Cyclooxygenase-2-derived Prostaglandin E₂ Directs Oocyte Maturation by Differentially Influencing Multiple Signaling Pathways

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The process of oocyte maturation, which impacts ovulation and fertilization, is complex and requires an integration of the endocrine, paracrine, juxtacrine, and autocrine signaling pathways. This process involves an intimate interaction between the oocyte and encircling cumulus cells within a follicle, a unique venue for somatic and germ cell communication. Cumulus cell expansion and resumption of meiosis with germinal vesicle breakdown are major events in oocyte maturation. Cyclooxygenase-2 (COX-2)-derived prostaglandin E₂ (PGE₂) is a known critical mediator of oocyte maturation, but the diverse function of this lipid mediator in oocyte maturation, ovulation, and fertilization has not been fully appreciated. We show here that gonadotropins in coordination with PGE₂ signaling via its cell surface G-protein-coupled EP2 and EP4 receptor subtypes direct cumulus cell expansion and survival and oocyte meiotic maturation by differentially impacting cAMP-dependent protein kinase, MAPK, NF-κB, and phosphatidylinositol-3 kinase/Akt pathways. This study is unique in the sense that it provides evidence for new site- and event-specific involvement of these signaling pathways under the influence of COX-2-derived PGE₂ during the critical stages of this somatic-germ cell interaction, an absolute requirement for oocyte maturation.

Prostaglandins (PGs)4 are involved in various female reproductive functions, including ovulation, fertilization, and implantation (1, 2). The cyclooxygenase (COX) isoforms COX-1 and COX-2 are the primary producers of PGs. Although COX-1 is considered to be constitutive, COX-2 is induced by inflammatory stimuli, including cytokines and growth factors (3). Gene targeting experiments in mice have revealed distinct functions of these isoforms. COX-1-deficient females are mostly fertile but show parturition defects. In contrast, COX-2−/− females are mostly infertile because of severely impaired ovulation, fertilization, and implantation (4). Although ovulation failure in COX-2−/− mice is rescued by PGE₂ supplementation (5), implantation defects are improved by prostacyclin (PGI₂) working via peroxisome proliferator-activated receptor-δ (6). Effects of PGE₂ are normally mediated through G protein-coupled receptors EP1, EP2, EP3, and EP4 (7). Gene targeting studies showed that although EP2-deficient females have ovulation and fertilization defects similar to those in COX-2−/− females (8–10), EP1- and EP3-deficient mice are apparently fertile (11). In contrast, most EP4−/− pups die shortly after birth because of patent ductus arteriosus, precluding studies on reproductive phenotypes (12, 13).

Oocytes are arrested at the germinal vesicle stage before the surge of pituitary luteinizing hormone. This surge induces meiotic resumption of oocytes acting via its receptors on theca or granulosa cells, because luteinizing hormone receptors are scant in cumulus cells (14). Although luteinizing hormone signaling in theca or granulosa cells is not clearly understood, recent findings show that epidermal growth factor-like growth factors, amphiregulin, epiregulin, and betacellulin, are induced by hCG, and these growth factors promote meiotic resumption and cumulus expansion by depositing cell matrix through expression of hyaluronan synthase-2, COX-2, and tumor necrosis factor-induced protein-6 (TSG-6) (15). Moreover, oocyte meiotic maturation is associated with cumulus expansion induced by gonadotropins (16). Cumulus expansion is important for ovulation, fertilization, and subsequent embryonic development (4, 5, 8).

PGE₂ induces oocyte meiotic maturation in cultured mouse oocytes (17), and indomethacin, a nonspecific COX inhibitor, attenuates gonadotropin-induced cumulus expansion and germinal vesicle breakdown (GVBD) of oocytes (18–20). However, indomethacin, which blocked the release of PGs, failed to inhibit GVBD induced by gonadotropin-releasing hormone and angiotensin II (21, 22), suggesting complex roles of PGs in oocyte maturation.

We have shown that ovulated oocytes from Cox-2−/− mice on the C57BL/6J/129 background had very few first polar bod-
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Oocytes have normal development compared with wild-type oocytes when matured spontaneously in vitro (10). Moreover, normal GVBD is observed in Cox-2$^{-/-}$ mice after superovulation (5). In contrast, oocytes with signs of fragmentation are frequently observed in Cox-2$^{-/-}$ mice on CD1 background on day 2 of pregnancy, suggesting defective oocyte maturation, resulting in fertilization failure. In the absence of definitive roles of PGs in oocyte maturation, we initiated an in-depth investigation on the roles of PGs in oocyte maturation in vivo and in vitro using Cox-2$^{-/-}$ mice on the CD1 background and the signaling pathways involved in this process.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Minimum essential medium and fetal bovine serum were purchased from Invitrogen. Human follicle-stimulating hormone (hFSH) was a gift from National Hormone and Peptide Program (NIDDK, National Institutes of Health, Bethesda, MD). PGE$_2$, butaprost, sulprostone, 11-deoxy-prostaglandin E$_1$ (11-deoxy-PGE$_1$), prostaglandin A$_1$ (PGA$_1$), and AH6809 were purchased from Cayman Chemicals (Ann Arbor, MI). H-89, U0126, SB203580, and SP60012 were purchased from Calbiochem. LY294002 and antibodies for ERK1/2, phospho-ERK1/2, Akt, and phospho-Akt were purchased from Cell Signaling Technologies (Danvers, MA). Antibodies for COX-2, EP1, EP2, EP3, and EP4 were purchased from Cayman Chemicals. Antibodies for actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for cleaved caspase-3 was purchased from Promega (Madison, WI). Antibody for Bcl-2 was purchased from GeneTex (San Antonio, TX). Fluorescein isothiocyanate-, rhodamine (TRITC)-, or peroxidase-conjugated secondary antibody was purchased from Jackson Immunoresearch (West Grove, PA). Other reagents were obtained from Sigma unless otherwise indicated.

