0888-8809/06/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 20(6):1352–1365

doi: 10.1210/me.2005-0504

Paracrine and Autocrine Regulation of Epidermal Growth Factor-Like Factors in Cumulus Oocyte Complexes and Granulosa Cells: Key Roles for Prostaglandin Synthase 2 and Progesterone Receptor

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The molecular bridges that link the LH surge with functional changes in cumulus cells that possess few LH receptors are being unraveled. Herein we document that epidermal growth factor (EGF)-like factors amphiregulin (*Areg*), epiregulin (*Ereg*), and betacellulin (*Btc*) are induced in cumulus oocyte complexes (COCs) by autocrine and paracrine mechanisms that involve the actions of prostaglandins (PGs) and progesterone receptor (PGR). *Areg* and *Ereg* mRNA and protein levels were reduced significantly in COCs and ovaries collected from prostaglandin synthase 2 (*Ptgs2*) null mice and *Pgr* null (PRKO) mice at 4 h and 8 h after human chorionic gonadotropin, respectively. In cultured COCs, FSH/forskolin induced *Areg* mRNA within 0.5 h that peaked at 4 h, a process blocked by inhibitors of p38MAPK (SB203580), MAPK kinase (MEK) 1 (PD98059), and PTGS2 (NS398) but not protein kinase A (PKA) (KT5720). Conversely, AREG but not FSH induced *Ptsg2* mRNA at 0.5 h with peak expression of *Ptgs2* and *Areg* mRNAs at 4 h, processes blocked by the EGF receptor tyrosine kinase inhibitor AG1478 (AG), PD98059, and NS398. PGE2 reversed the inhibitory effects of AG on AREG-induced expression of *Areg* but not *Ptgs2*, placing *Ptgs2* downstream of EGF-R signaling. Phorbol 12-myristate 13-acetate (PMA) and adenovirally expressed PGRA synergistically induced *Areg* mRNA in granulosa cells. In COCs, AREG not only induced genes that impact matrix formation but also genes involved in steroidogenesis (*StAR*, *Cyp11a1*) and immune cell-like functions (*Pdcd1*, *Runx1*, *Cd52*). Collectively, FSH-mediated induction of *Areg* mRNA via p38MAPK precedes AREG induction of *Ptgs2* mRNA via ERK1/2. PGs acting via PTGER2 in cumulus cells provide a secondary, autocrine pathway to regulate expression of *Areg* in COCs showing critical functional links between G protein-coupled receptor and growth factor receptor pathways in ovulating follicles. (*Molecular Endocrinology* 20: 1352–1365, 2006)

THE SURGE OF LH induces marked functional (endocrine, biochemical, and molecular) changes in the preovulatory follicle. Estrogen concentrations decline in follicular fluid as a consequence of the reduced expression of aromatase (*Cyp19a1*) and Hsd17β1 (*Hsd17b1*) in granulosa and theca cells, respectively. Conversely, progesterone and prostaglandin (PG; principally PGE2) concentrations rise in follicular fluid in association with induction of P450scc (*Cyp11a1*) and StAR (*Star*) and prostaglandin synthase 2 (*Ptgs2;* also known as cyclooxygenase 2, *Cox2*), respectively (1–6). In addition the PGE2 receptor subtype (*Ptger2*; *EP2*) and progesterone receptor (*Pgr*; *Nr3c3; PR*) are induced in granulosa cells of preovulatory (PO) follicles by LH (7–10). Induction of these genes is critical for fertility because mice null for *Ptgs2* and *EP2* exhibit impaired ovulation (11, 12). Female mice null for *Pgr* (PRKO) fail to ovulate, even in response to exogenous hormones (13, 14). Thus, LH induction of PGE2- and progesterone-dependent pathways in granulosa cells mediate critical events during the ovulation process.

First Published Online March 16, 2006

Abbreviations: AG, AG1478; AREG, amphiregulin; BTC, betacellulin; COCs, cumulus oocyte complexes; CRE, cAMP response element; eCG, equine chorionic gonadotropin; EGF, epidermal growth factor; EREG, epiregulin; hCG, human chorionic gonadotropin; *Has2*, hyaluronan synthase 2; MEK, MAPK kinase; *Pdcd1*, programmed cell death 1; PMA, phorbol 12-myristate 13-acetate; PG, prostaglandin; PGE2, prostaglandin E2; PGR, progesterone receptor; PKA, protein kinase A; PO, preovulatory; PRKO, *Pgr* null; *Ptgs2*, prostaglandin synthase 2.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Before ovulation, cumulus cells surrounding oocytes synthesize the glycosoaminoglycan polymer hyaluronan, specific matrix-associated factors as well as many other proteins/proteoglycans during a process termed cumulus expansion (15–17). Some of the matrix-associated genes that have identified encode hyaluronan synthase 2 [*Has2*; (18, 19)], TNFα-induced protein 6 [*Tnfaip6* also known as TSG6 (20, 21)], chondroitin proteoglycan sulfate2 [*Cgs2* also known as versican (22–24)]. TNFAIP6 binds to hyaluronan backbone and interacts with the serum-derived factor IαI heavy chain to stabilize matrix formation (19). Versican is also coexpressed with hyaluronan at many sites of inflammation (25). In mice null for the prostaglandin E receptor 2 (*Ptger2*) or *Ptgs2*, the expression levels of *Tnfaip6* mRNA and protein in cumulus cells is significantly reduced compared with wild-type mice (20). Additionally, the PR antagonist RU486 has been shown to suppress cumulus expansion when porcine COCs were cultured with FSH and LH (26). These reports provide evidence that LH induction of PGE2 and progesterone signaling pathways impact cumulus cell function during ovulation process.

Because cumulus cells of mouse and rat possess few if any LH receptors (27), the molecular and biochemical mechanisms by which LH impacts COC expansion have remained ill defined. However, earlier studies indicated that factors other than LH can induce cumulus expansion and oocyte maturation in culture, including prostaglandins (20) and epidermal growth factor (EGF) (28, 29). Furthermore, EGF-like activity was reported in porcine follicular fluid (30). A possible role for EGF or EGF-like factors in the ovulation process has been strengthened by recent observations showing that LH induces in mouse and rat preovulatory follicles the expression mRNAs encoding the EGF-like factors amphiregulin (AREG), epiregulin (EREG) (31–33), and betacellulin (BTC) (33) in temporal and spatial patterns similar to those of *Ptgs2* and *Pgr* (32, 33). Furthermore, an EGF-receptor tyrosine kinase inhibitor blocked LH-mediated COC expansion and oocyte maturation in explanted whole follicles in culture (33) and in PO follicles *in vivo* (34). Conti and colleagues (35, 36) have proposed an attractive and novel paracrine mechanism to account for the roles of LH and EGF-like factors in cumulus expansion and oocyte maturation. Specifically, they put forward the notion that LH induction of *Areg*, *Ereg*, and *Btc* in granulosa cells of PO follicles provides ligands that activate EGF receptors in cumulus cells leading to the induction of factors essential for COC expansion; namely, *Has2*, *Ptgs2*, and *Tnfaip6* mRNA. Attractive as this paracrine EGF activation hypothesis is, the molecular mechanisms controlling the induction and function of *Areg*, *Ereg*, and *Btc* directly in cumulus cells, granulosa cells, or COCs was not analyzed.

To characterize genes that are expressed specifically in cumulus cells before and after ovulation, we have recently performed microarray analyses on RNA prepared from nonexpanded COCs isolated from PO follicles of equine chorionic gonadotropin (eCG)-primed immature mice, expanded COCs isolated from PO follicles 8 h after human chorionic gonadotropin (hCG) treatment and ovulated COCs collected from the oviduct 16 h after hCG. Data from these gene profiling studies showed that mRNAs encoding the EGF-like factors *Areg*, *Ereg*, and *Btc* were induced markedly in intact COCs isolated 8 h after hCG *in vivo* (37). These results indicated that cumulus cells, as well as granulosa cells, produce these EGF-like ligands, and therefore expression of these factors in cumulus cells might provide an autocrine mechanism to regulate cumulus cell function. However, the molecular mechanism(s) by which COCs acquire the ability to express of these EGF-like factor genes is not easily explained. Although Park *et al.* (33) showed that EGF-like factors stimulated *Ptgs2* mRNA expression in whole follicle explants, little information was provided concerning the molecular mechanisms by which *Areg*, *Ereg*, or *Btc* mRNA were induced by LH/hCG. Other investigators analyzing different cell systems (38) have shown that AREG is expressed in the mouse uterus and in this tissue is induced specifically by a progesterone- and PGR-dependent pathway (38). In colon cancer cells, PGE2 activated *Areg* gene promoter activity by cAMP-dependent regulation of factors binding to a critical cAMP response element (CRE) and/or guanine: cytosine-rich Sp1 binding domains (39, 40). Therefore, we hypothesized that the EGF-like factors induced by LH in granulosa cells might induce a secondary signaling cascade(s) that would act in a manner similar to that of LH or FSH. One such rapid LH-induced signaling cascade involves *Ptgs2* and *Ptger2* and the actions of PGE2. Another gene rapidly induced by LH is *Prg*. Thus, we hypothesized that LH induction of EGF-like factor expression in granulosa cells and cumulus cells might involve PGE2- and/or progesterone-mediated pathways.

