Sustained Activity of the EGF Receptor Is an Absolute Requisite for LH-Induced Oocyte Maturation and Cumulus Expansion

Yitzhak Reizel,* Judith Elbaz,* and Nava Dekel

Department of Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel

Mammalian reproduction depends on the release of a mature oocyte from the ovarian follicle. Maturation of the oocyte and rupture of the follicle wall constitute part of the responses to the preovulatory surge of LH, which also include cumulus expansion and granulosa cell luteinization. It was previously shown that the epidermal growth factor receptor (EGFR) mediates the ovulatory response to LH in the ovarian follicle. We hypothesized that it is a sustained activity of the EGFR that generates oocyte maturation and cumulus expansion. We demonstrated that, whereas a transient exposure of rat isolated, intact, preovulatory follicles to either LH or forskolin was sufficient to induce oocyte maturation and cumulus expansion, these LH-induced responses were only generated upon a prolonged activity of the EGFR. In addition, the continuous activity of the EGFR is essential for the chronic phosphorylation of the ERK1/2 downstream signaling molecules, which were shown to be essential for oocyte maturation and cumulus expansion. Interestingly, EGFR-sustained activity was also necessary to maintain the up-regulation of Ptgs2, a gene essential for cumulus expansion. The unusual prolonged duration of ERK1/2 activity may possibly be attributed to the late induction of the ERK-specific phosphatase 3, demonstrated herein. These new data shed light on the unique characteristics of EGFR-ERK1/2 activity in the ovarian follicle and emphasize the fact that the ovulatory process involves a nonclassical activation of this pathway.

(Molecular Endocrinology 24: 402–411, 2010)
The role of ERK1/2 in gonadotropin-induced oocyte maturation and cumulus expansion was first demonstrated in mouse cumulus oocyte complexes (COCs) (13). It was later shown in rat ovarian follicles that ERK1/2 mediates the immediate effect of LH on gap junctional closure in granulosa cells (14). This stops the somatic cAMP influx to the oocyte, leading to a subsequent drop of the intracellular cAMP level, to allow the resumption of meiosis (15, 16). Additionally, it was recently shown that a genetically manipulated mouse, in which the granulosa ERK1 and ERK2 were depleted, did not ovulate (4). Hormonal administration in such mice failed to induce resumption of meiosis, cumulus expansion, and luteinization.

Two decades ago, we found that, similar to LH, the epidermal growth factor (EGF) stimulates rat large antral follicles and thereby promotes maturation of the oocyte (17), a fact that was further confirmed in mouse oocytes (18). We later demonstrated that the EGF-induced maturation produced fertilizable eggs (19). More recently, the epidermal growth factor (EGF) receptor (EGFR) was shown to mediate the effect induced by LH on oocyte maturation, cumulus expansion, and luteinization in mouse ovarian follicles (20, 21). These reports showed that LH increases the transcription of the epiregulin, amphiregulin, and betacellulin EGF-like molecules. These data were extended to the rat, further showing that in explanted follicles, metalloproteinases mediate the activation of the EGFR by LH (22). Furthermore, these authors have demonstrated that EGFR and metalloproteinases are involved in ovulation in vivo. The essential role of EGFR in mediating LH action was further confirmed in transgenic mice expressing a mutated EGFR, in which preovulatory follicles failed to respond to LH (20). Taken together, these data establish the indispensability of the EGFR pathway in the LH-induced ovulatory responses.

It was previously proposed that due to the lower density of LH receptors on the cumulus cells as compared with mural granulosa cells (23), the EGF-related growth factors produced at the periphery may serve as paracrine mediators that propagate the LH signal toward the center of the follicle (21). However, the fact that the EGF-like molecules are up-regulated similarly in granulosa and cumulus cells questions this possibility (24).