**Animals and Treatments**—The disruption of the Cox-2 gene in mice was described previously (23). PCR of genomic DNA determined the genotypes (4). Adult female mice on the CD1 background (2–6 months old) were used for all experiments. Generation of CD1 Cox-2$^{-/-}$ mice was described previously (24). All mice used were housed in the Institutional Animal Care Facility according to National Institutes of Health and institutional guidelines for laboratory animals. To induce superovulation, mice received intraperitoneal injections of 5 IU of pregnant mare serum gonadotropin (PMSG) followed by injections of 5 IU of hCG 48 h later as described previously (10, 24). To examine whether PGE$_2$ can rescue normal oocyte maturation in CD1 Cox-2$^{-/-}$ mice, wild-type or Cox-2 mutant females received subcutaneous injections of vehicle (3% ethanol in saline) or PGE$_2$ (40 µg/mouse) concurrent with hCG administration and followed by a second injection 4 h later (5). Ovulated cumulus-oocyte complexes were recovered from the oviduct 14–15 h after hCG injection. Cumulus cells were removed by a brief hyaluronidase treatment, and denuded oocytes were fixed in 4% buffered formalin, mounted on a glass slide with glycerol/phosphate-buffered saline (1:1) solution containing 2 µg/ml Hoechst 33258, and examined under a fluorescence microscope. Oocyte meiotic status was classified as follows: germinal vesicle, MI, or MII. To assess cumulus expansion, images of COCs were captured under an inverted microscope, and the projected cumulus areas of COCs were calculated using the Image J Imaging System software version 1.3 (National Institutes of Health, Bethesda, MD). In some experiments, the degree of cumulus expansion was determined as 0 (no expansion) to 4 (complete expansion) (25).

**In Vitro Fertilization**—To assess whether PGE$_2$ can restore normal fertilizing capacity of Cox-2 null oocytes, we performed in vitro fertilization experiments in both wild-type and Cox-2 mutant mice receiving vehicle or PGE$_2$ injections (5, 10). PGE$_2$ was given subcutaneously at a dose of 40 µg/mouse concurrently with hCG injection followed by a second injection 4 h later. Ovulated COCs were collected from the oviduct after 14–16 h of hCG injection, placed into droplets of 200 µl of HTF medium, and cultured in 5% CO$_2$ in air at 37 °C until sperm insemination. Sperm were collected from the cauda epididymis of mature wild-type mice and were preincubated for 2 h in 400 µl of HTF medium to allow capacitation. After capacitation, sperm were introduced into 200 µl droplets containing COCs. Six hours after insemination, COCs were transferred into droplets of 100 µl of HTF medium, and cumulus cells were removed by repeated pipetting. Fertilization was assessed by the appearance of two pronuclei in oocytes that were further cultured in KSOM medium for 5 days under 5% CO$_2$ in air at 37 °C. The number of blastocysts developed was recorded at the end of culture.

**Prostaglandin Assays**—Twenty COCs were cultured in 500-µl droplets of the HX media in the presence or absence of hFSH, and culture media were collected at 3, 6, and 18 h post-treatment for PG analysis by gas chromatography/negative ion chemical ionization mass spectrometric assay (6).

**Immunofluorescence**—Immunofluorescence was performed as described (26). Antibodies specific to COX-2 (1:300), TSG-6 (1:100), EP1 (1:100), EP2 (1:100), EP3 (1:100), EP4 (1:100), Akt (1:80), phospho-Akt (1:100), or cleaved caspase-3 (1:100) were used for in vitro oocyte maturation. COCs were collected 46–47 h after PMSG injection. COCs with uniform compacted cumulus cells were used for in vitro culture. COCs were transferred into the medium containing 4 mM hypoxanthine (HX) to prevent spontaneous GVBD. The culture medium was bicarbonate-buffered minimum essential medium supplemented with 75 µg/liter penicillin G, 50 µg/liter streptomycin sulfate, and 3 µg/ml crystallized lyophilized bovine serum albumin (baseline medium). To examine FSH-induced oocyte meiotic maturation, groups of 20–30 COCs were cultured in 100-µl droplets of basal medium containing 4 mM HX and 100 IU/liter hFSH covered with mineral oil in a humidified atmosphere of 5% CO$_2$ in air at 37 °C. For cumulus expansion experiments, 1% fetal bovine serum instead of bovine serum albumin was added to the baseline media. For assessment of oocyte meiotic status, cumulus cells were removed by hyaluronidase digestion, and denuded oocytes were fixed in 4% buffered formalin, mounted on a glass slide with glycerol/phosphate-buffered saline (1:1) solution containing 2 µg/ml Hoechst 33258, and examined under a fluorescence microscope. Oocyte meiotic status was classified as follows: germinal vesicle, MI, or MII. To assess cumulus expansion, images of COCs were captured under an inverted microscope, and the projected cumulus areas of COCs were calculated using the Image J Imaging System software version 1.3 (National Institutes of Health, Bethesda, MD). In some experiments, the degree of cumulus expansion was determined as 0 (no expansion) to 4 (complete expansion) (25).
used. The images were captured using the Image J Imaging System software (version 1.3) under an inverted fluorescence microscope.

Immunoblotting—Thirty COCs or cumulus cells from 30 COCs were lysed, and lysates were analyzed by immunoblotting for TSG-6, total Akt, phospho-Akt (1:1000), Bcl-2, or actin as described (26). Protein bands were visualized using chemiluminescent detection reagents (Pierce) and quantitated using a densitometer.