To test this hypothesis, we have analyzed the induction of *Areg*, *Ereg*, and *Btc* mRNA in COCs using both *in vivo* and *in vitro* model systems. Our results show that, in isolated COCs largely devoid of granulosa cells, *Areg* mRNA is induced by either FSH or forskolin or by AREG itself by mechanisms that are sensitive to inhibition of prostaglandin biosynthesis and EGF-receptor tyrosine phosphorylation. In addition, the induction and expression pattern of *Areg* in COCs and granulosa cells *in vivo* was altered in mice null for *Ptgs2* and *Pgr*, respectively. Thus, we propose that the EGF-like factors are synthesized by cumulus cells as well as granulosa cells and mediate autocrine as well as paracrine events in preovulatory follicles.

RESULTS

Induction *in Vivo* by hCG of *Areg*, *Ereg*, and *Btc* mRNA in COCs and Granulosa Cells of PO Follicles Is Rapid

To analyze the induction of the EGF-like ligands *Areg*, *Ereg*, and *Btc* specifically in COCs, COCs as well as

granulosa cells were isolated from ovaries of eCG-primed mice before (0 h) and at 4, 8, and 12 h after hCG as well as from oviducts at 16 and 24 h after hCG (Fig. 1). Semiquantitative RT-PCR analyses of total RNA document dramatic induction of each gene in COCs within 4 h after hCG, confirming microarray data that mRNA encoding these factors is present and induced in COCs of periovulatory follicles (37) and *in situ* hybridization data in rat periovulatory follicles 8 h after hCG (Ref. 31; our unpublished observations; and supplemental Fig. 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Expression levels of *Areg* and *Btc* declined progressively thereafter, whereas expression levels of *Ereg* mRNA persisted until 12 h but declined significantly in the ovulated complexes. This pattern of *Areg*, *Ereg*, and *Btc* mRNA induction in COCs was similar to that observed in granulosa cells (Fig. 1 and Refs. 31–33).

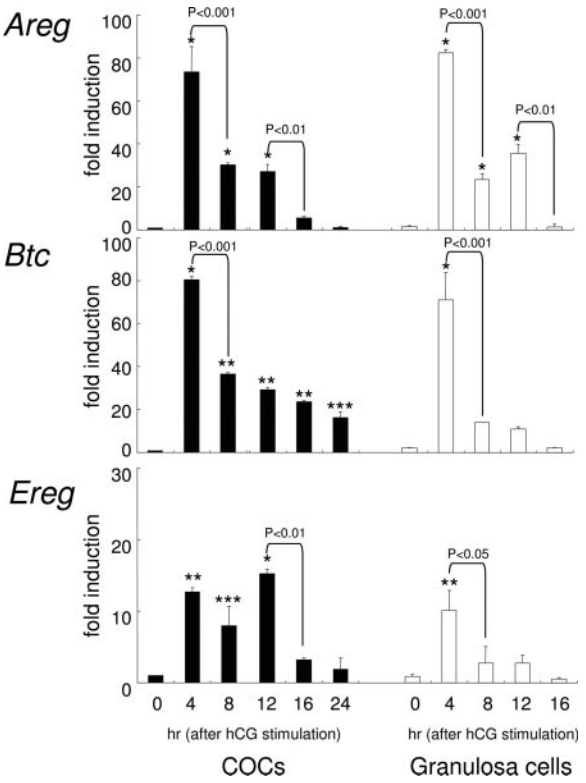

Fig. 1. Induction of *Areg*, *Btc*, and *Ereg* mRNA in Murine COCs and Granulosa Cells

Total RNA was isolated from COCs and granulosa cells harvested from ovaries of mice treated with eCG for 48 h to stimulate growth of preovulatory follicles (0 h) followed by hCG to stimulate ovulation. Ovulated COCs were collected from the oviducts of mice at 16 and 24 h after hCG. Two separate pools of RNA were analyzed by semiquantiative RT-PCR using specific primers as shown in Table 1. Mean ± SD were calculated and analyzed by one-way ANOVA and Neuman-Keuls Multiple Comparison (GraphPad Prism). Changes were significant if $P < 0.05$. Comparisons are as indicated in the figures. *, $P < 0.01$; **, $P < 0.05$; ***, $P < 0.07$.

To analyze AREG protein, whole ovarian samples were prepared and subjected to Western blot analyses using a specific rat antimouse monoclonal AREG antibody (Fig. 2). An immunoreactive band at approximately 60 kDa corresponds to the cellular, nonprocessed form of AREG, whereas the smaller product may be a soluble form (41). The approximately 60-kDa band was present in low amounts in eCG-primed mouse ovaries but increased dramatically at 4 and 8 h after hCG and declined thereafter, indicating that AREG protein was induced but more stable than *Areg* mRNA that had declined significantly in granulosa cells and COCs by 8 h.

Immunofluorescent studies using the same AREG-specific antibody document intense staining of AREG to the surface of cumulus cells in COCs isolated at 4 and 8 h after hCG compared with COCs isolated at 0 h (Fig. 2B, a–f).

Induction of Areg mRNA in COCs and Granulosa Cells Is Dependent in Part on Prostaglandin Production

Because EGF has been shown to induce cumulus expansion (28, 42) and genes associated with this process (17, 33), we sought to determine whether expression of EGF-like factor mRNA was impaired in *Ptgs2* null mice that exhibit impaired cumulus expansion and ovulation (11, 20). For this, expression of *Areg*, *Ereg*, and *Btc* mRNA was analyzed in COCs and granulosa cells isolated from ovaries of eCG, hCG primed $Ptgs2^{+/-}$ and $Ptgs2^{-/-}$ mice. As shown in Fig. 3A, *Areg, Ereg*, and *Btc* mRNA was induced markedly in COCs and granulosa cells of $Ptgs2^{+/-}$ mice at 4 h. In contrast, induction of mRNA encoding *Areg* and *Ereg* mRNA but not that encoding *Btc* was impaired significantly in COCs and granulosa cells of the $Ptgs2^{-/-}$ mice at this time interval. Levels of *Areg* and *Ereg* mRNA in the $Ptgs2^{+/-}$ mice declined significantly by 8 h reaching levels similar to those of the $Ptgs2^{-/-}$ mice. Thus, no significant differences in the expression of these genes was noted in COCs at 8 h (Fig. 3A).

Western blot analyses confirmed marked induction of AREG protein at 4 and 8 h in ovaries of $Ptgs2^{+/-}$ mice compared with reduced expression of AREG in ovaries of the $Ptgs2^{-/-}$ mice at these same time intervals (Fig. 3B). These data document that the rapid induction of *Areg* and *Ereg* mRNA and protein expression by hCG *in vivo* is dependent on expression of *Ptgs2*. Because previous studies had indicated that LH/hCG induction of *Areg* mRNA preceded that of *Ptgs2* (33), our results provided additional evidence that there might be a positive, autocrine feedback loop in which AREG acts to induce *Ptgs2* mRNA leading to PGE2 production that is critical for expression of the *Areg* mRNA.

