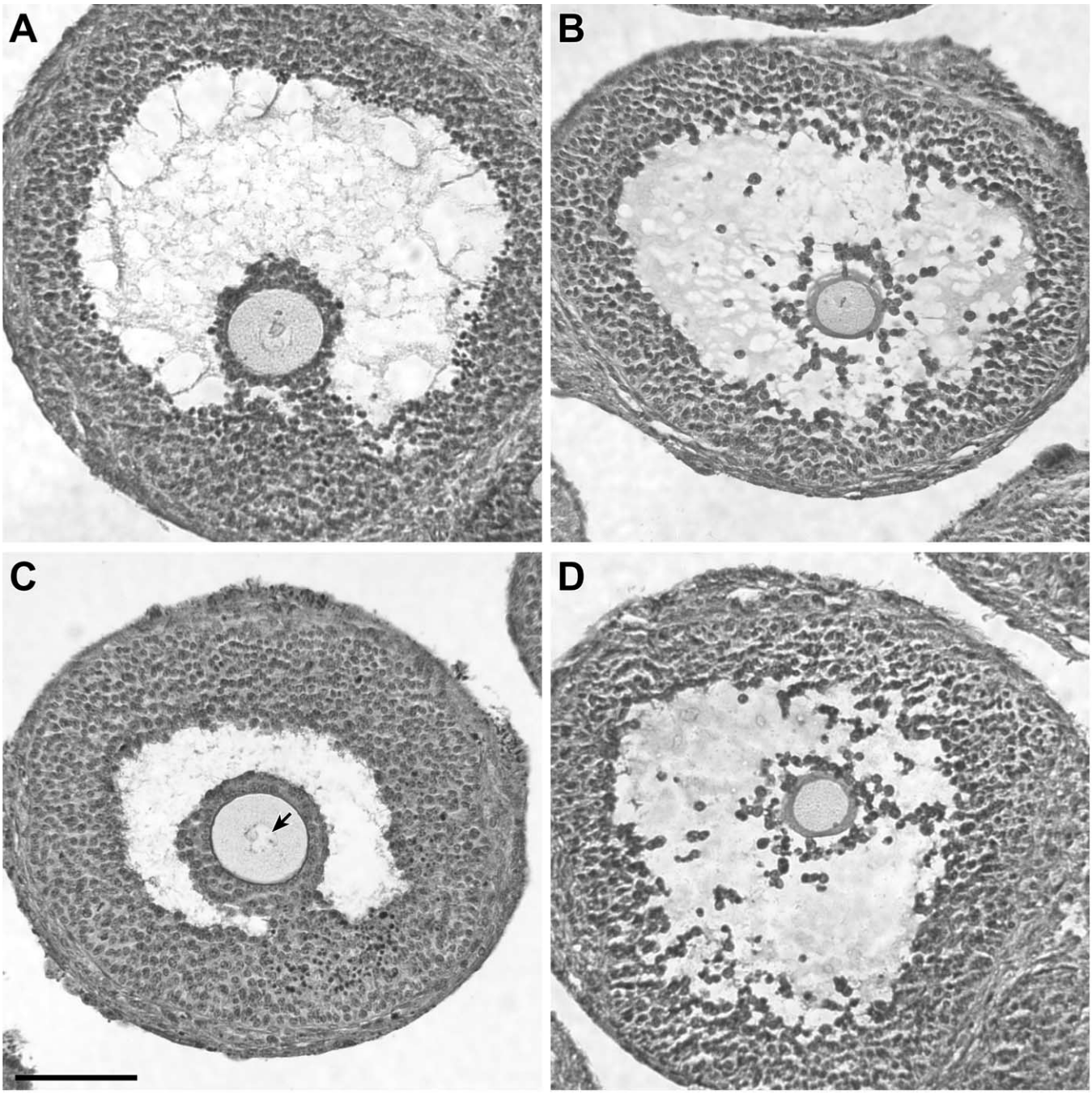

Fig. 2. MEK inhibitor blocks LH-induced cumulus expansion in mouse preovulatory follicles. Large antral follicles were cultured for 12 h. At the end of culture, follicles were fixed and sectioned for histological analysis. (A) Control follicle. (B) Follicle treated with 10 μg/ml LH; note the expanded cumulus oophorus. (C) Follicle treated with 100 μM U0126 plus 10 μg/ml LH. The MEK inhibitor blocked both cumulus expansion and GVB, note intact GV (arrow). (D) Follicle treated with 100 μM U0124 plus 10 μg/ml LH. The inactive analog of the MEK inhibitor (U0124) did not prevent LH-induced cumulus expansion or oocyte maturation. Scale bar, 100 μm.

induced expression of *Has2* and *Ptgs2* mRNA in the cumulus cells.