RNA Isolation and RT-PCR—Total RNA was extracted from cumulus cells using TRIzol reagents (Invitrogen) according to the manufacturer’s instructions. Reverse transcription with oligo(dT) primers generated cDNAs from 5 μg of total RNA using Superscript II. DNA amplification was carried out with a Taq polymerase (Invitrogen) using the following primers: Ptgerep1 (148 bp), 5'-GGGCGTGAACCTTAAGTC-3' and 5'-TGACTCCAGGGTAAGA-3'; Ptgerep2 (401 bp), 5'-AGGACTTGATGGCAGAGGAGAC-3' and 5'-CCTGGATTGCTACCATTTAGAGAC3'; Ptgerep3 (262 bp), 5'-TACCTTCCCATCACTGAGGAC-3' and 5'-CAAAGTCTTGAGGCTGGAG-3'; Ptgerep4 (389 bp), 5'-CCCTTCTCCTTCTCGG-3' and 5'-ACAACCTCATCACAACAGG-3'.

RESULTS


differential signaling by PGE2 in oocyte maturation

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Gonadotropin-induced Oocyte Maturation and Cumulus Expansion Are Compromised in Cox-2-/- Mice—Previous observations of ovulation and fertilization defects in Cox-2-/- mice on the C57BL/6J/129 background suggested that COX-2-derived ovarian PGs are critical to these events (4, 10). Furthermore, significantly improved ovulation with frequently observed fragmented eggs in CD1 Cox-2-/- females indicated that the processes of oocyte maturation and follicular rupture during ovulation have differential requirements for COX-derived PGs (24). In fact, we observed in CD1 Cox-2-/- females that an early induction of COX-1 by gonadotropin in mural granulosa and theca cells partially offsets the loss of COX-2 for completion of follicular rupture, whereas cumulus cells surrounding-

Apoptosis Detection—DNA fragmentation was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique using DeadEndTM colorimetric or Fluorometric TUNEL Systems (Promega, Madison, WI; catalog numbers G7130 and G3250) according to the manufacturer’s instructions.

Statistical Analysis—Each oocyte maturation experiment was conducted at least three times independently with 20–30 oocytes per treatment group. Differences between means were assessed by Student’s t test or were calculated by one-way analysis of variance followed by Fisher LSD post hoc test using StatView software (Abacus Concepts, Berkeley, CA). Each experiment was repeated at least three times unless stated otherwise. Values are shown as mean ± S.E. Data expressed as percentages were analyzed by using χ² tests. Significant differences are defined as p < 0.05.
Because oocyte meiosis resumption and progression, including GVBD and MI to MII transition, are temporally regulated dynamic events, we next examined the kinetics of in vivo oocyte maturation induced by hCG. As illustrated in Fig. 1A, although all wild-type oocytes underwent GVBD (the first visible sign of meiosis resumption) at 6 h post-hCG stimulation, Cox-2\(^{-/-}\) oocytes showed delayed resumption of meiosis, most of which exhibited GVBD at 15 h post-hCG administration. Under normal conditions, in parallel to meiotic oocyte maturation, the surrounding cumulus matrices underwent physical and physiological changes termed cumulus expansion preparing for “soon-to-occur” sperm-oocyte fertilization events. Thus, we compared the cumulus expansion status between wild-type and Cox-2\(^{-/-}\) mice in vivo. Again, we observed deferred and compromised cumulus expansion in Cox-2\(^{-/-}\) mice (Fig. 1B). For example, wild-type COCs showed visible signs of expansion at 3 h after hCG injection and reached full expansion at 12 h. In contrast, the expansion of Cox-2\(^{-/-}\) COCs was first noted at 9 h post-hCG administration with a largely reduced expanded area compared with that of wild-type COCs. Collectively, the results suggest that oocyte maturation is lessened in response to ovulation stimulus in Cox-2\(^{-/-}\) mice. This finding led us to examine whether gonadotropin-induced oocyte maturation is impaired in vitro in the absence of COX-2.

We have shown previously that in vitro maturation of C57BL/6J/129 Cox-2\(^{-/-}\) oocytes in the presence of fetal bovine serum and FSH is comparable with wild-type oocytes (10). Under the same culture condition, we further observed that there were no differences between the wild-type and CD1 Cox-2\(^{-/-}\) oocytes in their ability to undergo GVBD and progression to the MII (\(~80\%) stage (data not shown). This observation indicates that serum-derived factors compensate for the loss of COX-2 or, alternatively, COX-2-derived PGs are not always necessary for oocyte maturation in vitro. To address this issue, we next used a serum-free medium containing 4 mM HX, a non-specific phosphodiesterase inhibitor that prevents spontaneous meiosis. Interestingly, a compromised oocyte meiotic maturation and cumulus expansion evaluated by GVBD ratio and projected area of cumulus expansion, respectively, were observed when Cox-2\(^{-/-}\) COCs were cultured under serum-free conditions and challenged with FSH (Fig. 1, C and D, and supplemental Fig. 1C). In addition, the
percentage of \( \text{Cox-2}^{-/-} \) oocytes progressing to the MII stage in response to FSH stimulation was significantly lower in comparison to wild-type mice (supplemental Fig. 1D). These results faithfully phenocopied the \textit{in vivo} observation of defective oocyte maturation in \( \text{Cox-2}^{-/-} \) females and provoked us to further explore the underlying molecular mechanisms and signaling pathways associated with gonadotrophins and COX-2-derived PGs during oocyte maturation using this simply defined culture system.