To examine this possible autocrine feedback mechanism more directly, a COC culture system was used. For this, nonexpanded COCs were isolated from ova-

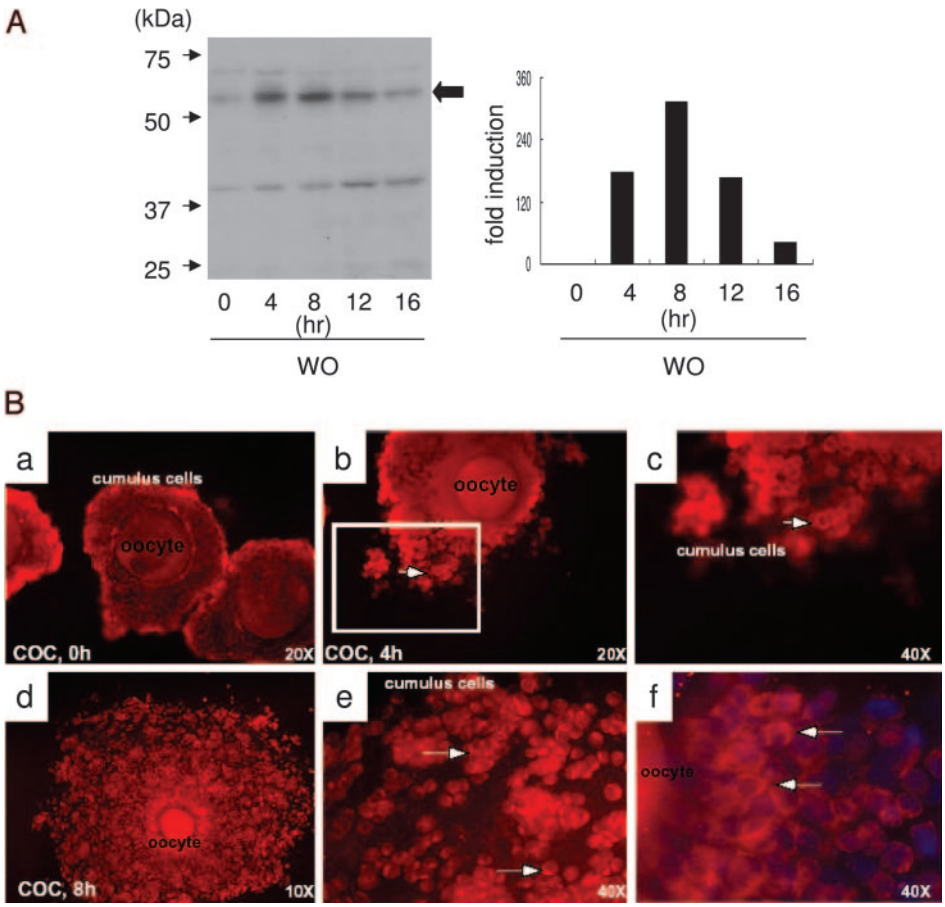

Fig. 2. Levels of AREG Protein Increase in Murine Ovaries and COCs

AREG protein was analyzed by Western blot (A) and immunofluorescence (B) using a specific anti-AREG antibody at 1:500 and 1:50, respectively. The approximately 60-kDa band noted in the Western blot represents nonprocessed cellular AREG and was quantified by densitometric analyses using a Molecular Dynamics Personal Densitometer (A). Note the increase in immunostaining in the ovary and COCs (*arrows*) at 4–8 h (B). WO, Whole ovary.

ries of eCG-primed immature mice and placed in defined medium containing 1% serum. When FSH (100 ng/ml) or AREG (250 ng/ml) was added to the COCs, expansion was observed 16 h later, confirming many previous studies (Refs. 20 and 33 and data not shown). Furthermore, FSH and AREG induced expression of *Areg* and *Ereg* mRNA to similar high levels within 4 h (Fig. 4A). Expression of *Areg* mRNA declined rapidly by 8 h, whereas the decline in *Ereg* mRNA levels was more gradual but significant ($P < 0.001$) by 16 h (Fig. 4A). To monitor the time-course of *Areg* induction in more detail, additional COCs were cultured in the presence of either forskolin (10 μM) or AREG (250 ng/ml) for 0, 0.5, 1, and 4 h (Fig. 4B). As shown, the induction of *Areg* mRNA occurred more rapidly in response to forskolin than to AREG with significant increases occurring within 0.5 h. In contrast, increases in *Ereg* and *Btc* mRNA were not observed until 4 h with either agonist. When induction of *Ptgs2* mRNA was analyzed in these same samples, a significant response to AREG but not to forskolin occurred at 0.5 and 1 h, whereas by 4 h the induction by AREG and forskolin was similar (Fig. 4B). These results show that forskolin as well as FSH is a potent stimulator of *Areg* expression in COCs and that AREG is a potent inducer of the *Ptgs2* gene, thereby indicating that induction of *Ptgs2* mRNA by forskolin/FSH is mediated at least in part, by induction of *Areg* mRNA and subsequent AREG activation of the EGF receptor signaling.

To analyze the signaling cascades by which FSH and AREG regulate gene expression in COCs, inhibitors of several kinases were used in the COC culture system. Induction of *Areg*, *Ereg*, and *Btc* mRNA by either FSH or AREG at 4 h was blocked by AG, an inhibitor of EGF receptor tyrosine kinase activity (Fig. 5A). Furthermore, induction of *Areg* and *Ereg* mRNA by FSH or AREG was blocked by the PTGS2 inhibitor NS398. Additional experiments showed that PGE2 alone induced *Areg*, *Ptgs2*, and *Has2* mRNA (Fig. 5B). Furthermore, PGE2 reversed the inhibitory effect of AG on AREG mediated cumulus expansion (data not shown) and induction of *Areg* and *Has2* mRNA but not that of *Ptgs2* (Fig. 5B). These results clearly place the induction of *Ptgs2* mRNA and PGE2 action down-

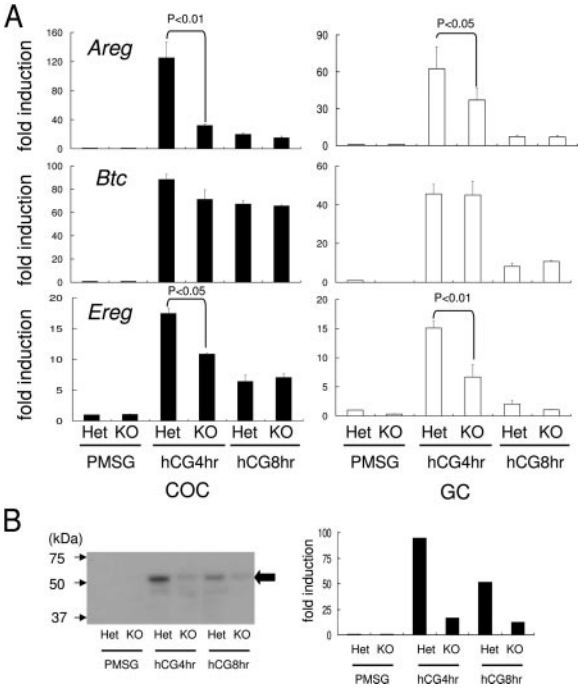

Fig. 3. Expression of *Areg* and *Ereg* mRNA Is Impaired in *Ptgs2* Null Mice

Total RNA from COCs and granulosa cells (A) and whole ovarian protein (B) were isolated and analyzed by semiquantitative RT-PCR and Western blot as indicated. Although EGF-like factor mRNA was induced at 4 h in *Ptgs2*$^{+/-}$ mice, levels of *Areg* and *Ereg* but not *Btc* were reduced significantly at 4 h in *Ptgs2*$^{-/-}$ mice. Levels of AREG protein were also reduced at 4 and 8 h in *Ptgs2*$^{-/-}$ mice compared with *Ptgs2*$^{+/-}$ littermates as shown by Western blot and densitometric analyses as in Fig. 2. Statistics were as described in the legend of Fig. 1. KO, Knockout; GC, granulosa cell.