First, to test the involvement of MAPK in LH-induced gene expression, large antral follicles were initially treated with 100 μM U0126 or U0124 for 1 h; LH was then added into the medium at a final concentration of 10 μg/ml and follicles were cultured for 6 h. At the end of culture, cumulus cells were isolated and the steady-state levels of *Has2* and *Ptgs2* mRNA were determined. Very low levels of *Has2* and *Ptgs2* mRNA were detected in the control group; however, LH stimulated mRNA expression of both genes in the cumulus cells, with 1.8-and 6-fold increase, respectively (Fig. 3B and C). Moreover, U0126 (but not U0124) inhibited LH-stimulated expression of *Has2* and *Ptgs2* mRNA,

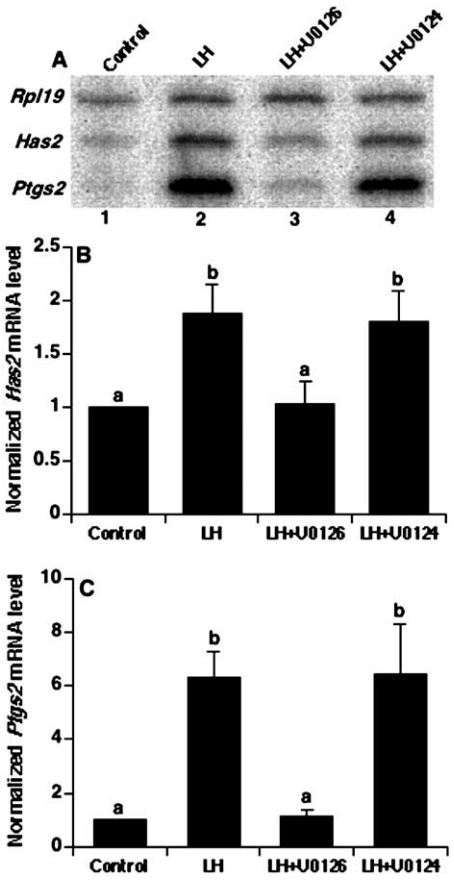

Fig. 3. MEK inhibitor blocks LH-induced *Has2* and *Ptgs2* mRNA expression in cumulus cells of preovulatory follicles. Large antral follicles were cultured for 6 h with no treatment (control), 10 μg/ml LH (LH), 10 μg/ml LH plus 100 μM U0126 (LH+U0126), or 10 μg/ml LH plus 100 μM U0124 (LH+U0124). At the end of culture, the cumulus cells were isolated and the steady state levels of *Rpl19*, *Has2*, and *Ptgs2* mRNA assessed by RNase protection assay. *Has2* and *Ptgs2* mRNA values were first corrected to *Rpl19* values to adjust for differences in loading between samples. Then, for each experiment, the control values were arbitrarily set at 1 and experimental groups were calculated relative to this value to allow unbiased comparisons of groups among experiments. All the RNase protection data shown in subsequent figures are presented in this manner. (A) A representative phosphorimage (Fuji Photo Film Co., Ltd.) of *Rpl19*, *Has2*, and *Ptgs2* mRNA. (B) Normalized steady-state levels of *Has2* mRNA in cumulus cells. (C) Normalized steady-sate levels of *Ptgs2* mRNA in cumulus cells. The values shown are the mean (±SEM) values from four experimental replicates. Where there are no common letters over the bars, the groups were significantly different ($P < 0.05$).

resulting in the same levels of expression as in control groups.

Second, to test the involvement of a MAPK-dependent pathway in the regulation of steady state expression of *Has2* and *Ptgs2* mRNA in cumulus cells stimulated by FSH, isolated OCCs were initially treated with 10 μM of U0126 or U0124 for 30 min before FSH was added to the culture medium at a final concentration of 100 IU/liter. After a 6-h culture, cumulus cells were stripped from the oocyte and the expression of *Has2* and *Ptgs2* in the cumulus cells determined. Low levels of *Has2* and *Ptgs2* mRNA were detected in the control group. FSH stimulated expression of both *Has2* and *Ptgs2* mRNA in cumulus cells with 3.2-and 3.4-fold increase, respectively (Fig. 4B and C); however, this stimulatory effect was inhibited by 10 μM of U0126, but not U0124. The levels of *Has2* and *Ptgs2* mRNA in the group treated with U0126+FSH were the same as that in control group without FSH, while the levels in the group treated with U0124+FSH were the same as that in the group treated with FSH.

Requirement of MAPK activity for elevation of Has2 and Ptgs2 steady-state mRNA expression in FSH-stimulated cumulus cells cocultured with oocytes and in cumulus cells treated with GDF-9

It was previously shown that both the oocyte and GDF-9 promote cumulus expansion as well as *Has2* and *Ptgs2*