\textit{PGE_2} Differentially Regulates Oocyte Maturation and Cumulus Expansion \textit{in Vitro—}Previous studies have demonstrated a spatiotemporal expression of COX-2 in mural granulosa and cumulus cells in response to preovulatory gonadotropin stimulation in mice (24, 27, 28). To further elucidate the physiological significance of COX-2-derived PGs in oocyte maturation \textit{in vitro}, we first assessed COX-2 expression by immunofluorescence in wild-type COCs in culture. As shown in Fig. 2A, COX-2 proteins are progressively induced in cumulus cells by FSH stimulation; first detectable expression was seen as early as 4 h with sustained high levels until termination of the culture at 18 h. This temporal induction of COX-2 in COCs is well correlated with PG accumulation in the culture medium. We noted that \( \text{PGE}_2 \) is the major PG secreted upon induction of COX-2 by FSH in wild-type COCs with low to undetectable levels in \( \text{Cox-2}^{-/-} \) COCs similarly cultured (Fig. 2B). These results further support the previous view that COX-2-derived \( \text{PGE}_2 \), but not COX-1, is a key player in regulating oocyte maturation and cumulus expansion (8, 24, 27).

Because \( \text{PGE}_2 \) is the primary PG produced by cumulus cells in response to gonadotropin both \textit{in vivo} and \textit{in vitro}, we subsequently tested whether \( \text{PGE}_2 \) could rescue defective oocyte maturation and cumulus expansion in \( \text{Cox-2}^{-/-} \) mice. With respect to meiotic maturation, although FSH was fully functional in stimulating wild-type oocyte GVBD and progression into the MII stage, \( \text{PGE}_2 \) alone was also able to induce meiotic resumption but to a lesser extent. A combined treatment with FSH and \( \text{PGE}_2 \) exerted a similar effect as FSH alone (Fig. 3, A and B). This observation is consistent with our findings described above showing FSH-induced COX-2 expression and PG secretion in cumulus cells. However, our observation of less efficient meiotic resumption by \( \text{PGE}_2 \) is suggestive of alternative pathways under FSH regulation, namely the epidermal growth factor family of growth factors, ovarian steroids, and sterols (15, 29–33). We thus speculated that FSH and \( \text{PGE}_2 \) have additive effects in inducing maturation of \( \text{Cox-2}^{-/-} \) oocytes in culture. Indeed, it was interesting to note that FSH or \( \text{PGE}_2 \) alone had a modest stimulatory role in inducing GVBD and MI to MII transition in null oocytes, whereas FSH and \( \text{PGE}_2 \) cotreatment additively improved oocyte meiotic maturation in the absence of COX-2 (Fig. 3, A and B). In the same context, we also examined cumulus expansion in culture. It is interesting to note that \( \text{PGE}_2 \) alone was sufficient to induce nearly normal cumulus expansion in both wild-type and \( \text{Cox-2}^{-/-} \) COCs (Fig. 3, C and D). No additive effects were observed when COCs of either genotype were cotreated with FSH and \( \text{PGE}_2 \), although FSH did exert some stimulatory effects on mutant COC cumulus expansion. It is conceivable that gonadotropin-induced alternative signaling molecules in addition to \( \text{PGE}_2 \) are involved in regulating oocyte meiotic maturation as well as cumulus expansion. Nonetheless,
the results reinforce the concept that de novo synthesis of PGE₂ via COX-2 in cumulus cells is essential for the functional integrity of COCs during oocyte maturation and cumulus expansion.

These findings provoked us to elucidate further the mechanism by which COX-2-derived PGE₂ induces cumulus expansion.

PGE₂ Restores Normal Cumulus Expansion via Up-regulating TSG-6 Expression in Cox-2⁻/⁻ COCs in Culture—Cumulus expansion involves matrix remodeling, and TSG-6 is considered as an important player in this event (34). Previous investigation has shown that in vivo and in vitro expression of TSG-6 in Cox-2⁻/⁻ COCs is lower as compared with wild-type mice (35). We wanted to confirm this earlier observation in our experiments that used mice on the CD1 background by immunofluorescence and immunoblotting. Immunofluorescence of TSG-6 was strongly detected in the matrix of COCs collected from superovulated wild-type mice after 15 h of hCG injection (Fig. 4A). In contrast, only a faint immunofluorescence of TSG-6 was detected in cumulus cells, but not in the matrix, of Cox-2⁻/⁻ COCs (Fig. 4A). This down-regulated TSG-6 expression in Cox-2⁻/⁻ COCs was further confirmed by immunoblotting. Antibodies against TSG-6 recognized two bands, a 36-kDa band, corresponding to free TSG-6, and a 120-kDa band, representing inter/trypsin inhibitor-TSG-6 complex. We found that Cox-2⁻/⁻ COCs recovered from superovulated females had less TSG-6 proteins of either type when compared with wild-type COCs receiving the same treatment (Fig. 4B). We speculated that this remarkable reduction of TSG-6 expression and incorporation into the cumulus matrix compromises the structural integrity of the matrix, leading to cumulus cell death. Thus, we examined the apoptosis status of superovulated wild-type and Cox-2⁻/⁻ COCs. We noted that a substantial number of cumulus cells undergo apoptosis (TUNEL-positive cells) upon the loss of COX-2 (Fig. 4C). This increased cumulus cell death in Cox-2⁻/⁻ mice was associated with down-regulation of Bcl-2, an anti-apoptotic protein (36, 37), but an up-regulation of caspase-3, a key mediator of apo-
we examined the regulatory effects of FSH and PGE2 on TSG-6 cumulus cell death. To explore further insights to this defect, because of down-regulated TSG-6 function and enhanced Cox-2, the defective cumulus expansion in PGE2 is an upstream inducer of TSG-6 expression in cumulus giving a smeared staining pattern. This suggests that although into the cumulus matrix of COCs in both genotypes (Fig. 4, exhibited strong TSG-6 expression, but also its incorporation expression and cell apoptosis status in cultured COCs. As shown in Fig. 4, G–J, FSH or PGE2 alone was sufficient to induce TSG-6 expression and facilitate cell survival in wild-type COCs in culture, whereas PGE2, but not FSH, was capable of up-regulating TSG-6 expression and restoring normal cumulus expansion in the absence of COX-2, although a modest role of FSH in restoring cell survival was observed in mutant COCs. It is noteworthy that cotreatment with FSH and PGE2 not only exhibits strong TSG-6 expression, but also its incorporation into the cumulus matrix of COCs in both genotypes (Fig. 4G), giving a smeared staining pattern. This suggests that although PGE2 is an upstream inducer of TSG-6 expression in cumulus cells, other signaling molecules under FSH stimulation in collaboration with PGE2 further stimulate TSG-6 expression and incorporation into the COC matrix. It was also interesting to note that even the basal level of cumulus cell apoptotic activity was higher in Cox-2 mice in comparison with that of wild-type mice. Nonetheless, the results suggest that TSG-6 expression is under the regulation of COX-2-PGE2 signaling in response to gonadotropin during oocyte maturation and cumulus expansion. Our next objective was to see which of the four PGE2 receptor subtypes, EP1, EP2, EP3, and EP4, mediate PGE2 signaling in these events.