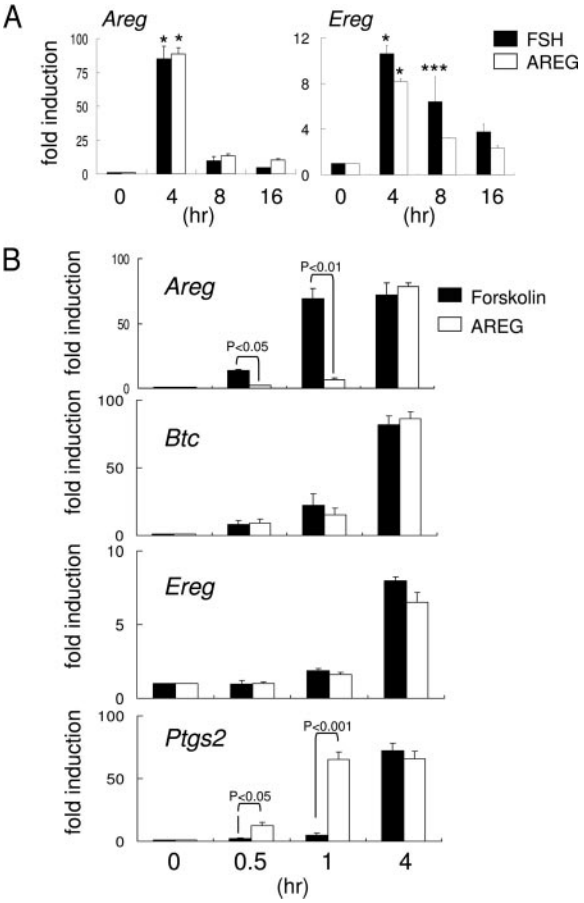

Fig. 4. Induction of *Areg* and *Ptgs2* mRNA by FSH/Forskolin or AREG Exhibit Distinct Temporal Patterns

A, COCs were cultured in the presence of FSH (100 ng/ml) or AREG (250 ng/ml) for 0–16 h in defined medium. Total RNA was extracted and analyzed by semiquantitative RT-PCR. FSH and AREG induced expression of *Areg* mRNA with maximal levels at 4 h. *Ereg* mRNA expression was elevated at 4 and 8 h with FSH and at 4 h with AREG. *, $P < 0.01$; ***$P < 0.05$. B, Additional COCs were cultured with either forskolin (10 μM) or AREG (250 ng/ml) for the indicated time intervals. Note that forskolin mediated more rapid induction of *Areg* mRNA (0.5 h) than does AREG, whereas AREG induced expression of *Ptgs2* mRNA more rapidly (0.5 h) than forskolin. Statistical analyses were as described in Fig. 1.

stream of AREG and EGFR activation but also show that PGs feedback to increase *Areg* mRNA expression.

To determine what cell signaling cascade(s) might mediate the induction of *Ptgs2* mRNA, COCs were cultured with FSH or AREG in the absence or presence of inhibitors of putative downstream targets of FSH and EGFR signaling, namely protein kinase A, PKA (KT5720), p38MAPK/MAPK14 (SB203580), or MEK1 (PD98059) (Fig. 6A). The most potent inhibitor of FSH or AREG induction of *Ptgs2* mRNA was PD98059, providing strong evidence that the MAPK/ERK1/2 pathway was activated by these agonists. In addition, the induction by FSH of mRNAs for *Ptgs2* and *Areg* was dependent on p38MAPK and MEK1 activation but not PKA. Western blot analyses confirmed that FSH and AREG stimulated phosphorylation of both p38MAPK and ERK1/2 in COCs at 4 h culture (Fig. 6B). When a more detailed time-course analysis was performed using cultured granulosa cells, forskolin rapidly phosphorylated p38MAPK within 5 min, whereas 60 min was required for increased phosphorylation of p38MAPK by AREG. However, AREG was more potent than forskolin in stimulating rapid ERK1/2 phosphorylation. Furthermore, whereas FSH or forskolin-mediated phosphorylation of p38MAPK in COCs (Fig. 6B) and granulosa cells (not shown) was blocked completely by SB203580, it was not blocked by the EGF receptor tyrosine kinase inhibitor AG. PGE2 also stimulated marked increases in phospho-p38MAPK in COCs and granulosa cells within 5–15 min (data not shown). In contrast, AREG-mediated phosphorylation of p38MAPK was sensitive to AG. AG strongly suppressed ERK1/2 phosphorylation by both FSH/forskolin and AREG, indicating that FSH (forskolin and PGE) mediated phosphorylation of p38MAPK is independent of AREG, whereas FSH-mediated phosphoryla-

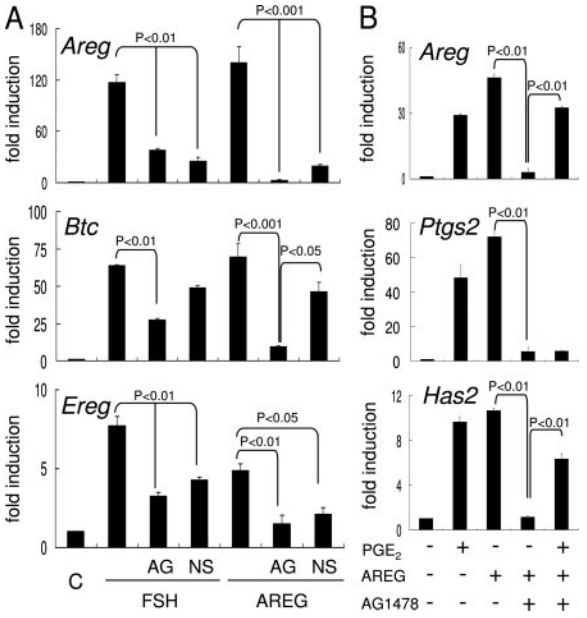

Fig. 5. Regulated Expression of *Areg* and *Ereg* mRNA by FSH and AREG in Cultured COCs Is Blocked by Inhibition of EGF Receptor Tyrosine Kinase Activity or PTGS2 Activity

A, COCs were cultured with FSH or AREG as in panel A with or without inhibitors of EGF-receptor tyrosine kinase (AG; 10 μM) and PTGS2 (NS; NS398, 10 μM). B, COCs were cultured with PGE2 (500 ng/ml), AREG, AREG with AG or AREG with AG and PGE2 for 4 h. Statistical analyses were as described in Fig. 1.

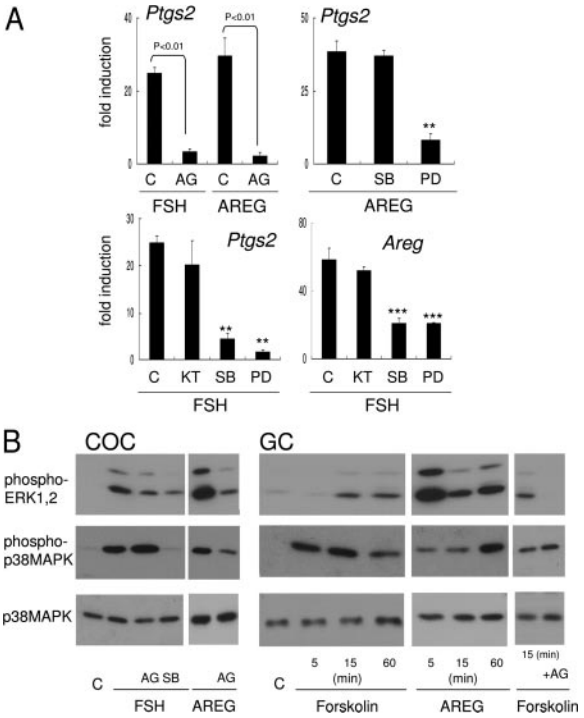

Fig. 6. Induction of *Ptgs2* mRNA and *Areg* mRNA Is Dependent on Activation of p38MAPK and ERK1/2

A, COCs were cultured with FSH or AREG as in Fig. 4 with or without inhibitors of PKA (KT; KT5720, 10 μM), p38MAPK (SB203580, 20 μM) or MEK1 (PD; PD98059, 20 μM). **, $P < 0.01$; ***, $P < 0.05$. B, COCs cultured for 4 h with FSH or AREG without or with AG or SB203580. Granulosa cells were cultured for 0–60 min without or with AG. COC and granulosa cell extracts were prepared and analyzed by Western blot using 30 mg of protein and antibodies specific to phospho- and non-phospho forms of p38MAPK and ERK1/2. C, Control; GC, granulosa cell.

tion of MAPK is likely the consequence of FSH induction and activation of AREG at these early time points.