It is commonly established that the EGFR pathway is rapidly shut down (25, 26). However, in the ovary, EGFR and ERK1/2 are phosphorylated for few hours after LH stimulation (27, 28). In this study, we hypothesized that the sustained activity of the EGFR is required to mediate LH action in the ovary. We confirm herein that a short-term exposure of ovarian follicles to LH is sufficient to trigger the response to this gonadotropin (29). We further show that a forskolin-induced transient activation of the adenyl cyclase is sufficient to generate the same responses. By contrast, we indeed demonstrate that termination of the activity of the EGFR at time points that are earlier than 3 h of exposure to LH severely impairs oocyte maturation and cumulus expansion. In addition, we report that the continuous activity of the EGFR is essential for the phosphorylation of its downstream effectors ERK1/2 and for maintaining these kinases in a phosphorylated state. A sustained activity of the EGFR is also required for the increase of the transcription of Ptgs2, an essential gene for cumulus expansion. Finally, in search for a mechanism that may be responsible for the prolonged ERK1/2 activity, we screened for the mRNA expression of MAPK phosphatases (MKPs) in the ovarian follicle upon exposure to LH, pointing toward MKP-3 as the potential enzyme responsible for ERK1/2 shutdown in this system.

Results
A transient exposure to LH is sufficient to induce oocyte maturation and cumulus expansion

Our first experiment aimed at determining the time window during which the presence of LH is required for the induction of oocyte maturation and cumulus expansion. For this purpose, isolated intact ovarian follicles from 25-d-old pregnant mare’s serum gonadotropin (PMSG)-primed rats were exposed to LH for 20 min, after which time they were washed and placed in a hormone-free medium for a total incubation period of 10 h. Both germinal vesicle breakdown (GVB) and cumulus expansion (30) should have occurred at this time point. At the end of incubation, the follicles were incised and COCs were monitored for the meiotic status of the oocyte, as indicated by presence or the absence of a GV, as well as for the extent of the cumulus expansion. As positive and negative controls, ovarian follicles were exposed for 10 h to LH or vehicle, respectively. No significant difference was detected between the two groups incubated with LH, demonstrating that a 20-min pulse of LH is sufficient to induce resumption of meiosis and cumulus expansion (Fig. 1, A and B). Because these responses might be attributed to the classical, well-known, irreversible nature of ligand-receptor interaction, the effect of a transient activation of the adenyl cyclase, the direct downstream effector of LH, was examined. Preovulatory follicles were incubated for 4 min, 20 min, and 10 h with forskolin, a reversible adenyl cyclase activator. We found that exposure of follicles to forskolin for 20 min was sufficient to induce oocyte maturation and cumulus expansion in a maximal fraction of the incubated follicles (Fig. 1, C and
D). Incubation with forskolin for only 4 min did not generate the same output, demonstrating in addition that the wash was indeed effective. Taken together, these data reveal that a short exposure to either LH or cAMP is sufficient to generate an irreversible commitment of this system for ovulatory changes.

A sustained activity of the EGFR is necessary to mediate the LH-induced oocyte maturation and cumulus expansion

It was previously reported for mouse isolated intact follicles (27, 28), and herein confirmed for the rat (supplemental Fig. 1 published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org), that in the ovary, EGFR stays phosphorylated for few hours after LH stimulation. To characterize the duration of the EGFR activity that is required to generate the response to LH, ovarian follicles were monitored for oocyte maturation, as indicated by GVB (A and C) and for the extent of the cumulus expansion. The fraction of expanded, out of the total cumuli examined, is presented in panels B and D. The histograms show means ± SE of three independent experiments. Columns with different superscripts differ significantly (P < 0.005 in A–C; P < 0.05 in D).

A sustained activity of the EGFR is necessary to mediate the LH-induced oocyte maturation and cumulus expansion

It was previously reported for mouse isolated intact follicles (27, 28), and herein confirmed for the rat (supplemental Fig. 1 published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org), that in the ovary, EGFR stays phosphorylated for few hours after LH stimulation. To characterize the duration of the EGFR activity that is required to generate the response to LH, ovarian follicles were incubated with LH for 10 h, and the EGFR antagonist AG1478 (AG) was added at different time points. As a positive control, AG1478 was not added to the incubation medium and as negative controls, ovarian follicles were incubated without LH or preincubated with AG1478 for 1 h before the addition of LH (lane 2). At the end of the incubation period, the follicles were incised, and the COCs were monitored for oocyte maturation, as indicated by GVB (A) and for the extent of cumulus expansion (B). The histograms show means ± SE of at least three independent experiments. Columns with different superscripts differ significantly (P < 0.05).