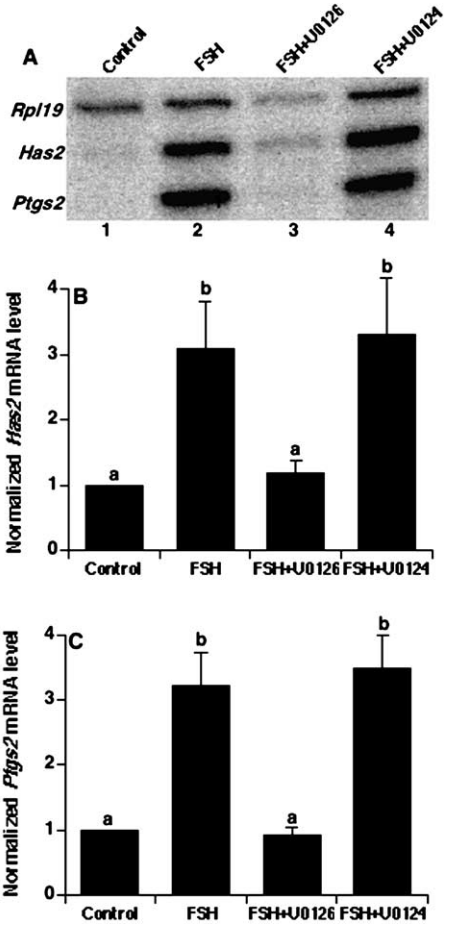

Fig. 4. MEK inhibitor blocks FSH-induced *Has2* and *Ptgs2* mRNA expression in the cumulus cells of isolated OCCs. OCCs were cultured for 6 h with no treatment (control), with 100 units/liter FSH (FSH), 100 units/liter FSH plus 10 μM U0126 (FSH+U0126), or 100 units/liter FSH plus 10 μM U0124 (FSH+U0124). At the end of the culture, cumulus cells were isolated and the steady state levels of *Rpl19*, *Has2*, and *Ptgs2* mRNA assessed as described in the legend to Fig. 3. (A) Normalized levels *Has2* mRNA in cumulus cells. (B) Normalized levels of *Ptgs2* mRNA in cumulus cells. The values shown are the mean (±SEM) values of three independent experiments. Where there are no common letters over the bars, the groups were significantly different ($P < 0.05$).

expression in the cumulus cells (Buccione et al., 1990; Elvin et al., 1999, 2000; Joyce et al., 2001; Su et al., 2002; Tirone et al., 1997, 1993). Moreover, the MEK inhibitor is known to block GDF-9-induced cumulus expansion (Su et al., 2002). Therefore, it is possible that the oocyte and/or GDF-9 might regulate the MAPK-dependent cumulus expansion process. The following experiments tested this hypothesis. Isolated cumulus cells either were cocultured with the fully grown GV-stage oocytes at a density of 2 oocytes/μl and treated with 100 IU/liter FSH plus 10 μM U0126 or U0124; or were cultured alone and treated with 100 ng/ml of GDF-9 plus 10 μM U0126 or U0124. After 6 h of culture, cumulus cells were collected and their steady-state levels of *Has2* and *Ptgs2* mRNA were determined. In the control group, where isolated cumulus cells were treated with FSH alone, low level of *Has2* mRNA and almost no *Ptgs2* mRNA were detected (Fig. 5). When oocytes were added to the culture, relatively high levels of both *Has2* and *Ptgs2* mRNA were detected in the cumulus cells. However, treatment with 10 μM U0126, but not U0124, almost totally blocked these increases in the steady state levels of *Has2* and *Ptgs2* mRNA. Similarly, recombinant GDF-9, in the absence of FSH, elevated the expression of both *Has2* and *Ptgs2* in isolated cumulus cells. The stimulatory effect of GDF-9 was completely blocked by U0126, but not U0124 (Fig. 6). Therefore, elevation of the steady-state levels of *Has2* and *Ptgs2* mRNA requires activation of MAPK by the oocyte with FSH, or by GDF-9 without FSH.

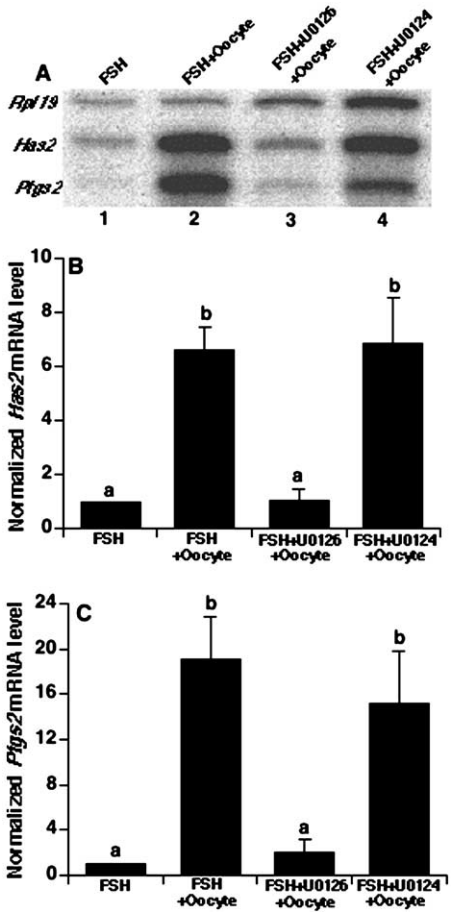

Fig. 5. MEK inhibitor blocks oocyte-enabled *Has2* and *Ptgs2* mRNA expression in isolated cumulus cells treated with FSH. Isolated mouse cumulus cells were either cultured alone and treated with 100 units/liter FSH (FSH) for 6 h or cocultured with fully grown oocytes, at a density of 2 oocytes/μl, and treated with 100 units/liter FSH (FSH+Oocyte), 100 units/liter FSH plus 10 μM U0126 (FSH+U0126+Oocyte), or 100 units/liter FSH plus 10 μM U0124 (FSH+U0124+Oocyte) for 6 h. At the end of the culture, cumulus cells were isolated and the steady state levels of *Rpl19*, *Has2*, and *Ptgs2* mRNA assessed as described in the legend to Fig. 3. (A) Normalized levels of Has2 mRNA in cumulus cells. (B) Normalized levels of *Ptgs2* mRNA in cumulus cells. The values shown are the mean ($\pm$SEM) values of four independent experiments. Where there are no common letters over the bars, the groups were significantly different ($P < 0.05$).