Induction of EGF-Like Ligands Also Depends on the PR/PGR

Because $Pgr^{-/-}$ (PRKO) mice exhibit impaired ovulation, the expression patterns of *Areg*, *Ereg*, and *Btc* mRNA were analyzed in RNA samples prepared from granulosa cells of mice primed with eCG and hCG. Induction of each gene was similar in $Pgr^{+/-}$ and $Pgr^{-/-}$ granulosa cells at 4 h, whereas in the $Pgr^{-/-}$ cells expression of *Areg* and *Ereg* mRNA and protein (but not that of *Btc*) was reduced significantly at 8 h (Fig. 7, A and B). These results suggested that these EGF-like factors might be targets of PGR. To analyze this in more detail, granulosa cells were harvested from eCG-primed mice and placed in culture in serum-free defined medium. The cells were infected with adenovirus expressing Myc-tagged PGRA or control andenovirus (Ref. 43; provided by V. Sriraman) overnight and were then stimulated with forskolin or PMA for 4 h, a regimen known to induce other genes (43). Total RNA was prepared and analyzed by semiquantitative RT-PCR. As shown, forskolin stimulated *Areg*, *Ereg*, and *Btc* mRNA, whereas PMA was ineffective (Fig. 7C). Expression of PGRA alone did not induce EGF-like factor mRNA, nor did it enhance the effect of forskolin. Rather, PGRA-Myc enhanced significantly the effect of PMA on EGF factor (*Areg* and *Ereg*) induction. Thus, PGR can modulate but not induce expression of the *Areg* and *Ereg* genes.

AREG Can Induce Expression of Specific Genes in COCs

Recent microarray data indicated that many genes in addition to *Ptgs2, Has2*, and *Tnaip6* were induced in COCs *in vivo* at 8 h after hCG (37). Therefore, we sought to determine whether some of these genes might be targets of AREG and thereby define functions for AREG in addition to its ability to impact COC expansion and matrix formation. As expected, AREG induced *Ptgs2*, *Has2*, and *Tnfaip6* expression in cultured COCs at 4 h. In addition, genes involved in steroidogenesis *Star* and *Cyp11a1* were induced by AREG alone, a process reduced by the EGF receptor tyrosine kinase blocker AG (Fig. 8A). These results show that COCs acquire steroidogenic capabilities and can produce progesterone. These steroidogenic

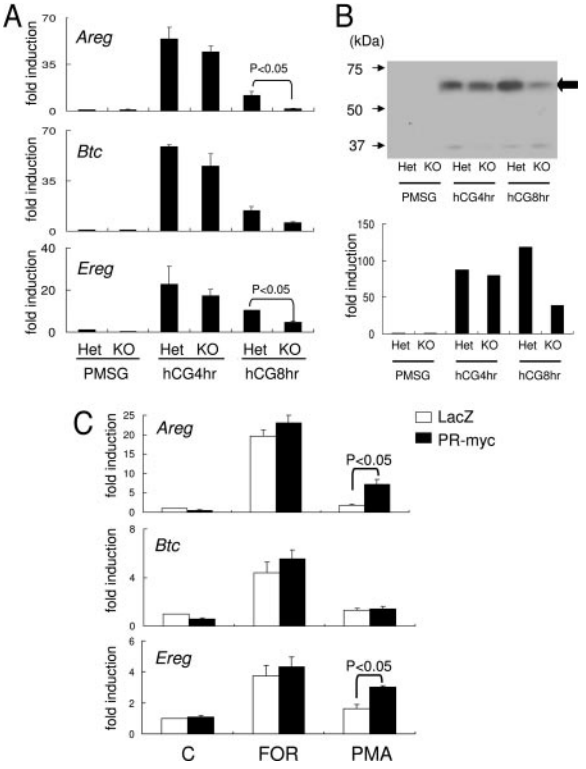

Fig. 7. Induction of *Areg* and *Ereg* mRNA *in Vivo* and *in Vitro* Is Modulated by PGR

Granulosa cells were harvested from ovaries of heterozygous (Het; $Pgr^{+/-}$) and *Pgr* Null (KO; $PGR^{-/-}$) mice and used to prepare total RNA (Panel A) or protein (Panel B). As previously, EGF-like factors were induced by hCG *in vivo* with maximal levels in Het and KO samples at 4 h. However, at 8 h, *Areg* and *Ereg* mRNA declined more precipitously ($P < 0.05$) in the KO than Het samples. Similarly, levels of PGR protein as determined by Western blot were less in granulosa cells of the KO than Het mice at 8 h (panel B). C, An adenoviral vector expressing PGR-A modulates the induction of *Areg* and *Ereg* mRNA by PMA but not forskolin. Granulosa cells were infected with adenovirus expressing PGR-A or LacZ and cultured overnight. The cells were stimulated with forskolin (FOR) (10 μM) or PMA (20 nM) for 4 h. Total RNA was isolated and analyzed by semiquantitative RT-PCR. Each treatment was done in triplicate and repeated twice. PMSG, Pregnant mare's serum gonadotropin; C, control.

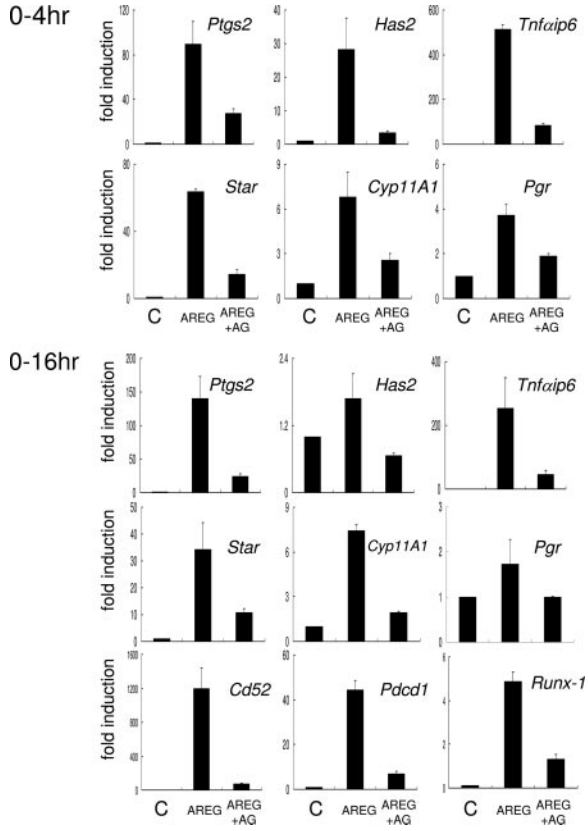

Fig. 8. AREG Induces the Expression of Genes Involved in Matrix Formation, Steroidogenesis, and Immune Cell Function in COCs

COCs were cultured with AREG without or with AG to block EGF receptor activity for 4 h (*upper panels*) or 16 h (*lower panels*). Total RNA was prepared and analyzed by semiquantitative RT-PCR using specific primers (Table 1). Significant increases ($P < 0.01$) were observed with each gene at 4 and 16 h, responses that were significantly reduced ($P < 0.05$) by AG.

genes continue to be expressed at 16 h by EGF receptor-dependent mechanisms (Fig. 8B). In addition, genes that have been shown to be selectively expressed in COCs compared with granulosa cells [programmed cell death 1 (*Pdcd1*), *Cd52* and *Runx1* (37)] were also induced by AREG, providing confirmation that genes induced in COCs *in vivo* are also induced in COCs in culture and that the EGF receptor pathway is required. Lastly, ovulated COCs were isolated from oviducts of mice 16 h after hCG and cultured for an additional 8 h without or with AREG to determine whether these factors remain critical for stability of expanded COCs after ovulation. As shown in Fig. 9, AREG had little or no effect on the expression of either *Cd52* or *Pdcd1* transcripts during this time but did exert a slight increase in expression of *Has2*, *Ptgs2*, and *Tnfaip6* mRNAs. However, when the morphology of COCs was examined, those cultured with AREG for 16–24 h appeared similar to COCs isolated from the oviducts at 24 h, whereas COCs cultured in the absence of AREG had begun to disintegrate. Thus, AREG likely regulates additional factors at these times.