Sustained activity of the EGFR is required for the LH-induced up-regulation of Ptgs2 expression

To decipher the mechanism involved in the requirement for the prolonged activity of EGFR, we examined the expression of Ptgs2, an essential gene for cumulus expansion (10, 11), which is up-regulated in response to LH. Consistent with previous publications (8), the expression of Ptgs2 was up-regulated at 1 h after LH stimulation and stayed elevated for at least 3 h (Fig. 3). The EGFR inhibitor was added 2 h after LH stimulation, a time that represents the midpoint of the ascending part of Ptgs2 expression curve. Ovarian follicles were therefore exposed to LH for 2 h, at which time either AG1478 or vehicle was added for different time intervals. At the end of incubation, RNA was extracted from these follicles; cDNA was synthesized and subjected to quantitative real-
time PCR (Q-PCR). We found that the addition of AG1478 to the culture medium 2 h after LH stimulation for either 15 or 30 min (referred to in Fig. 3 as 2.25 and 2.5 h, respectively) blocked further up-regulation of Ptgs2; incubation with AG1478 for 1 h (referred to in Fig. 3 as 3 h) significantly reduced Ptgs2 mRNA level (Fig. 3). These observations reveal that termination of EGFR activity after 2 h immediately blocks further transcription of Ptgs2. Therefore, a continuous activity of the EGFR is required to achieve a full Ptgs2 up-regulation, pointing at the fact that EGFR activity is tightly coupled to cumulus expansion via the transcription Ptgs2.

A sustained activity of the EGFR induces prolonged ERK1/2 phosphorylation

We have previously shown that after LH stimulation, ERK1/2 is activated within minutes and remains active for at least 4 h (27). It was further demonstrated that the EGFR is essential for the LH-induced ERK1/2 phosphorylation (28). The experiment described herein determines whether the long duration of ERK1/2 phosphorylation requires the continuous activity of the EGFR. For this purpose, preovulatory follicles were incubated with LH for different time intervals, and AG1478 was added at the last 15 min of incubation. The follicles were then lysed, and the extracted proteins were subjected to SDS-PAGE, followed by Western blot analysis using antibodies recognizing the phosphorylated, active ERK1/2 (pERK1/2) and those raised against general ERK1/2 (gERK1/2). B, Densitometric quantification of the results normalized against gERK1/2 protein levels. For quantification, intensity values of the bands were measured from three different repeats using image J program. The histograms show means ± se of three independent experiments (*, \( P < 0.05 \) vs. corresponding LH treatment).

The chronic ERK1/2 phosphorylation is exclusively maintained by EGFR

We showed that the prolonged duration of the ERK1/2 phosphorylation is dependent on the continuous activity of the EGFR. The aim of the present experiment was to examine whether, over time, an alternative pathway may compensate for EGFR inactivation to induce ERK1/2 phosphorylation. For this purpose, ovarian follicles were subjected to LH for 1 h, at which time AG1478 or vehicle was added for 15, 30, and 90 min. ERK1/2 phosphorylation was inhibited when EGFR was blocked for either 15, 30, or 90 min (Fig. 5). These results rule out the presence of a redundant pathway that bypasses the EGFR, to mediate the phosphorylation of ERK1/2 by LH.
A sustained activity of ERK1/2 is necessary for the LH-induced oocyte maturation and cumulus expansion

We have demonstrated that after LH stimulation, an active EGFR is essential to maintain ERK1/2 in its phosphorylated state. To further examine whether this prolonged ERK1/2 phosphorylation is essential for oocyte maturation and cumulus expansion, ovarian follicles incubated for different time periods with LH were subjected to UO126, a specific inhibitor of MAPK kinase (MEK), the direct upstream activator of ERK1/2. The follicles were incised after a total of 10 h incubation, and the recovered COCs were examined for meiosis resumption and cumulus expansion. We found that blocking ERK1/2 during the first 2 h after LH stimulation partially inhibits oocyte maturation and cumulus expansion (Fig. 6, A and B). It is only after 3 h of a sustained ERK1/2 activity that the fraction of GVB oocytes and that of expanded cumuli were similar to that of ovarian follicles incubated without the ERK1/2 inhibitor throughout the 10-h incubation.