Effect of oocytes on FSH-induced activation of MAPK in cumulus cells

Observations that the MEK inhibitor blocked oocyte plus FSH induced *Has-2* and *Ptgs2* mRNA expression in the cumulus cells suggest that the oocyte may also participate in the regulation of MAPK activity in the cumulus cells. This hypothesis was tested, by comparing MAPK activity in response to FSH by the cumulus cells of OCCs relative to isolated cumulus cells. We found that FSH promotes the activation of MAPK in cumulus cells of intact OCCs within 30 min (Fig. 7), but there was little activation of MAPK in cumulus cells in the absence of the oocyte. Moreover, coculture of cumulus cells with denuded oocytes (4–5 fully grown GV-stage oocytes/μl) maintains the ability of FSH to promote MAPK activation (Fig. 7). These data indicate that a paracrine factor produced by oocytes enables cumulus cells to activate MAPK in response to gonadotropic stimulation.

The ability of oocytes to enable isolated cumulus cells to respond to FSH with stimulation of MAPK activation is dependent on the density of the oocytes cocultured with the cumulus cells (Fig. 8A). When isolated cumulus cells were cocultured with oocytes at a density of 1.5 oocytes/μl of culture medium, MAPK activity in FSH-treated isolated cumulus cells began to increase, and the highest levels of MAPK activity in the cumulus cells were detected when they were cocultured with oocytes at a density of 2–4 oocytes/μl of culture medium.

To assess whether the ability of oocytes to enable activation of MAPK in response to FSH depends on the developmental stage of the oocytes, cumulus cells were cocultured with partly grown GV-stage oocytes isolated from preantral follicles of 12-day-old mice, with fully grown, meiotically competent GV-stage oocytes from 22-day-old mice, or with metaphase II oocytes isolated from the oviducts of superovulated 22-day-old mice. Oocytes at all stages tested enabled FSH-induced activation of MAPK in cumulus cells (Fig. 8B).

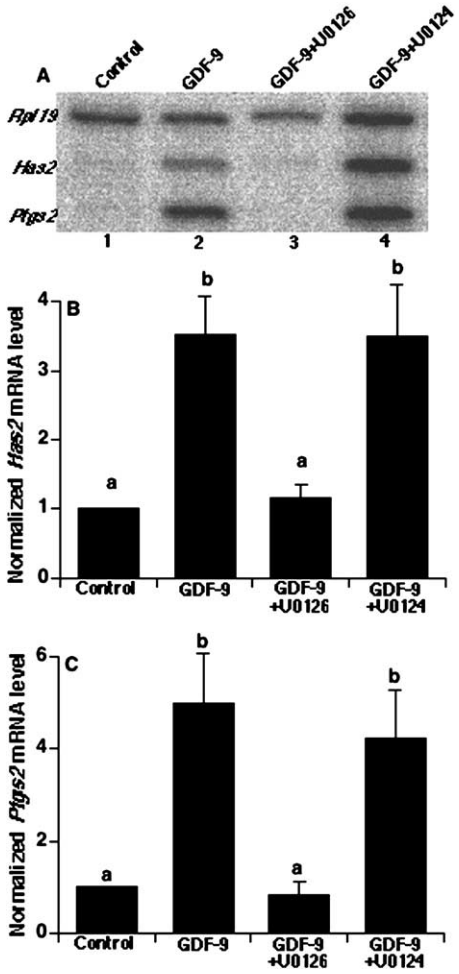

Fig. 6. MEK inhibitor blocks GDF-9-induced *Has2* and *Ptgs2* mRNA expression in isolated cumulus cells. Isolated cumulus cells were cultured for 6 h in control medium or medium supplemented with 100 ng/ml GDF-9 (GDF-9), 100 ng/ml GDF-9 plus 10 μM U0126 (GDF-9+U0126), or 100 ng/ml GDF-9 plus 10 μM U0124 (GDF-9+U0124). At the end of the culture, cumulus cells were collected and the steady-state levels of *Rpl19*, *Has2*, and *Ptgs2* mRNA assessed as described in the legend to Fig. 3. (A) Normalized levels *Has2* mRNA in cumulus cells. (B) Normalized levels of *Ptgs2* mRNA in cumulus cells. The values shown are the mean (±SEM) values of three independent experiments. Where there are no common letters over the bars, the groups were significantly different ($P < 0.05$).