DISCUSSION

The LH surge initiates a remarkable reprogramming of granulosa cell and cumulus cell gene expression during the ovulatory cascade that impact cumulus expansion and oocyte maturation (37). During this process, many signaling cascades are induced and activated (17, 33, 35, 36, 44). The first evidence that EGF-like factors might be involved in cumulus cell function was

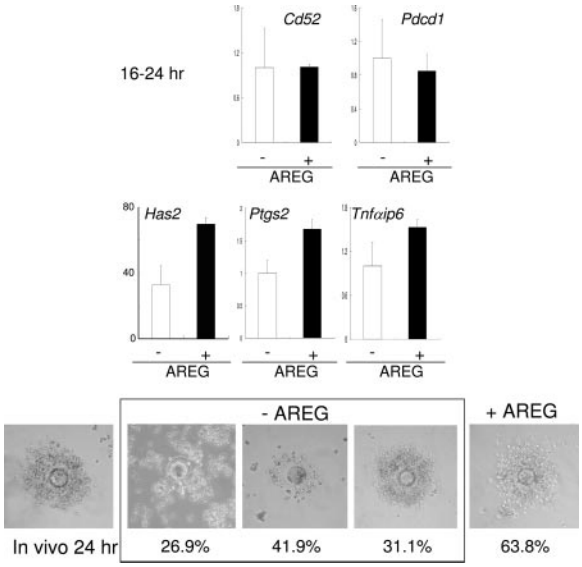

Fig. 9. COCs Collected from the Oviducts at 16 h Depend on AREG for Structural Integrity

COCs were isolated from the oviducts of mice 16 h after hCG and cultured an additional 8 h in the presence or absence of AREG. COCs were scored for morphological appearance and used for the isolation of total RNA. Semiquantitative RT-PCR and morphological images are shown.

reported by Downs (28) and Dekel and Sherizly (29). Specifically, when COCs are cultured in defined medium, cumulus expansion was induced by FSH and/or LH (42), as well as by EGF alone (28, 29). More recently Park *et al.* (33) showed that LH induction of cumulus expansion in explanted whole follicles was dramatically suppressed by the EGF receptor tyrosine kinase inhibitor AG. In addition, they and other groups have reported that LH induces expression of EGF-like factors, *Areg*, *Btc*, and *Ereg* in granulosa cells of preovulatory follicles during the ovulation process (31, 32). Conti *et al*. (36) have presented the hypothesis that LH acts in a paracrine manner to regulate cumulus expansion. In their model, the LH surge induces in granulosa cells expression of the EGF like factors that, when shed from the cell surface, act on EGF receptors present in cumulus cells to mediate cumulus expansion.

Herein, we confirm previous reports (31–33) that *Areg*, *Btc*, and *Ereg* mRNA levels increase rapidly in granulosa cells after hCG stimulation *in vivo*. However, in this study, we also provide the novel observation that mRNAs encoding these EGF-like factors are induced markedly in cumulus cells *in vivo* and *in vitro*. Because cumulus cells have low or undetectable levels of LH receptor (27), other stimulatory factors appear to be required to induce expression of EGF-like factors in cumulus cells. Because the promoters of the *Areg* and *Ereg* genes can be transactivated by cAMP-dependent mechanisms (39, 45) and because one target of LH and is *Ptgs2*, we hypothesized that prostaglandins, specifically PGE2 (46) might be produced locally and via the EP2 receptor PTGER2 (7) serve as the intermediary stimulus. In support of this notion, we document that *Areg* and *Ereg* but not *Btc* expression levels were markedly reduced in COCs and granulosa cells of *Ptgs2* null mice. Furthermore, the accumulation of AREG protein within ovary was also reduced in *Ptgs2* null mice. Additionally, both FSH and AREG induced *Areg* and *Ereg* expression within 4 h in cultured COCs, actions that were completely suppressed by the PTGS2 selective inhibitor, NS398, suggesting that PGs mediate the effects of these agonists on EGF-like factor expression. Moreover, PGE2 reversed the inhibition of AG on AREG-mediated induction of *Areg* and *Has2* mRNA but not that of *Ptgs2*, clearly placing induction of *Ptgs2* mRNA downstream of EGFR activation. Time course studies further showed that forskolin accelerated *Areg* mRNA expression, but not that of *Btc* or *Ereg*, whereas AREG accelerated *Ptgs2* expression more rapidly than did forskolin. These results suggested that FSH mediated induction of *Areg* mRNA precedes AREG induction of *Ptgs2* and PGE2 production that, via its receptors in cumulus cells, provides an secondary, autocrine pathway to regulate expression of *Areg* in cumulus cells (Fig. 10). Hence, there is a critical functional link between the G protein-coupled receptor pathway and growth factor receptor pathways.

Although binding of LH, FSH, and PGE to their cognate receptors is known to stimulate cAMP production and protein kinase A activation (47, 48), these agonists also activate other signaling pathways (49). Gonzalez-Robayna *et al.* (50) and others (51) have shown that, in granulosa cells, LH and FSH increase p38MAPK phosphorylation and activation by a PKA-independent mechanism that may involve exchange protein activated by cAMP (50, 52, 53). Phosphorylation of p38MAPK by FSH in cumulus cells has also been documented and linked to expansion of porcine and murine COCs (20, 54). ERK1/2, members of MAPK family, were phosphorylated by MEK1 in cumulus cells of mouse and porcine COCs during oocyte maturation (55, 56) and are essential for cumulus cell functions, including cumulus expansion (20, 55, 56). Herein, we show that when COCs were cultured with p38MAPK inhibitor or the MEK1 inhibitor, FSH induction of *Ptgs2* expression was not detected. On the other hand, induction of *Ptgs2* expression by AREG was suppressed by the MEK1 inhibitor but not the p38MAPK inhibitor. Moreover, the p38MAPK inhibitor suppressed FSH-induction of *Areg* mRNA, suggesting that FSH activation of p38MAPK plays a critical role in controlling transcriptional activation of the *Areg* gene in cumulus cells and that AREG in turns activates the MAPK pathway. In support of these observations, FSH or AREG stimulation of ERK 1/2 phosphorylation is suppressed by the EGF receptor tyrosine kinase inhibitor, AG. In contrast, FSH-induced phosphorylation of p38MAPK was not reduced by AG and AREG stimulated low levels of p38MAPK phosphorylation. Because the *Areg* gene contains a CRE critical for activation in

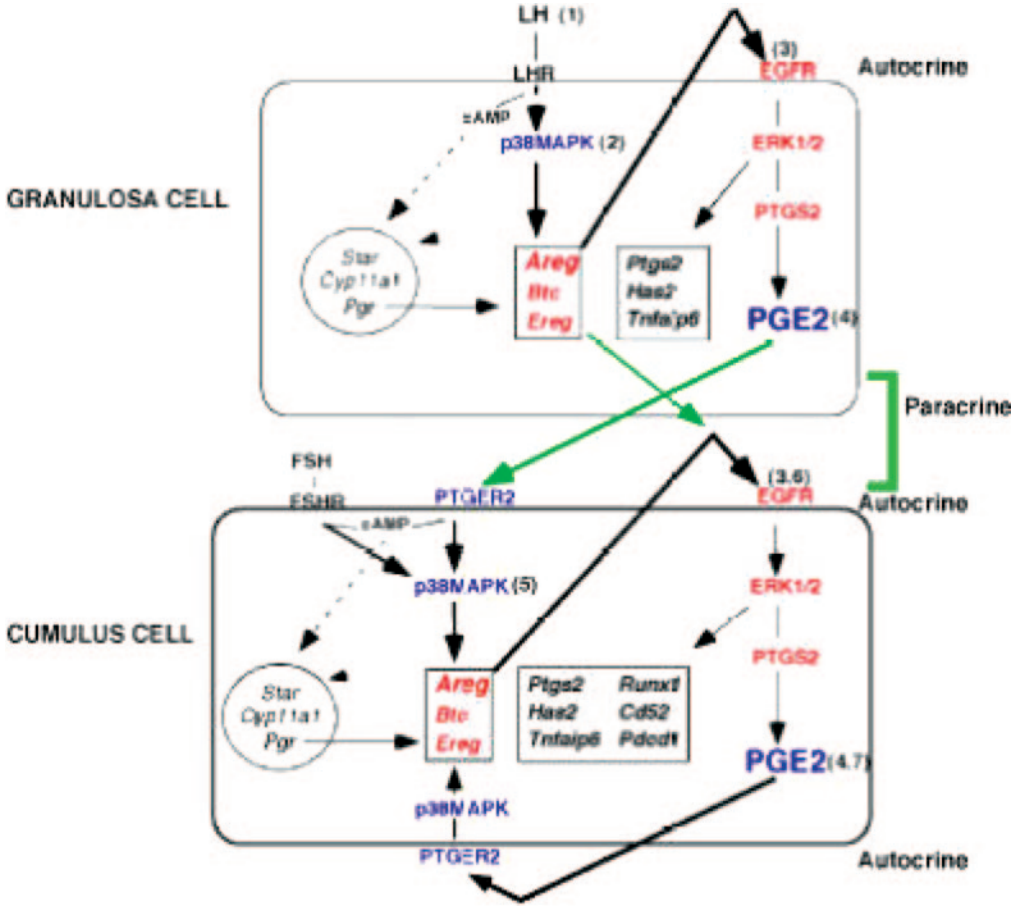

Fig. 10. Schematic Showing Potential Paracrine and Autocrine Pathways by Which Expression of EGF-Like Factors, *Ptgs2*, and Other Genes Expressed Are Regulated in Granulosa Cells and COCs of Ovulating Follicles