Expression of different members of the MKP family upon LH stimulation

The continuous mode of ERK1/2 activity showed herein in the ovarian follicle is unusual, because in many other systems, the EGFR-induced ERK1/2 phosphorylation is rapidly reversed. It is well established that ERK1/2 signaling is mainly attenuated by the inducible family of MKPs, an atypical class of dual-specificity phosphatases (DUSPs), which specifically dephosphorylate the active ERK1/2. It was previously shown in many systems that MKPs are regulated at the transcriptional level (31). Taking this information into consideration, we screened the known MKPs that could possibly be up-regulated in temporal association with ERK1/2 dephosphorylation (4 h after LH exposure). For this purpose, ovarian follicles were exposed to LH for different periods, at the end of which RNA was extracted and the synthesized cDNA was subjected to Q-PCR for MKP-1 (DUSP1), MKP-2 (DUSP4), MKP-3 (DUSP6), and MKP-X (DUSP7) transcripts, as well as PACAP-preferring type 1 (PAC1; DUSP2), which inactivates ERK1/2 (32). This experiment revealed for the first time that upon LH stimulation, MKP-1, 2, 3 and PAC1 mRNA levels were significantly elevated whereas the expression MKP-X remained unchanged (Fig. 7). It is noteworthy that these phosphatases are differently expressed as follows: MKP-1 mRNA level is rapidly elevated, peaks at 1 h of exposure to LH, and is subsequently quickly reduced. Elevation of the MKP-2 mRNA is less abrupt, reaching a 14-fold induction within 2 h of exposure to LH with a reduced but still high level for at least 4 h. In a different manner, MKP-3 is up-regulated progressively to attain a 17-fold increase at 4 h after exposure to LH. These observations reveal that most MKPs are rapidly up-regulated in the ovarian follicle after LH stimulation, when ERK1/2 is strongly phosphorylated, whereas MKP-3 up-regula-
tion after 4 h is temporally correlated with ERK1/2 dephosphorylation.

**Discussion**

We found that whereas the transient exposure to LH and the forskolin-induced short-term activation of the adenylyl cyclase are sufficient to induce oocyte maturation and cumulus expansion in rat preovulatory follicles, a prolonged activity of the EGFR is absolutely required to generate the same responses. We further show that the sustained nature of EGFR activity is also essential for downstream responses such as up-regulation of the Ptgs2 expression, as well as for the ERK1/2 phosphorylation. Interestingly, it is also a chronic phosphorylation of ERK1/2 that is essential for oocyte maturation and cumulus expansion. In addition, our data demonstrate that ERK1/2 dephosphorylation is temporally correlated with MKP-3 up-regulation, suggesting that this phosphatase may be responsible for shutting down the ERK1/2 cascade in the ovarian follicle. Taken together, this study reveals that the ovulatory response is contingent on a nonclassical, sustained activity of the EGFR-ERK pathway.

**A nonclassical mode of activity of the EGFR-ERK1/2 pathway**

It is well established that the EGFR undergoes internalization within minutes after ligand binding, which is followed by its degradation (25, 26). This negative regulatory mechanism, which shuts down the EGFR-signaling pathway, apparently acts to protect the cells from excessive, harmful effect of the members of the EGF-like family. Indeed, various cancers are associated with mutations of the EGFR, which bring about its constitutive activity (33). The common transient nature of EGFR activity is associated with a phosphorylation of its downstream ERK1/2 that is immediately followed by their dephosphorylation (34). Surprisingly, in the ovary, the continuous activity of EGFR that lasts for at least 3 h is necessary to generate physiological responses such as oocyte maturation and cumulus expansion. This ovarian prolonged activity of the EGFR keeps ERK1/2 phosphorylated for an extended period of time. To our knowledge, it is the first time that the EGFR is shown to continuously activate ERK1/2.