PGE2 Functions through EP2 and EP4 Receptors during Oocyte Maturation and Cumulus Expansion in Vitro—Although previous pharmacological and genetic evidence has shown the importance of EP2 in mediating PGE2 function during oocyte maturation (8, 10, 35), the participation of other EP receptors in this process remained unexplored. In this study, we addressed this question using RT-PCR and immunofluorescence analysis. COCs were collected after 15 h of hCG injection and were subjected to immunofluorescence analysis for EP receptors. For RT-PCR analysis, cumulus cells dispersed from oocytes by hyaluronidase digestion were used. As shown in Fig. 5, A and B, cumulus cells, but not oocytes, expressed EP2, EP3, and EP4. To characterize the role of these receptors in cumulus expansion in vitro, wild-type COCs were cultured with butaprost (an EP2 agonist), sulprostone (an EP1/EP3 mixed agonist), or 11-deoxy-PGE1 (an EP2/EP4 mixed agonist) for 18 h. As shown in Fig. 5, C and D, 11-deoxy-PGE1, but not butaprost or sulprostone, mimicked the role of native PGE2 in inducing cumulus expansion. The results suggest that both EP2 and EP4 subtypes are required for cumulus expansion in vitro. To provide further evidence on the involvement of EP2 and EP4 receptors in oocyte meiotic resumption and cumulus expansion, we used EP receptor antagonists in culture, such as AH6809 (an EP1/EP2 mixed antagonist) and AH23848 (an EP4 antagonist). Wild-type COCs were cultured in the presence or absence of AH6809, AH23848, or a combination of AH6809 and AH23848 for 1 h before FSH treatment. It was interesting to observe that blockade of either EP2 or EP4 activity did not substantially influence FSH-induced oocyte meiotic maturation (Fig. 6, A and B), but significantly inhibited FSH- or PGE2-triggered cumulus expansion (Fig. 6, C and D). The results are consistent with our above observations that cumulus cells, but not oocytes, are the primary target of PGE2 signaling. Moreover, this impaired cumulus expansion in response to FSH by EP2/EP4 antagonists is associated with significantly less induction of TSG-6 (Fig. 6E), reinforcing that TSG-6 is a downstream target of PGE2. However, a modest inhibitory effect on oocyte meiotic maturation was observed when COCs were cotreated with AH6809 and AH23848. We speculate that this is a secondary effect that resulted from severely restrained cumulus expansion under the cotreatment condition, suggesting that reciprocal oocyte-somatic cell interactions are critical for normal oocyte maturation. More importantly, the results add new evidence that PGE2 signaling is primarily mediated by both EP2 and EP4 subtypes.

PGE2 Directs Cumulus Cell Expansion and Survival during Oocyte Maturation by Using Different Signaling Cascades—We next asked which PGE2 downstream signaling pathways are involved in oocyte maturation and cumulus expansion. Increasing evidence points toward the involvement of the PKA signaling cascade in gonadotropin-induced oocyte meiotic maturation (40, 41). Thus, we first examined the

FIGURE 5. EP2 and EP4 receptors are important for cumulus expansion. A and B, PGE2 receptor expression in COCs. EP1, EP2, EP3, and EP4 mRNAs and proteins were analyzed by RT-PCR and immunofluorescence, respectively. Mouse kidney RNA was used as a positive control for the EP1, +, −, presence or absence of RT reaction. M, marker. C and D, activation of EP2 and EP4 induces cumulus expansion in culture. COCs were cultured in hypoxanthine medium containing butaprost, sulprostone, or 11-deoxy-PGE2 for 18 h. Numbers inside bars indicate numbers of expanded COCs/total number of COCs examined. *, significantly different from other groups (p < 0.01). PI, propidium iodide. Bar, 100 μm.
expression of phospho-Akt in COCs in wild-type and Cox-2−/− mice during in vivo oocyte maturation. Phospho-Akt was detected as early as 3 h after hCG injection in both oocytes and cumulus cells of wild-type mice, but not in those from Cox-2 mutant mice as assessed by immunofluorescence and immuno-blotting (supplemental Fig. 2, A–C). Interestingly, at 6 h post hCG injection, phospho-Akt signals were detected in all oocytes examined in wild-type mice as opposed to only 50% of Cox-2−/− oocytes showing phospho-Akt signals (supplemental Fig. 2, A and B). This timely activation of Akt signaling in maturing oocytes is well correlated with the timing of the GVBD event as illustrated in Fig. 1 and suggests that Akt signaling is an important pathway for ensuring normal oocyte meiotic maturation. In contrast, its deferred activation in the absence of COX-2 leads to defective oocyte maturation in vivo. This finding led us to examine Akt activation in oocytes during in vitro maturation.