Effect of GDF-9 on activation of MAPK in cumulus cells

GDF-9, an oocyte-specific member of the TGFβ superfamily (McGrath et al., 1995), is thought to mediate several of the effects of oocytes on granulosa cell differentiation and function, including cumulus expansion (Eppig, 2001; Matzuk et al., 2002). We assessed the effects of recombinant GDF-9 on activation of MAPK in cumulus cells. GDF-9 stimulated the activation of MAPK in the absence of FSH (Fig. 8C). When isolated cumulus cells were treated with GDF-9, a dose-dependent effect on MAPK activity was observed (Fig. 8C). An increase in MAPK activity was observed when the cumulus cells were treated with 5 ng/ml GDF-9, and the highest levels of MAPK activity were detected when the cumulus cells were treated with 20–100 ng/ml GDF-9.

Effect of recombinant GDF-9 on the resumption of meiosis by cumulus cell-enclosed oocytes

Since GDF-9 induced MAPK activation in cumulus cells, the ability of GDF-9 to induce the resumption of meiosis was determined. Cumulus cell-enclosed oocytes were maintained in meiotic arrest by hypoxanthine (all groups) and treated with GDF-9, together with FSH, or with GDF-9 plus FSH. There were two control groups of cumulus cell-enclosed oocytes not treated with either GDF-9 or FSH: one group maintained in the standard culture medium used throughout this study and the other in medium containing the same proportion of conditioned medium as the recombinant GDF-9-treated group, but conditioned by untransfected cells. GDF-9 did not stimulate the resumption of meiosis, nor did it increase the frequency of GVB induced by FSH (Fig. 9).

Inhibition of MAPK activation does not prevent FSH-induced elevation of cAMP levels in cumulus cells

Previous studies showed that the membrane permeable analog of cAMP, 8Br-cAMP, induced GVB and that the MEK1/2 inhibitor U0126 blocked this effect of cAMP on oocyte maturation (Su et al., 2002). These results suggest that GVB is promoted by elevation of cAMP in the cumulus cells and that the effect of cAMP is mediated by activation

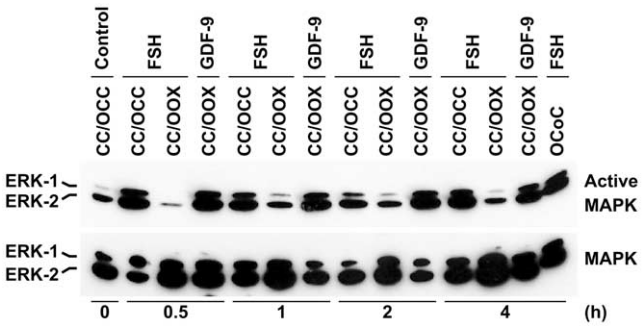

Fig. 7. Activation of MAPK in cumulus cells: effects of FSH, oocytes, and recombinant GDF-9. Mouse OCCs or isolated cumulus cells were treated with FSH or GDF-9 respectively, and cultured for 1–4 h in vitro. In addition, cumulus cells from 30 OCCs were collected immediately at the beginning of the culture to work as the 0-h control. At the end of culture, cumulus cells were collected and MAPK activity was assessed indirectly by western blot analysis using a specific anti-active MAPK monoclonal antibody (indicated in the top panels). The total amounts of MAPK protein (indicated in the bottom panels) were determined by reprobing the same blot using an anti-MAPK polyclonal antibody. Cumulus cells from 30 OCCs were loaded into each lane. The positions of the two MAPK isoforms (ERK1 and-2) are indicated on the left of each gel. The experiment was repeated three times with similar results, and a representative gel is shown. CC/OCC, cumulus cells of OCCs. CC/OOX, isolated cumulus cells. OCoC, isolated cumulus cells cocultured with denuded oocytes (4 oocytes/μl of medium) isolated from 22-day-old mice.