Prolonged activity of ERK1/2, which has been demonstrated in PC12 cells, was triggered by nerve growth factor and led to differentiation. However, the effect of EGF in this system brought about a transient ERK1/2 activity leading to proliferation (35). Transient vs. sustained ERK1/2 activity that generates different outputs was also shown in other cell types (Refs. 36 and 37; reviewed by Ref. 38).

The prolonged EGFR activity in the ovary is also associated with a unique, sustained up-regulation of Ptgs2 that differs from the transient duration of the EGF-induced, Ptgs2-elevated expression in WISH cell line (39). A short kinetics of EGFR-induced up-regulation was also described for the early growth response factor 1 (EGR1), a zinc finger transcription factor. This gene was shown to be up-regulated within 20 min and further down-regulated 40 min after EGF stimulation in cell cultures (34), whereas an elevated expression that lasts for several hours was described previously in the ovary (40). Taken together, these findings point at the unique mode of regulation of the EGFR-signaling cascade in the ovarian physiology leading to ovulation.

**The sustained activity of the EGFR maintains the transient LH stimulation**

Ovulation is a tightly orchestrated process, in which a multitude of cells respond simultaneously to the surge of LH to release, 12 h later, mature oocytes surrounded by expanded cumuli. The fact that a short-term exposure to this gonadotropin generates this remote response agrees with the classical concept of ligand-receptor interaction. However, the fact that a transient exposure to forskolin is sufficient to stimulate oocyte maturation and cumulus expansion confirms that the response to LH is induced by a brief activation of adenylyl cyclase. It is the first time that a short pulse of forskolin is shown to bring about oocyte maturation and cumulus expansion to an extent that is similar to that obtained by a long-term exposure to this adenylyl cyclase activator. However, unlike the transient nature of LH action, a prolonged activity of the EGFR was found to be essential to induce oocyte maturation and cumulus expansion. We propose that, upon
LH stimulation, the prolonged activity of EGFR in the granulosa cells maintains and synchronizes the many complex events that finally converge to ovulation. This mechanism allows translation of the short systemic surge of LH into a deferred response that involves the spatiotemporal coordination of the many processes, collectively defined as ovulation. According to this idea, the surge of LH could be compared with the starter of a car, the EGFR with the fuel, and the ERK1/2 with the engine; their combined activity will move ovulation forward.

It is interesting to note that the prolonged activity of EGFR-ERK1/2 appears to be specific to the female gonad because LH was shown to transiently activate the EGFR in a steroidogenic Leydig cell line (41).

**EGFR activity and ERK1/2 phosphorylation are tightly coupled**

We have shown that inhibition of the LH-induced EGFR activity for 15 min during the first hour of LH stimulation induced partial ERK1/2 dephosphorylation. However, a total ERK1/2 inactivation was obtained at later intervals of incubation. These findings may suggest that during the first hour of incubation with LH, the ERK1/2 phosphatases (MKPs) have not yet reached their maximal level of expression and are not fully effective. Alternatively, after the first hour, ERK1/2 could be exclusively phosphorylated by the EGFR, whereas during the first hour, another pathway may partially stimulate ERK1/2 phosphorylation. PKA might be responsible for the partial ERK1/2 phosphorylation during the first hour of LH stimulation, a possibility that remains to be validated. It is interesting to note that the immediate reduction of ERK1/2 phosphorylation upon EGFR inhibition suggests the presence of active phosphatases that rapidly dephosphorylate ERK1/2 in the absence of EGFR activation.

We noted that ERK1/2 reaches its maximal phosphorylation level within minutes of LH stimulation and that this phosphorylation depends on EGFR activity. Such a rapid response is unlikely to occur as a consequence of transcription of the EGFR-like agonists. It seems that during the first minutes of exposure to LH, EGFR is activated as a consequence of metalloproteinases activity, which sheds the already existing membrane-bound EGF-like factors, allowing activation of the receptor (42). This initial effect could possibly be prolonged by further synthesis of EGF-like factors. The identity of the metalloproteinases and their mechanism of activation remain to be explored.