As shown in Fig. 7A, we observed peri-nuclear signals for phospho-Akt after 8 h in culture. Signals were initially scattered but concentrated at the center of oocytes after 10 h, corresponding to germinal vesicle breakdown. Interestingly, barrel-shaped signals of phospho-Akt were observed after 12 h in culture. The fluorescence intensity of phospho-Akt in Cox-2−/− oocytes was less than those of wild-type oocytes and so was the percentage of oocytes positive for phospho-Akt (Fig. 7B).

Again, it was interesting to note that a combined treatment of FSH and PGE2 significantly increased the percentage of phospho-Akt positive oocytes in Cox-2−/− mice compared with FSH treatment alone, indicating that substantially restored cumulus expansion by PGE2 in null COCs improves oocyte meiotic maturation.

To provide further evidence on the participation of the Akt pathway during oocyte maturation, we employed a pharmacological approach using LY294002 to selectively block PI3K and thus Akt signaling in culture. As shown in Fig. 8, A–C, LY294002 treatment in a dose-dependent manner inhibited FSH-induced oocyteAkt activation as well as meiosis resumption and progression, which was not corrected by PGE2 cotreatment. This is consistent with our observation of a lack of EP receptor expression in oocytes, suggesting that oocyte Akt phosphorylation is activated by an alternative stimulus independent of the COX-2-PGE2 signaling axis. Because gonadotropin also induces Akt phosphorylation in cumulus cells during in vivo oocyte maturation (supplemental Fig. 2), the physiological significance of this signaling cascade in these cells remains unexplored. Therefore, we examined cumulus cell expansion in response to LY294002. As shown in Fig. 8D, we observed a significant decrease in FSH-induced cumulus expansion in the presence of LY294002, which was not restored by PGE2 cotreatment, indicating that PI3K-Akt signaling in regulating cumulus cell function lies downstream of COX-2-derived PGE2. This is consistent with our subsequent observation of more prominent Akt phosphorylation by FSH in wild-type cumulus cells as compared with Cox-2−/− cells (Fig. 8E) and of Akt activation by PGE2 alone in cumulus cells in culture (Fig. 8F). In addition, alteration of TSG-6, but not COX-2 expression, in response to FSH by LY294002 (Fig. 8, G and H) further places the PI3K-Akt signaling pathway downstream of the COX-2-PGE2 signaling axis during cumulus expansion.

Because PGE2 restores normal cumulus cell expansion (Fig. 3C) as well as cumulus cell survival (Fig. 4H) in Cox-2−/− females, we further asked whether these processes under the influence of PGE2 utilize differential signaling cas-
cles during oocyte maturation. Early studies have demonstrated that cumulus expansion in response to gonadotropin or other stimuli depends on multiple signaling pathways, including cAMP/PKA (42–45), ERK1/2 (34, 46–49), p38 MAPK (34, 49), as well as PI3K-Akt signaling (40, 41). Thus, we next used selective antagonists of these pathways and tested their effects under PGE2 stimulation in cumulus cells. As depicted in Fig. 8, we surprisingly observed that attenuation of ERK1/2, p38 MAPK, or nuclear factor \(\text{kB} (\text{NF-kB})\) by U0126, SB203580, or PGA1, respectively, failed to show any effect on PGE2-stimulated cumulus cell survival, but profoundly blocked PGE2-induced cumulus expansion. It is conceivable that PGE2 functions through alternative pathways in regulating cell survival. Indeed, we noted that blockade of the PI3K-Akt pathway by LY294002 substantially dampened cumulus cell survival, perhaps resulting in modest impairment of cumulus expansion seen in response to PGE2. Similar effects were also noted when PKA or c-Jun N-terminal kinase (c-JNK), which is known to participate in Xenopus oocyte meiotic maturation (50, 51), was selectively inhibited by H-89 or SP60012, respectively (Fig. 8). It is interesting to note that PGE2 up-regulated the expression of its own synthesizing enzyme COX-2 in cumulus cells during oocyte maturation (Fig. 8). We speculated that this positive feedback regulation of COX-2 contributes to de novo amplification of PGE2 signaling and consequently to the regulation of cumulus expansion. To test this hypothesis, we analyzed the consequence of inhibition of differential signaling cascades on COX-2 expression. As illustrated in Fig. 8, we observed that blockade of the ERK1/2, p38 MAPK, or NF-kB signaling cascade that leads to defective cumulus expansion, but not PI3K-Akt, PKA, or c-JNK pathway, antagonized the PGE2-induced up-regulation of COX-2 expression. This finding reinforces the idea that PGE2 by a feed-forward mechanism regulates cumulus cell expansion via ERK1/2, p38 MAPK, or NF-kB signaling, although its effects on cell survival are mediated through the PI3K-Akt, PKA, or c-JNK pathway. Nonetheless, the results highlight the diversity of PGE2 signaling in regulating cumulus cell expansion and survival via different downstream pathways.