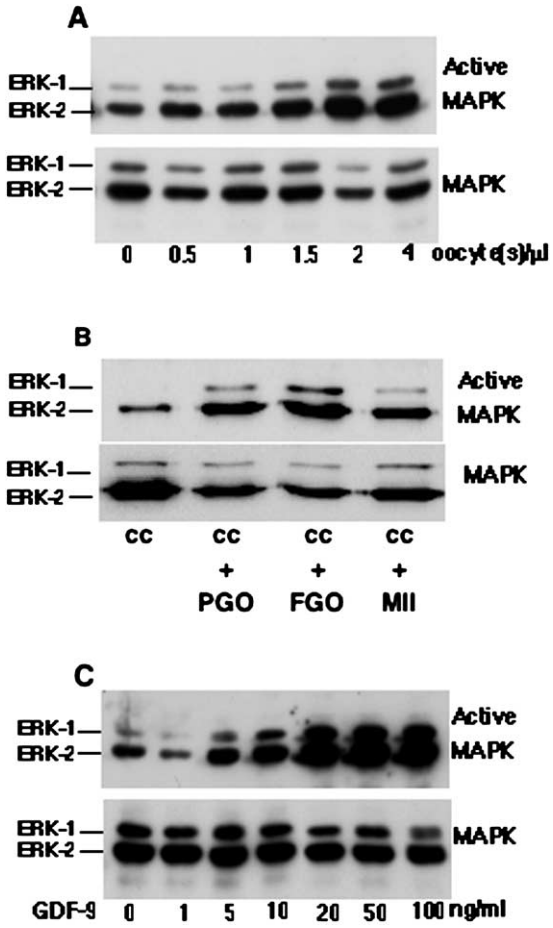

Fig. 8. Oocytes and GDF-9 promote the activation of MAPK in isolated cumulus cells. (A) Isolated mouse cumulus cells were cocultured with fully grown oocytes isolated from 22-day-old mice at a density of 0–4 oocyte(s)/μl and treated with FSH for 4 h. (B) Isolated cumulus cells stimulated with FSH and cocultured with partly grown oocytes (PGO) (10 oocytes/μl) isolated from the preantral follicles of 12-day-old mice, fully grown oocytes (FGO) (5 oocytes/μl), and metaphase II oocytes (MII) (5 oocytes/μl). (C) Isolated cumulus cells were treated with various doses of GDF-9 (0–100 ng/ml) and cultured for 4 h. The experiment was repeated three times with similar results, and a representative gel is shown.

of MAPK. However, it remained possible that inhibition of MAPK activity prevents FSH-induced elevation of cAMP levels in cumulus cells and that FSH induces GVB by a cAMP-independent pathway. However, U0126 had no effect on elevation of cAMP levels in cumulus cells stimulated by FSH (Fig. 10), supporting the hypothesis that the pathway for gonadotropin-induced resumption of meiosis is mediated sequentially by cAMP then MAPK.

Discussion

A preovulatory surge of LH stimulates the maturation of the oocyte–cumulus cell complex, processes that include both the resumption of meiosis in the oocyte and the mucification and expansion of the cumulus oophorus. However, neither of these processes took place in intact follicles cultured with LH and a highly specific MEK1/2 inhibitor that blocks the activation of MAPK. Therefore, since activation of MAPK in mammalian oocytes is not required for the resumption of meiosis (GVB) (Araki et al., 1996; Phillips et al., 2002; Su et al., 2001, 2002; Verlhac et al., 1996), LH-induced GVB requires the activation of MAPK in the somatic cell compartment of the follicle. Moreover, although it was previously shown that activation of MAPK is required for cumulus expansion (Su et al., 2002), the present experiments demonstrate that MAPK activation is required for the transcription of two genes (*Has2* and *Ptgs2*) essential for cumulus expansion. This does not exclude the possibility that posttranscriptional processes are also affected.

It was previously known that expansion of the mouse cumulus oophorus requires a factor secreted by the oocyte, the cumulus expansion enabling factor (CEEF) (Buccione et al., 1990), and that FSH-stimulated cumulus expansion in vitro requires activation of MAPK in the cumulus cells of isolated OCCs (Su et al., 2002). However, it was not known whether oocytes are required for gonadotropin-dependent activation of MAPK in the cumulus cells or whether this activation is upstream of the oocyte-dependent pathway required for cumulus expansion. Here, we show that denuded oocytes cocultured with isolated cumulus cells greatly enhanced MAPK activity in cumulus cells stimulated by FSH, while MAPK activity was low in the absence of oocytes. This indicates that oocytes secrete one or more paracrine factors needed to promote MAPK activity in cumulus cells. Moreover, since elevated MAPK activity in cumulus cells is required for the maturation of both the oocyte and the cumulus oophorus, the oocyte itself thus

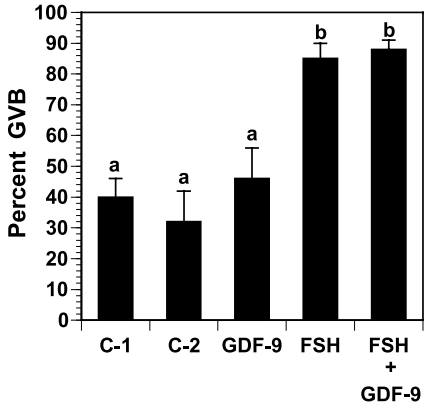

Fig. 9. FSH, but not GDF-9, stimulates the resumption of meiosis (GVB) in hypoxanthine-treated OCCs. OCCs were cultured in medium containing 4 mM hypoxanthine. Control-1 (C-1) was the standard Minimum Essential Medium α, and Control-2 (C-2) included the same proportion of conditioned medium as in the GDF-9-treated group, but conditioned by untransfected cells. GVB was assessed after 15 h of culture. Results are presented as the mean and standard errors of the mean of three independent experiments, and approximately 40 OCC were included in each group in each experiment. Where there are no common letters over the bars, the groups were significantly different ($P < 0.05$).

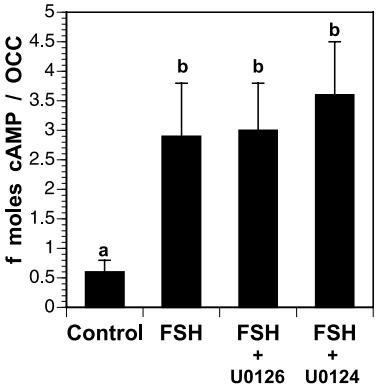

Fig. 10. Inhibition of MAPK activation does not prevent FSH-induced elevation of cAMP. Complexes were incubated for 3 h before assessment of cAMP levels by radioimmunoassay. Results are presented as the mean and standard errors of the mean of three independent experiments. Where there are no common letters over the bars, the groups were significantly different ($P < 0.05$).

plays a key role in enabling, or licensing, both of these maturational processes.