**Presence of MKPs in the ovarian follicle**

MKPs, which are induced by ERK activation, consist of a subclass of the DUSPs that selectively dephosphorylate ERKs. Some MKPs, including MKP-2 (DUSP4), MKP-3 (DUSP6), and -X (DUSP7) as well as PAC-1 (DUSP2) inactivate specifically ERK1/2 (reviewed by Refs. 43 and 44). In EGF-stimulated HeLa cells, MKPs are induced rapidly (MKP-2 peaks at 30 min whereas MKP-3 peaks at 2 h) (34), whereas in the mammalian preovulatory follicle, the expressions of MKP-2 and 3 are delayed. These data may explain the prolonged phosphorylation of ERK1/2 in the ovary.

Interestingly, 4 h after LH exposure, MKP-3 mRNA levels are substantially elevated, which temporally coincides with the time point of ERK1/2 dephosphorylation described herein. It suggests that, in the ovarian follicle, MKP-3 may be the phosphatase that plays a major role in shutting down the ERK1/2 cascade. It is noteworthy that MKP-3 is the most specific cytoplasm ERK1/2 phosphatase. Because its up-regulation is temporally correlated with ERK1/2 dephosphorylation, we propose that this phosphatase is the dominant regulator of ERK1/2 in the ovarian follicle.

MKP-1, which is considered as an immediate early gene (45), is also rapidly up-regulated in the ovarian follicle. The early up-regulation of MKP-1 and -2 after LH stimulation, which does not coincide with the ERK1/2 dephosphorylation, is surprising. Nevertheless, because MKP-1 and -2 are nuclear, cytoplasmatic ERK1/2 might be unaffected by these phosphatases. In addition, it is important to note that MKP-1 may be more specific to c-Jun N-terminal kinase and p38 than ERK1/2. The role of these phosphatases in the ovary remains to be clarified.

In conclusion, our results emphasize the different mode of action of the hormones controlling the ovulatory responses. Whereas a transient exposure to LH is sufficient to induce oocyte maturation and cumulus expansion in rat preovulatory follicles, a chronic activity of EGFR is necessary to generate the same responses. These findings raise the interesting novel notion that the physiological surge of LH requires a local sustained activity of the EGFR to not only mediate but also maintain its switch-like stimulation. Specifically, the EGFR continuously controls transcription, as reflected by the immediate decrease in Ptsg2 up-regulation upon inhibition of the EGFR. Equally interesting, we demonstrate that upon exposure to LH, EGFR continuous activity is essential for the ERK1/2-sustained phosphorylation. We further reveal that, 4 h after LH stimulation, ERK1/2 dephosphorylation temporally coincides with MKP-3 up-regulation. Collectively, we point at the fact that oocyte maturation and cumulus expansion involve a nonclassical, prolonged activity of the EGFR-ERK pathway.
Materials and Methods

Reagents

Leibovitz’s L-15 tissue culture medium and fetal bovine serum were purchased from Biological Industries (Kibbutz Beit Hemeek, Israel). Antibiotics were purchased from Bio-Lab Ltd. (Jerusalem, Israel). PMSG was supplied by ChronoGest Intervet (Boxmeer, The Netherlands), and ovine LH (o-LH-26) was purchased from the National Hormone and Pituitary Program (Harbor-University of California Los Angeles Medical Center, Torrance, CA). The MEK inhibitor UO126 was supplied by Axxora (San Diego, CA), the EGFR blocker AG1478 was purchased from Calbiochem (San Diego, CA), and the adenylyl cyclase activator forskolin was supplied by Sigma-Aldrich Corp. (St. Louis, MO). Protease inhibitor cocktail, phenylmethylsulfonylfluoride, leupeptin, and pepstatin were from Sigma-Aldrich. Anti-phospho-ERK1/2, antigeneral ERK1/2, and anti-EGFR (sc-03) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-EGFR antibody (tyr1086) was purchased from Cell Signaling Technology (Beverly, MA). The secondary antibodies were purchased from The Jackson Laboratory (Bar Harbor, ME).