The proposed sequence is as follows: 1) LH binds to its cognate receptor localized to granulosa cells stimulating cAMP production as well as 2) p38MAPK phosphorylation and activation (52, 53). As a consequence, *Areg*, *Btc*, and *Ereg* mRNAs are induced rapidly providing ligands that 3) bind EGF-receptors on granulosa cells (autocrine) as well as cumulus cells (paracrine) leading to activation of ERK1/2 and induced expression of *Ptgs2* in both cell types. 4) Increased production of prostaglandins (PGs; PGE) then provides ligands that bind PTGER2 on cumulus cells (paracrine and autocrine) that (like the FSH and LH receptors) activate p38MAPK. By the PGE/PTGER2 pathway, *Areg* and *Ereg* mRNA can be induced in cumulus cells independently of granulosa cells leading to EGFR activation (6) and induction of *Ptgs2* and PG production (7) by autocrine mechanisms. Lastly, induction of genes involved in steroidogenesis and steroid (progesterone) hormone action as well as those linked to immune cell function are regulated by AREG-dependent pathways, providing additional functional links between of these signaling cascades as well as potential novel functions for cumulus cells during the ovulation process.

other tissues (39, 40), it is tempting to speculate that factors binding to this region of the *Areg* promoter in cumulus cells are targets of the p38MAPK pathway as well as possibly the PKA pathway that has not been ruled out. Likewise, factors binding to the *Ptgs2* promoter are likely targets of ERK1/2 (57) as well as PKA.

Not only enzymes required for PG production but also those required for steroid production are induced in COCs of ovulating and ovulated follicles *in vivo* (37). Furthermore, we show herein that AREG is a potent stimulator of *Cyp11a1* and *StAR* mRNA expression in COCs, indicating that these acquired steroidogenic capabilities are dependent, in part, on EGF receptor-mediated events. Because AREG induces *Ptgs2*, leading to PG production in COCs and granulosa cells, the effects of AREG may be mediated in part by the PTGER2 pathway. The importance of progesterone in the ovulating follicle is evident by the infertility of the *Pgr* null mice (14). Although the specific targets of *Pgr* that control ovulation are not yet entirely clear, we show herein that the *Areg* gene may be one target because expression of these EGF-like factors was repressed in granulosa cells from $Pgr^{-/-}$ mice compared with $Pg^{+/-}$ at 8 h after hCG. Thus, PGR appears to exert a modulatory role in maintaining expression of these genes just before ovulation. Although acute regulation of the *Areg* gene requires an intact CRE region in the promoter of this gene (39, 40), the *Areg* promoter also contains an Sp1 element responsible for the basal transcription of this gene (39). Furthermore, the promoter of the *Ereg* gene (that is also PGR responsive) has three Sp1 sites that are essential for transcription

(58). The importance of Sp1 sites in the regulation of genes by PGR in ovarian cells has been highlighted by recent studies showing PGR enhances promoter activity of the *Adamts1* (59) and *Snap-25* (our unpublished data) genes via Sp1/Sp3 binding sites. Other genes regulated by PGR in granulosa cells, such as cyclic-dependent protein kinase II (cGKII, *Prkg2*) (43) and cathpsinL [*Ctsl*; (60)] have at least one Sp1/Sp3 site. Furthermore, Sp1 coprecipitated with ligand-activated hPGRA or hPGRB in the decidual cell nuclear extracts (61). The modulatory role of PGR in regulating EGF-like factor expression is supported by our observations that *Areg* and *Ereg* expression was enhanced by infection of mouse granulosa cells with adenoviral expressing PGRA-myc but only in the presence of PMA. In a similar manner, when mouse granulosa cells were coinfected/transfected with adenoviral PGRA and a *Snap-25* promoter luciferase-reported construct, PMA enhanced promoter activity through Sp1/Sp3 binding sites (our unpublished data). Thus, we hypothesize that PGR regulates the basal level of EGF-like factor gene expression, at least in part, via Sp1/Sp3 binding sites in a PMA-dependent but cAMP-independent manner.

Although expression of EGF-like factor mRNA and protein is highest at 4 h and impacts induction of cumulus cell expansion at the early stages, mRNA and protein continue to be expressed at significant levels in ovary and COCs, respectively, at 8–16 h after hCG. Herein, we show for the first time that the EGF-like factors can maintain cumulus cell function in the postovulatory period. When nonexpanded COCs were isolated and cultured with AREG for 4 and 16 h, AREG stimulated not only genes involved in induction of cumulus expansion (*Ptgs2*, *Has2*, and *Tnfaip6*) but also genes regulating steroidogenesis and steroid hormone action (*StAR*, *Cyp11a1*, and *Pgr*) as well as immune-like functions. The immune-related genes, *Pdcd1, Runx1*, and *Cd52*, were selectively expressed in cumulus cells just before and after ovulation *in vivo* (37). Furthermore, FSH-mediated induction of these genes in cultured COCs was dependent on EGF-R tyrosine kinase activity, p38MAPK, and ERK1/2 activity, but not on PTGS2 (37), indicating that genes in addition to *Ptgs2* are targets of EGF receptor signaling in COCs. When ovulated COCs were isolated at 16 h and cultured for 8 h with AREG, enhanced integrity of the expanded complexes was observed. Although, at this time AREG did not impact expression of immune cell-related (*Pdcd1*, *Runx1*, and *Cd52*) genes, it did exert a small but nonsignificant increase in matrix-related (*Has2*, *Ptgs2*, and *Tnfaip6*) genes. These results suggest that the rapid induction of *Areg (Ereg* and/or *Btc*) expression is essential for initiating altered gene expression profiles in COCs leading to the expansion process. However, in ovulated COCs, AREG may regulate the expression of other genes.

In summary, we propose the following scenario by which EGF-like factors are induced in cumulus cells *in vivo* and how they regulate specific cumulus cell functions (Fig. 10). LH induces EGF like factor expression in granulosa cells (33), most probably via the p38MAPK pathway (52, 53). By autocrine and paracrine mechanisms, respectively, these EGF-like factors induce *Ptgs2* expression in these cells and COCs, leading to increased PGE2 production. PGE2 binds PTGER2 on cumulus cells (7) activating p38MAPK (20). Activation of this signaling pathway and later that regulated by PGR leads to transcriptional activation of the *Areg* and *Ereg* genes in cumulus cells. AREG binds to EGF receptor activating downstream pathways, critically ERK 1/2 in cumulus cells. Thus, the p38MAPK and ERK1/2 pathways act synergistically and sequentially and are essential for cumulus cell functions, such as cumulus expansion during ovulation process.

MATERIALS AND METHODS

Materials

Gestyl (eCG) was purchased from Professional Compounding Center of America (Houston, TX). Pregnyl (hCG) was purchased from Organon Special Chemicals (West Orange, NJ) and FSH (oFSH-16) was a gift from the National Hormone and Pituitary Program (Rockville, MD). Forskolin, PMA, KT5720, PD98059, SB203580, AG, and NS398 were purchased from Calbiochem (San Diego, CA). PGE2 was from Sigma (St. Louis, MO). MEM, DMEM:F12, and penicillin-streptomycin were from Invitrogen (Carlsbad, CA). Fetal bovine serum was obtained from Hyclone (Logan, UT). Oligonucleotide poly-(deoxythymidine) was purchased from Amersham Pharmacia Biotech (Newark, NJ), and avian myeloblastosis virus reverse transcriptase, *Taq* polymerase were from Promega (Madison, WI). Radiolabeled [^{32}P]deoxy (d)-CTP was purchased from ICN (Los Angeles, CA). Oligonucleotide primers for RT-PCRs were from Sigma-Genosys (Houston, TX). Routine chemicals and reagents were obtained from Fisher Scientific (Pittsburgh, PA) or Sigma. Antibodies for AREG was purchased from R&D systems (Minneapolis, MN); phospho-p38MAPK, p38MAPK, and phospho-ERK1/2 were purchased from Cell Signaling Technologies (Danvers, MA).