Gonadotropin-dependent activation of MAPK is downstream of elevation of granulosa cell cAMP levels and the activation of PKA (Cameron et al., 1996; Das et al., 1996; Seger et al., 2001). In the cumulus cells, the membrane permeable cAMP analog, 8Br-cAMP, stimulates activation of MAPK. Moreover, inhibition of MAPK activity blocked both 8-Br-cAMP-induced oocyte GVB and cumulus expansion (Su et al., 2002). Thus, the cAMP-PKA pathway regulates these functions of cumulus cells by activation of MAPK, although the relationship of the PKA and MAPK pathways is not understood. FSH-dependent elevation of cumulus cell cAMP levels is independent of oocyte paracrine factors arid cumulus cell MAPK activation since FSH induces cAMP production to the same levels in cumulus cells in the absence of the oocyte as in intact oocyte-cumulus cell complexes (Buccione et al., 1990), and this elevation of cAMP levels is not affected by the MEK1/2 inhibitor (as shown in this paper). These results also demonstrated that separation of cumulus cells from oocytes and inhibition of MAPK activity in the cumulus cells do not affect the function of FSH receptors. Therefore, the likely path of gonadotropin action begins with the elevation of cAMP levels in granulosa cells promoting the activation of MAPK followed by the events leading to GVB and cumulus expansion. Importantly, in cumulus cells, cAMP-dependent activation of MAPK, which is necessary for GVB and cumulus expansion, requires the presence of one or more factors secreted by oocytes.

Is activation of cumulus cell MAPK sufficient to launch the specific processes leading to oocyte resumption of meiosis and cumulus expansion? Two lines of evidence suggest that it is not. First, in conjunction with FSH, paracrine factors from partly grown oocytes isolated from preantral follicles were able to activate MAPK in cumulus cells. Yet, cumulus expansion does not occur in this case (Vanderhyden et al., 1990) nor does increased *Ptgs2* mRNA expression (Joyce et al., 2001). Second, recombinant GDF-9 promotes the activation of MAPK in cumulus cells (Su et al., 2002, and this study). Yet GDF-9, alone or in combination with FSH, does not promote GVB (in the presence of hypoxanthine). Nevertheless, recombinant GDF-9 promotes *Has2* and *Ptgs2* expression and cumulus expansion in the absence of FSH, and this stimulation required MAPK activation (Elvin et al., 1999; Su et al., 2002, and this study). Thus, activation of MAPK in cumulus cells is necessary but not sufficient for launching the pathways leading either to the induction of GVB in oocytes or to cumulus expansion. In fact, these maturational pathways appear distinct from each other, and the differences may be in events downstream from MAPK activation (Fig. 11). However, it cannot be excluded that there are independent, parallel pathways leading to MAPK activation.

Partly grown oocytes isolated from preantral follicles enabled FSH-dependent activation of MAPK in cumulus cells, but, unlike fully grown oocytes, did not enable cumulus expansion (Vanderhyden et al., 1990) or increased *Ptgs2* mRNA expression (Joyce et al., 2001). This suggests that paracrine factors secreted by oocytes at the two stages of development are different. The nature of this difference is unknown. It is possible that a specific factor acting downstream of, or parallel to, the MAPK-activation enabling factor is secreted only by fully grown oocytes. It also seemed possible that partly grown oocytes might secrete a factor that blocks the action of the cumulus expansion enabling factor. However, in unpublished experiments, partly grown oocytes were co-cultured with fully grown oocytes, with the partly grown oocytes in great numerical excess, and did not interfere with the ability of the fully grown oocytes to enable cumulus expansion. This suggests that partly grown oocytes do not secrete a blocking factor that could prevent the activity of a cumulus expansion enabling factor.

It is puzzling that recombinant GDF-9 promotes expression of *Has2* and other genes required for cumulus expansion and induces the MAPK activation required for cumulus expansion, but, unlike native paracrine factors secreted by oocytes, does not require FSH to induce cumulus expansion. Perhaps FSH promotes cumulus cell production of a factor that modifies the structure, and therefore regulates the function, of native GDF-9 produced by oocytes. Recombinant GDF-9 may be produced in this modified form and therefore not require the FSH-dependent factor produced by cumulus cells. Resolution of this issue must await further studies.