Animals

Sexually immature 25-d-old Wistar female rats were purchased from Harlan Laboratories (Rehovot, Israel) and handled according to the guidelines of the National Institute of Health and of the Weizmann Institute for management of laboratory animals. The rats were housed in a light- and temperature-controlled room, with food and water provided ad libitum.

Culture of follicles

The aforementioned rats were injected sc with 10 IU PMSG for induction of follicular development and killed 48 h later. The ovaries were removed and placed in L-15 medium, supplemented with 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). The experiments assessing EGFR phosphorylation were conducted in serum-free medium. Isolated intact rat ovarian preovulatory follicles were separated and grown in suspension in L-15 tissue culture medium containing 5% fetal bovine serum in 25-ml flasks gassed with 50% O2-50% N2 as described previously (46). Each group comprised at least 35 preovulatory follicles. Incubations were carried out at 37°C in an oscillating water bath in the presence or absence of either 1 μg/ml LH, with or without 10 μM AG1478 or 10 μM U0126, doses that were previously shown to give an optimal effect in this system (14, 17, 22). At the end of the incubation period, the follicles were incised, and the oocyte COCs were recovered and microscopically examined for cumulus expansion using differential interference contrast optics as described by us previously (47). The oocytes were monitored for reinitiation of meiosis, as indicated by the disappearance of the visible large nucleus called germinal vesicle (GV).

RNA extraction and cDNA preparation

Liquid nitrogen frozen follicles were homogenized in 500 μl Tri-Reagent (Sigma Aldrich). Glycogen (10 μl) (Roche Applied Science, Mannheim, Germany) was added to allow better precipitation of RNA. After 100 μl of chloroform was added to allow phase separation by centrifugation (at 4°C), 250 μl of isopropanol was added to the aqueous phase. After several hours in −20°C, the RNA was precipitated by centrifugation. The pellet was washed in cold 70% ethanol and then dried. The RNA pellet was resuspended in deoxyribonucleic acid–ribonucleic acid-free water, and deoxyribonucleic acid treatment was performed according to manufacturer’s instructions (Ambion, Austin, TX). Quality and quantity of total RNA extracted were assessed using Nanodrop spectrophotometer. RNA samples (1.5 μg) were reverse transcribed using high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA) as indicated in the manufacturer’s protocol. The cDNA was then diluted in a 1:100 ratio.

Quantitative real-time PCR (Q-PCR)

The reversed transcribed diluted cDNA was amplified by Q-PCR. The primers were chosen using primer express software (Applied Biosystems). They were checked by the NCBI-BLAST program for their specificity. Each of these primer pairs yielded only one sharp band of amplified product with the molecular weight of the desired amplicon. The nucleotide sequences for the different PCR primer pairs are as follows. MKP-1 (NM_053769): forward (F), GGACAACACAGAGCGACA; reverse (R), CAGTGCAAAACACCTTCTCT; MKP-2 (NM_022199): F, AGACTGC-CACAATCACCTTGA; R, CGATGGCTTCAATGACCACCA; MKP-3 (NM_053883): F, CTGCAGGCGGCTTCTCACTCT; R, CT-GACAGACCGCTTGATATTG; PAC1 (NM_001012089): F, GCACCTGCAAGCTGTATCTC; R, CAAATGCAAGGCCCTCTCATCCA; MKP-X (X91486): F, CCTACAAGCAATCCCATCTCT; R, GAGCGGCTTTACTATG. B2m (NM_012512): F, ACATCCTGGTCACACTGAA; R, AT-GTCTCGGTTCCCAGGT. PTgs2 (NM_017232): F, CCCT-GAAACCTTACTCATGT; R, TGGTACGTGTGTCTGGTAGA. Relative quantification of the mRNA was performed using the Stepone system v2.1 (Applied Biosystems). Q-PCRs (10 μl) were carried out using 5 μl of mix (Fast SYBR Green Master Mix; Applied Biosystems), 2 μl cDNA, and 2.5 pmol of each primer. β2-microglobulin (B2m) was used as internal control for normalization. The amplification process was monitored through the fluorescence of SYBR Green.