To further explore the physiological relevance of COX-2-derived PGE2 in oocyte meiotic maturation and their fertilizing capacity, we examined in vivo oocyte maturation in Cox-2 null mice receiving PGE2 injections. As shown in Table 1, PGE2 largely restores normal oocyte meiotic maturation in vivo in the absence of COX-2. Furthermore, these null oocytes with PGE2 pretreatment in vivo during the preovulatory period exhibit normal fertilizing capacity in vitro (Table 2). The results reinforce that COX-2-derived PGE2 is at least one of the key players in regulating the resumption and progression of oocyte meiotic...
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maturation during ovulation to further ensure their normal developmental potential.

**DISCUSSION**

In this investigation, we studied in-depth the roles of PGs in oocyte maturation using genetic, molecular, physiological, and pharmacological approaches. The highlights of our investigation are that COX-2-derived PGE2 in response to gonadotropin coordinates oocyte meiotic maturation, cumulus cell expansion, and survival by utilizing different downstream signaling cascades. Furthermore, this study elucidates a novel mechanism of COX-2 expression in cumulus cells by PGE2 that facilitates cumulus expansion. The findings of decreased accumulation of TSG-6 in the cumulus matrix with enhanced cumulus cell apoptosis in the absence of COX-2 provide evidence for the underlying cause of compromised fertilization in *Cox-2−/−* mice. In addition, here we provide pharmacological evidence that both EP2 and EP4 receptors are potentially important in mediating PGE2 bioactivity during oocyte maturation and cumulus expansion.

Activation and expansion of the cumulus oophorun as well as timely resumption of oocyte meiosis are critical to successful ovulation and fertilization (52). The participation of COX-2-derived PGE2 in these processes has been elucidated by a number of pharmacological and genetic studies. In this study, we demonstrate that FSH up-regulates cumulus COX-2 expression and thus PGE2 production in culture, which phenocopies the *in vivo* induction of COX-2 and PGE2 synthesis in cumulus cells in response to the preovulatory gonadotropin surge, suggesting its role in cumulus expansion and oocyte maturation. The rescue of defective cumulus expansion in *Cox-2−/−* mice in *vivo* or *in vitro* by PGE2 provides definitive evidence that this PG is critical to this process. TSG-6, which is expressed in cumulus and mural granulosa cells in preovulatory follicles (53–56), is known to modulate extracellular matrix remodeling and is a known marker of cumulus expansion, but not meiotic maturation of oocytes. Our observation of down-regulation of TSG-6 in cumulus cells in the absence of COX-2, but its up-regulation by PGE2, provides evidence that TSG-6 is a downstream target of PGE2. This is consistent with other studies showing down-regulation of hCG or FSH-induced TSG-6 in cumulus cells missing EP2 or COX-2 (34, 35). TSG-6 secreted into the cumulus cell matrix binds to hyaluronic acid and other hyaluronic binding proteins, such as PTX-3 or serum-derived inter-α-trypsin inhibitor, to stabilize the matrix bed. The stabilization of the matrix by these molecules is essential for cumulus expansion and ovulation. Gene-targeting experiments in mice have shown multiple female reproductive defects that include reduced ovulation, defective cumulus expansion, and fertilization failure in the absence of these factors (57–61).

Early studies have provided genetic evidence that EP2 is a key player in ovulation and fertilization (8, 9, 62). Similar to *Cox-2−/−* mice, EP2-deficient mice also show defective cumulus expansion in addition to reduced ovulation and fertilization.

**TABLE 1**

**PGE2 rescues oocyte meiotic maturation in Cox-2−/− mice in vivo**

Superovulated oocytes were collected from WT and *Cox-2−/−* mice 14–15 h after hCG injection. Oocytes were examined and classified as follows: GV, germinal vesicle; MI, metaphase I; MII, metaphase II by nuclear staining. The percentage of oocytes classified for each stage of meiosis for WT and *Cox-2−/−* mice was calculated by dividing the number of oocytes at each stage of meiosis by the total number of oocytes. Data are presented as mean ± S.E. Values with different superscript letters within the same column are statistically significant (*p* < 0.05; analysis of variance).

| Treatment                | No. of tested mice with ovulation | No. of oocytes with ovulation | GV | MI | MII |
|--------------------------|----------------------------------|--------------------------------|----|----|-----|
| WT + vehicle             | 12                               | 30 ± 3*                        | 0  | 2  | 4   |
| *Cox-2−/−* + vehicle     | 7                                | 16 ± 3*                        | 2  | 2  | 2   |
| *Cox-2−/−* + PGE2        | 5                                | 25 ± 4*                        | 0  | 2  | 2   |

* Statistical analysis was performed by χ² test; *p* < 0.01.

**TABLE 2**

**PGE2 restores normal fertilizing capacity of Cox-2−/− oocytes**

Superovulated COCs were subjected to *in vitro* fertilization. The appearance of two pronuclei (PN) indicated successful fertilization. The number of blastocysts formed from fertilized eggs was recorded after 5 days of culture.

| Treatment                | No. of tested mice | No. of 2-PN oocytes/total oocytes | No. of blastocysts/total 2-PN oocytes |
|--------------------------|--------------------|-----------------------------------|--------------------------------------|
| WT + vehicle             | 13                 | 269/347 (78%)                     | 254/269 (94%)                       |
| *Cox-2−/−* + vehicle     | 9                  | 73/172 (42%)*                     | 56/73 (77%)*                        |
| *Cox-2−/−* + PGE2        | 3                  | 44/66 (67%)                       | 39/44 (89%)                         |