In conclusion, this study demonstrates a remarkable interaction between oocytes and their companion cumulus cells that is essential for gonadotropin-induced maturation of both the somatic cell and germ cell compartments of the oocyte-cumulus cell complex (Fig. 11). In our current working model, gonadotropin induces both the resumption of meiosis and cumulus expansion by mechanisms requiring the activation of MAPK in the cumulus cells, but this

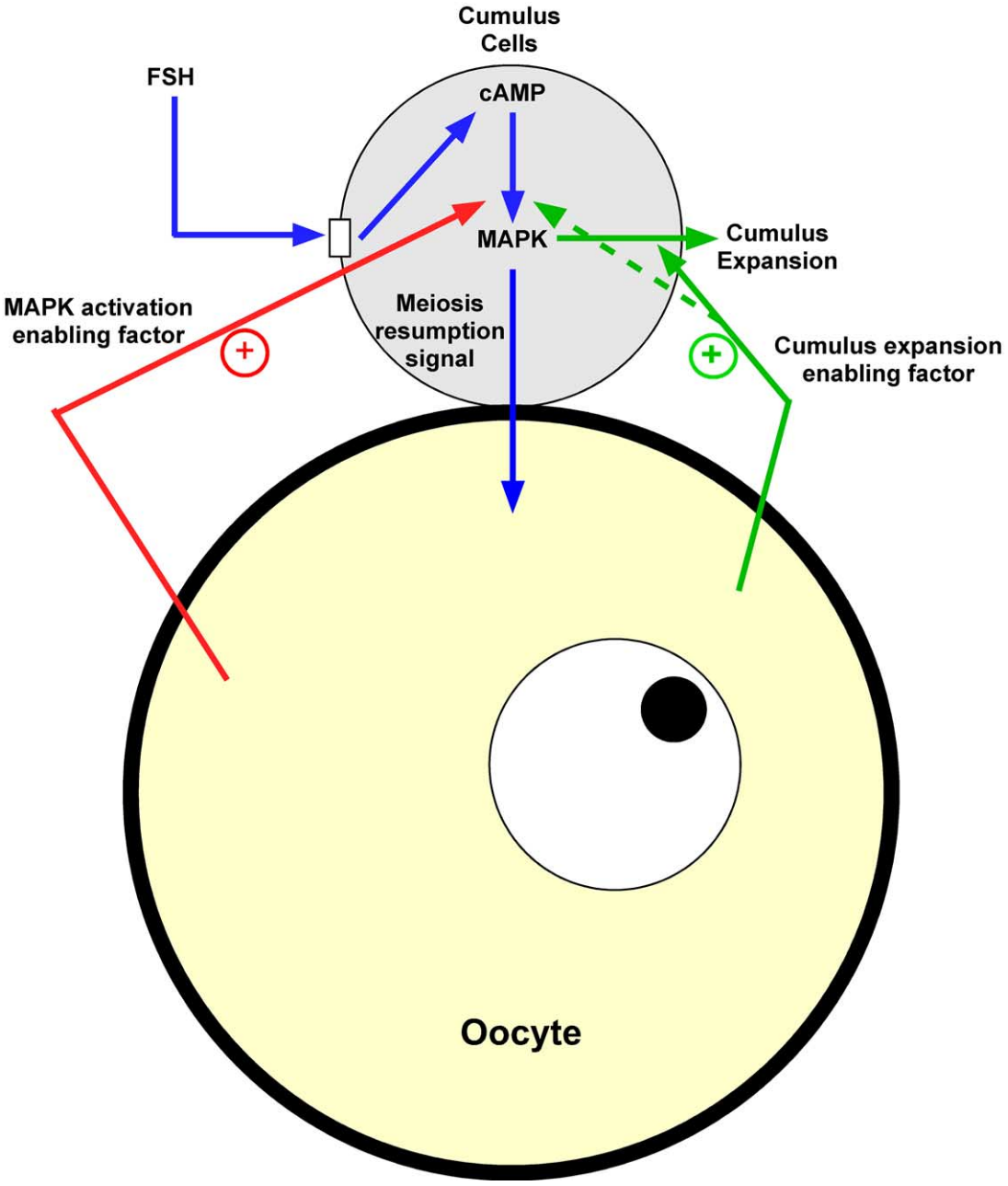

Fig. 11. A working hypothesis of the bidirectional communication between the oocyte and its companion somatic cells for the regulation of GVB and cumulus expansion in the isolated cumulus cell-enclosed oocyte model system. FSH induces the elevation of cAMP in the cumulus cells. In the presence of a meiosis resumption enabling factor (red arrow) produced by the oocyte, elevated cumulus cell cAMP promotes the activation of MAPK, which, in return, promotes the generation of a meiosis resumption inducing signal. The signal is communicated to the oocyte via gap junctions. The green arrows indicate a distinct cumulus expansion enabling factor (CEEF) produced by the oocyte that could enable cumulus expansion by promoting MAPK activity in the cumulus cells (dashed green arrow) or act downstream of cAMP-dependent activated MAPK (solid green arrow). The arrows may indicate multiple intermediary steps.

activation cannot occur in the absence of the oocyte, and is likely mediated by one or more paracrine factors. Thus, oocytes enable, or license, the cumulus cells to produce, in response to gonadotropin-induced elevation of granulosa cell cAMP, a return signal that induces the resumption of meiosis. It also appears that an oocyte-dependent pathway downstream from oocyte-enabled activation of MAPK, and distinct from that promoting the resumption of meiosis, governs cumulus expansion (Fig. 11).

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