Protein extraction and Western blot analysis

Total protein was extracted from cultured follicles (15–25 per sample) by homogenization in radioimmune precipitation assay buffer supplemented with 1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 2 μg/ml pepstatin, protease inhibitor (according to manufacturer’s instructions), and 400 μM NaVO3. The lysates were then centrifuged for 20 min, after which the supernatants were collected. The samples were dissolved in protein sample buffer [2% β-mercaptoethanol; 2% sodium dodecyl sulfate; 50 mM Tris-HCl (pH 6.8); 10% glycerol; and 0.01% bromophenol blue] and boiled. For ERK1/2, 15 μg of protein was loaded onto 12% SDS-PAGE. After electrophoretic separation, the proteins were transferred to polyvinylidene fluoride membranes (Millipore Corp, Bedford, MA), which were blocked for 1 h with a blocking solution [5% nonfat dry milk, 0.05% Tween in Tris-buffered saline (TBST)], and then incubated with primary antibodies (overnight, 4°C). Two anti-ERK1/2 antibodies were used for the Western blot analysis: one antibody immunoreacted with the phosphorylated (active) ERK1/2 (pERK1/2), whereas the second immunoreacted with both the active and inactive ERK1/2 (general eERK1/2). The relative amount of the pERK1/2 in each sample represents the extent of ERK1/2 activation. The membranes were then incubated with antirabbit horseradish per-
oxidase (HRP)-conjugated antibodies (1:4000, 1 h, room temperature).

For EGFR, 30 µg of protein was loaded onto 7% SDS-PAGE. After electrophoretic separation, the proteins were transferred to polyvinylidene fluoride membranes (Millipore), which were blocked for 1 h in TBST plus 5% nonfat dry milk, washed with TBST, probed with an anti-phospho-EGFR antibody (specific for autophosphorylation site Tyr1068) diluted 1:1000 in TBST plus 5% BSA overnight at 4°C, and then incubated for 1 h at room temperature with an antirabbit IgG HRP-conjugated antibody diluted in TBST plus 0.5% nonfat dry milk. Afterward, the same membranes were incubated in a solution containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, and 100 mM β-mercaptoethanol for 30 min at 50°C, washed in TBST, and reprobed for total EGFR. Membranes were blocked for 1 h at room temperature in TBST plus 5% nonfat dry milk, incubated with a polyclonal anti-EGFR antibody diluted 1:200 in TBST plus 0.2% nonfat dry milk overnight at 4°C, washed in TBST, and then incubated for 1 h at room temperature with an antirabbit IgG HRP-linked antibody diluted 1:5000 in TBST plus 0.2% nonfat dry milk.

Chemiluminescent signals were generated by incubation with the enhanced chemiluminesence reagent (Amersham, Buckinghamshire, UK). For quantification, intensity values of bands were measured from three different repeats for each experiment using Image J (National Institutes of Health, Bethesda, MD).

Statistical analysis
All experiments were repeated at least three times. All data were analyzed using Student’s unpaired two-tailed t test and presented as mean ± se. P < 0.05 was considered significant.

Acknowledgments
We thank Professor Alex Tsafriri, Professor Ronny Seger, and Dr. Yaara Zwang for helpful discussions and deep insight in this project.

Address all correspondence and requests for reprints to: Professor Nava Dekel, Weizmann Institute of Science, Department of Biological Regulation, Herzl Street 1, Rehovot 76100, Israel. E-mail: nava.dekel@weizmann.ac.il.

This work was supported by The Dwek Fund for Biomedical Research. N.D. is the incumbent of the Philip M. Klutnick Professorial Chair in Developmental Biology.

Disclosure Summary: The authors have nothing to disclose.

References
23. Lawrence TS, Dekel N, Beers WH 1980 Binding of human chori-
45. Sun H, Charles CH, Lai LF, Tonks NK 1993 MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell 75:487–493