**FIGURE 8.** PGE2 directs cumulus cell expansion and survival using different signaling cascades. A–D, inactivation of PI3K-Akt signaling inhibits FSH-induced oocyte meiotic maturation and cumulus expansion. WT COCs were cultured in medium containing HX, HX + 100 IU/liter FSH, HX + FSH + 1 μM PGE2 for 18 h. COCs were pretreated with various concentrations of LY294002 for 1 h before exposing to FSH or PGE2. Numbers inside bars indicate numbers of p-Akt-positive oocytes/total number of oocytes tested. F, FSH induces Akt phosphorylation in WT, but not in *Cox-2−/−*, cumulus cells. COCs were cultured in HX medium with 100 IU/liter FSH for 4 h. Cumulus cells were dispersed from COCs for immunoblotting. F, PGE2 activates Akt in cumulus cells. WT COCs were cultured without 1 μM PGE2. These experiments were repeated twice with similar results. G, and H, silencing of PI3K-Akt signaling attenuates FSH-induced TSG-6 but not COX-2 expression. J, PGE2 regulates cumulus cell expansion and survival through multiple signaling pathways. COCs were cultured in HX medium with 1 μM PGE2, with or without H-89, U0126, SB203580, SP60012, PGA1, or LY294002 for 18 h. J, PGE2 up-regulates COX-2 expression in cumulus cells. At least 30 COCs from each group were tested and repeated three times. Bars with different letters are significantly different (*p* < 0.05), PI, propidium iodide. BF, bright field. Bar, 100 μm.
However, our present observations provide pharmacological evidence that both EP2 and EP4 are important players in cumulus expansion for the following reasons. First, 11-deoxy-PGE1, a mixed agonist for EP2/EP4, but not butaprost, a selective EP2 agonist, induces cumulus expansion. Second, these processes are attenuated by a mixed EP2/EP4 antagonist. Third, EP2, EP3, and EP4 are expressed in cumulus cells, and hCG up-regulates EP2 and EP4 expression in these cells (28). However, the role of EP3 in cumulus cell function does not appear to be critical, because EP3 null females do not exhibit overt reproductive phenotypes (11). Because EP4 deficiency results in perinatal lethality (12, 13), it remains unanswered whether EP2 and EP4 have distinct and/or overlapping function in female reproduction.

Cumulus cell expansion in response to gonadotropins has been shown to be governed by multiple signaling pathways (52). However, our observation of increased apoptosis in addition to impaired expansion of cumulus cells missing COX-2 adds a new complexity to this process. Because PGE2 can simulate gonadotropins in cumulus expansion, we explored site- and event-specific participation of PKA, MAPK, NF-κB, and PI3K-Akt pathways during PGE2-induced oocyte maturation. Indeed, a remarkable diversification of these pathways in cumulus cell expansion and survival has emerged from this study (Fig. 9). For example, although ERK1/2, p38 MAPK, and NF-κB pathways are critical to PGE2-induced cumulus expansion, they are not essential for cumulus cell survival. In contrast, PI3K-Akt, PKA, and c-JNK pathways are more involved in cumulus cell survival with modest effects on cumulus expansion. These observations also suggest that PGE2 modulation of these signaling pathways lies downstream of gonadotropin-induced oocyte maturation. Another striking observation is the up-regulation of COX-2 by PGE2 involving the same signaling pathways that promote cumulus cell expansion. This would then suggest that COX-2-derived PGE2 occupies a pivotal place downstream of gonadotropins in amplifying its own signaling via a “feedforward-feedback” loop not only to initiate but also to sustain the oocyte maturation process after termination of the gonadotropin surge. Our study also reveals that direct targets of PGE2 signaling are the cumulus cells but not the oocytes that lack EP receptors. In conclusion, the present investigation underscores the diversity of PGE2 signaling during oocyte maturation. This study is clinically relevant to female fertility with special reference to women of reproductive ages who consume COX-2-selective inhibitors or other nonsteroidal anti-inflammatory steroids that are nonspecific inhibitors of COX-1 and COX-2.

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REFERENCES

1. Dey, S. K., Lim, H., Das, S. K., Reese, J., Paria, B. C., Daikoku, T., and Wang, H. (2004) Endocr. Rev. 25, 341–373
2. Wang, H., and Dey, S. K. (2005) Prostaglandins Other Lipid Mediat. 77, 84–102
3. Smith, W. L., and Dewitt, D. L. (1996) Adv. Immunol. 62, 167–215
4. Lim, H., Paria, B. C., Das, S. K., Dinchuk, J. E., Langenbach, R., Trzaskos, J. M., and Dey, S. K. (1997) Cell 91, 197–208
5. Davis, B. J., Lennard, D. E., Lee, C. A., Tiano, H. F., Morham, S. G., Wetsel, W. C., and Langenbach, R. (1999) Endocrinology 140, 2685–2695
6. Lim, H., Gupta, R. A., Ma, W. G., Paria, B. C., Möller, D. E., Morrow, J. D., DuBois, B. N., Trzaskos, J. M., and Dey, S. K. (1999) Genes Dev. 13, 1561–1574
7. Negishi, M., Sugimoto, Y., and Ichikawa, A. (1995) Biochim. Biophys. Acta 1259, 109–119
8. Hizaki, H., Segi, E., Sugimoto, Y., Hirose, M., Saji, T., Ushikubi, F., Matsuo, T., Noda, Y., Tanaka, T., Yoshida, N., Narumiya, S., and Ichikawa, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10501–10506
9. Kennedy, C. R., Zhang, Y., Brandon, S., Gu, Y., Coffee, K., Funk, C. D., Magnuson, M. A., Oates, J. A., Breier, M. D., and Breier, R. M. (1999) Nat. Med. 5, 217–220
10. Matsumoto, H., Ma, W., Smalley, W., Trzaskos, J., Breier, R. M., and Dey, S. K. (2001) Biol. Reprod. 64, 1557–1565
11. Ushikubi, F., Segi, E., Sugimoto, Y., Murata, T., Matsuo, K., Kobayashi,