Animals

Immature female C57BL/6 mice were obtained from Harlan, Inc. (Indianapolis, IN). On d 23 of age, female mice were injected ip with 4 IU of eCG (Pregnyl; Organon) to stimulate follicular growth followed 48 h later with 5 IU hCG (Gestyl) to stimulate ovulation and luteinization (14, 59). PRKO and *Ptgs2* null mice were used in selected experiments because follicles develop normally in response to eCG but fail to ovulate in response to hCG (11, 59). PRKO mice were generated as in Ref. 13. *Ptgs2* null mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed under a 16-h light/8-h dark schedule in the Center for Comparative Medicine at Baylor College of Medicine and provided food and water *ad libitum*. Animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee at Baylor College of Medicine.

Cumulus Oocyte Complex Isolation and Culture

Ovaries of immature mice primed with eCG for 48 h contain multiple preovulatory follicles. COCs cells were isolated from

these follicles by needle puncture and collected by pipette. Nonexpanded COCs were selected and then were cultured in separate wells of a Falcon 24-well plate (Becton Dickinson, Franklin Lakes, NJ) in 1.0 ml of defined medium (20) containing 1% fetal bovine serum without or with FSH (100 ng/ml), Amphiregulin (250 ng/ml), PGE2 (500 ng/ml) and various protein kinase inhibitors: PKA (KT5720), MEK1 (PD98059), p38MAPK (SB203580) and EGFR activation via tyrosine phosphorylation (AG) as well as a selective inhibitor of PTGS2 (NS398). After 4 or 16 h, the COCs total RNA was extracted (see below). Each pool represents RNA prepared from COCs of 12 mice. Each experiment generating a separate pool was repeated three times. In other experiments, ovulated COCs were collected from the oviduct 16 h after hCG injection of eCG primed mice. After ovulated COCs were cultured with or without 250 ng/ml of amphiregulin for 8 h, cumulus expansion was observed by microscopy, and total RNA purified from these COCs.

Granulosa Cell Culture

Granulosa cells were harvested by needle puncture from immature mice treated with eCG on d 23–25 of age as described previously (43, 59). Briefly, cells were cultured in 12-well culture plates in 1% serum-containing medium (DMEM:F12 containing penicillin and streptomycin). On the next day, cells were washed, then cultured for 4 h in fresh, serum-free medium containing forskolin (10 μM), which mimics FSH stimulation of cAMP production; PMA (20 nM), which activates diacylglycerol-mediated signaling; or both; and harvested for protein and RNA analysis. Forskolin and PMA have previously been used to mimic the effects of the LH surge for optimal induction of *Ptgs2* and *Pgr* mRNA in cultured rat granulosa cells (62, 63). Selected granulosa cell cultures were treated with amphiregulin (250 ng/ml). In other experiments, granulosa cells were infected with PRA-Myc and Lac-Z expressing adenoviral vectors for 14 h (overnight) at a multiplicity of infection of 2:1 before the addition of agonists (43).

RT-PCR Analyses

Total RNA was obtained from COCs or granulosa cells using the RNAeasy mini kit (QIAGEN Sciences, Germantown, MD) according to the manufacturer's instructions and semiquantitative RT-PCR analyses were performed as described previously (64). Briefly, total RNA was reverse transcribed using 500 ng poly-deoxythymidine (Amersham Pharmacia Biotech) and 0.25 U avian myeloblastosis virus-reverse transcriptase (Promega Corp., Madison, WI) at 42 C for 75 min and 95 C for 5 min. For the amplification of the cDNA products, specific primers pairs were selected and analyzed as indicated in Table 1. All PCRs contained [^{32}P]dCTP (ICN, Los Angeles, CA), *Taq* Polymerase and Thermocycle buffer (Promega Corp.) as in previous studies (64, 65). cDNA products were resolved on 5% polyacrylamide gels which were dried and exposed to film. The radioactive PCR product bands were quantified using a Storm 860 PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA). The authenticity of the PCR products was verified by sequencing.

Western Blot Analyses

Protein samples from whole ovaries or cultured granulosa cells were prepared either by homogenization in whole cell extract buffer (66) or boiling sodium dodecyl sulfate sample buffer (67) as indicated in the text and as described previously (67). Extracts (30 μg protein) were resolved by SDS-PAGE (8 or 10%) and transferred to Immobilon-P nylon membranes (Millipore Corp, Bedford, MA). Membranes were blocked in Tris-buffered saline and Tween 20 [TBST; 10 mM Tris (pH 7.5), 150 mM NaCl and 0.05% Tween 20] containing 5% nonfat Carnation instant milk (Nestle Co., Solon, OH). Blots were incubated primary antibody overnight at 4 C. After washing in TBST, enhanced chemiluminescence (ECL) detection was performed by using Pierce (Rockford, IL) Super Signal according the manufacturer's specifications and appropriate exposure of the blots to Kodak x-ray film. Specific

Table 1. List of Oligonucleotides Used for RT-PCRs

Gene	Forward Primer (F) and Reverse Primer (R), all 5′–3′	Size (bp)	PCR Cycle
Areg	F:TTCATGGCGAATGCAATGCAGATACA	402	27
	R:ATCCGAAAGCTCCACTTCCT		
Btc	F:CGGGTAGCAGTGTCAGCTC	432	27
	R:CGATGTTTCCGAAGAGGATG		
Cyp11a1	F:TCACATCCCAGGCAGCTGCATGGT	270	25
	R:GCAGCAGGCAACCTGGTG		
Ereg	F:AACTCAGGAACAATTTACGTCTCTG	338	26
	R:GCTTTGGTTCTCAGTATAGAGAGAGA		
Has-2	F:GCTTGACCCTGCCTCATCTGTGG	405	27
	R:CTGGTTCAGCCATCTCAGATATT		
L19	F:CTGAAGGTCAAAGGGAATGTG	196	23
	R:GGACACAGTCTTGATGATCTC		
Pdcd1	F:GTAACAGAGAGAATCCTGGAGACCT	335	27
	R:AGTGAAGACAATGGTGGCATATT		
Pgr	F:CCCACAGGAGTTTGTCAAGCT	328	27
	R:TAACTTCAGACATCATTCCGG		
Ptgs2	F:TGTACAAGCAGTGGCAAAGG	433	27
	R:GCTGTGGATCTTGCACATTG		
Runx1	F:CCAGCAAGCTGAGGAGCGGCG	294	28
	R:CCGACAAACCTGAGGTCGTTG		
Star	F:GCAGCAGGCAACCTGGTG	249	26
	R:TGATTGTCTTCGGCAGCC		
Tnfαip6	F:TTCCATGTCTGTGCTGCTGGATGG	330	27
	R:AGCCTGGATCATGTTCAAGGTCAAA		

bands were quantified by densitometric analyses using a Molecular Dynamics Personal Densitometer.

Immunofluorescence

For immunolocalization of proteins in COCs, COCs were isolated from ovaries of eCG-primed mice at 8 and 16 h after hCG. COCs were immobilized on polylysine-coated coverslips, fixed with 4% paraformadehyde, washed with PBS, and used directly or stored in 1% paraformaldehyde until use. COCs were incubated O/N at 4 C with primary antibodies. Antibody localization was visualized with fluorescein isothiocyanate-labeled antirabbit IgG and Streptavidine AlexaFluor 568 (Molecular Probes, Invitrogen, Eugene, OR). Nuclei were visualized by 4′,6-diamidino-2-phenylindole present in the Vectashield D mounting medium (Vector Laboratories, Burlingame, CA). Digital images were captured using an Axiphot microscope with ×5–40 objectives.

Statistics

The semiquantitative RT-PCR data are represented mean ± SD. Data were analyzed by using GraphPad (San Diego, CA) Prism programs (ANOVA or Student's *t* test and Neuman-Keuls multiple comparison tests) to determine significance. Values were considered significantly different if $P < 0.05$ or $P < 0.01$.

Acknowledgments

Received December 12, 2005. Accepted March 6, 2006.

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This work was supported, in part, by National Institutes of Health Grants HD-16229 and HD-07495 (Project III, Specialized Cooperative Program in Reproductive Research (SCPRR) (to J.S.R.), an Overseas Advanced Educational Research Practice Support Program of Japanese Ministry of Education, Culture, Sports Science and Technology (16-311) (to M.S.), and the Spanish Ministry of Education, Culture and Sport and Autonomous Community of Canary Islands (Consejeria de education, Cultura y Deportes) (to I.H.-G. and I.G.-R.).

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