Timely interaction between prostaglandin and chemokine signaling is a prerequisite for successful fertilization

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Timely interaction between the egg and sperm is required for successful fertilization; however, little is known about the signaling therein. Prostaglandin (PG) E receptor EP2-deficient (*Ptger2*$^{-/-}$) female mice exhibit a severe fertilization defect. We investigated the molecular events leading to this failure. We found increased gene expression for chemokines, such as *Ccl2*, *Ccl7*, and *Ccl9*, in *Ptger2*$^{-/-}$ cumulus cells (the somatic cells surrounding the egg) compared with wild-type cells. Furthermore, under physiological conditions, cumulus-derived chemokine signaling was found to have a dual action; CCL7 facilitates sperm migration to the cumulus–egg complex and integrin-mediated cumulus extracellular matrix (ECM) assembly to protect eggs. However, in the absence of PGE_2-EP2 signaling, chronic CCL7 signaling results in excessive integrin engagement to the ECM, making the cumulus ECM resistant to sperm hyaluronidase, thereby preventing sperm penetration. Our findings indicate that PGE_2-EP2 signaling negatively regulates the autocrine action of chemokines and prevents excessive cumulus ECM assembly. This interaction between PG and chemokine signaling is required for successful fertilization.

cumulus cells | extracellular matrix | fibronectin | integrin | prostanoid receptor EP2

Ovulation and fertilization are key processes in mammalian female reproduction, which are strictly regulated by pituitary gonadotropins, follicle-stimulating hormone, and luteinizing hormone. These hormones induce various preovulatory processes, including follicular development, oocyte maturation, and cumulus expansion, and trigger ovulation (1). Ovulated eggs move to the oviduct; subsequently the timely interaction between an egg and sperm leads to successful fertilization (2). Whereas various molecules are known to regulate preovulatory processes locally in the follicle under the control of gonadotropin (3), little is known about the regulation of postovulatory processes, including fertilization in the oviduct, which gonadotropin does not control.

One potential candidate for the local mediators regulating fertilization within the oviduct is chemokines. Chemokines constitute a family of structurally related, inducible peptides involved in various immune responses and inflammation, acting as chemoattractants and activators of specific types of leukocytes (4). Approximately 40 chemokines, grouped into distinct families and secreted by various cells, are currently known (5). It was recently reported that in humans, CCL5 (also called RANTES) is present in the follicular fluid and stimulates the *in vitro* migration of sperm, which express CCR1, a receptor for CCL5 (6). Based on these findings, it has been proposed that chemokines may participate in sperm guidance within the genital tract (7). The physiological significance of such chemokine signaling within the oviduct remains elusive, however.

The cumulus oophorus is composed of a group of closely associated granulosa cells that surround the oocyte in the antral follicle and are collectively called cumulus cells (8). In response to a surge in luteinizing hormone, the cumulus cells start to produce extracellular matrix (ECM) components, which are deposited into the intercellular space and are stabilized by accessory proteins. This phenomenon is called cumulus expansion. A major component of the ECM produced by the cumulus cells is hyaluronan, which provides the viscoelastic properties of the cumulus oophorus. The expanded cumulus oophorus (cumulus cells and ECM) makes a tight complex with an oocyte that is ovulated as the cumulus-oocyte complex (COC). During ovulation, the cumulus oophorus protects the oocyte from mechanical stress and from proteolytic enzymes present in the follicle and oviduct, and directs the oocyte into the oviduct by facilitating its capture by the ciliated epithelial cells of the infundibulum and its transport to the fertilization site (9). In the oviduct, the cumulus oophorus facilitates access of the sperm to the oocyte by trapping and selecting sperm for successful fertilization (10). Thus, the complex organization of the oocyte, cumulus cells, and ECM is essential for successful fertilization in the oviduct. Indeed, recent studies using mice null of several hyaluronan-binding proteins have shown that the cumulus ECM is required for successful fertilization *in vivo*; female mice deficient in these molecules are sterile, due to loss of the cumulus and ECM (11–13). However, once sperm reach the COC, the cumulus ECM must be disassembled so that the sperm can find a passage through the ECM layer. Although it has been suggested and is generally believed that the cumulus ECM is disassembled by sperm motility and sperm hyaluronidase (14), whether the disassembly of the cumulus ECM is induced by the force of the sperm alone or is regulated in an autocrine or paracrine manner by the cumulus itself remains unclear.

Prostaglandin E_2 (PGE_2), an arachidonate metabolite synthesized most abundantly within the follicle, is a key mediator in the ovulation-stimulating action of gonadotropin (15). PGE_2 exerts its actions by acting on four subtypes of the PGE receptor, EP1–EP4 (16). On gonadotropin stimulation, cyclooxygenase-2 (COX-2; *Ptgs2*), a rate-limiting enzyme of PG biosynthesis, is induced in all cells within the follicle (17), and a large amount of PGE_2 is released into the follicular fluid (15). Indeed, *Ptgs2* null mice demonstrate severely impaired ovulation (18). Similarly, mice lacking the PGE receptor EP2 (*Ptger2*), which is expressed in the cumulus cells, exhibit reduced ovulation (19). These findings clearly indicate that PGE_2-EP2 signaling in the cumulus plays a role in the ovulatory

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processes. Notably, however, *Ptger2*$^{-/-}$ mice show a higher rate of failure in fertilization than in ovulation; the ovulation number of *Ptger2*$^{-/-}$ mice is 80% of that of wild-type (WT) mice, whereas the fertilization rate of *Ptger2*$^{-/-}$ mice is ≈20% of that of WT mice. Both the *Ptgs2* and *Ptger2* genes are still highly expressed in cumulus cells even after ovulation. Intriguingly, COCs isolated from the *Ptger2*$^{-/-}$ oviduct show a significant reduction in *in vitro* fertilization with sperm compared with control WT COCs (19), suggesting that PGE_2-EP2 signaling contributes to fertilization-related processes, such as cumulus ECM disassembly and sperm penetration.

To gain insight into the mechanism causing fertilization failure in the *Ptger2*$^{-/-}$ cumulus, in this study we compared the gene expression profiles of WT and *Ptger2*$^{-/-}$ cumuli isolated from the oviduct. We found increased gene expression for chemokines, such as *Ccl2*, *Ccl7*, and *Ccl9*, in *Ptger2*$^{-/-}$ cumuli compared with WT cumuli. We further examined the physiological function of cumulus-derived chemokines in postovulatory processes and found that the chemokines facilitate both sperm attraction and compaction of the cumulus by integrin-mediated ECM assembly, the latter of which is down-regulated by PGE_2-EP2 signaling to allow sperm penetration for successful fertilization.

Results

Expression of Chemokine Genes in Ovulated Cumuli and Its Enhancement by *Ptger2* Deficiency. We previously reported that *Ptger2*$^{-/-}$ mice exhibit impaired fertilization and abortive expansion of the cumulus after ovulation; however, why *Ptger2* deficiency leads to a severe reduction in fertilization rate was unclear (19). Because *Ptger2* is induced in cumulus cells on gonadotropin stimulation, we hypothesized that the PGE_2-EP2 signaling in cumulus cells regulates the expression of unidentified molecules with important regulatory roles in fertilization. To identify such key molecules, we used oligonucleotide arrays as a screening tool. COCs were collected from the oviducts of WT and *Ptger2*$^{-/-}$ mice 14 h after injection of human CG (hCG) and were subjected to analysis after oocyte removal. In the initial screen of the ≈12,000 genes represented on the array, we identified 16 genes showing more than a fivefold increase and 7 genes showing more than a fivefold decrease in expression in the *Ptger2*$^{-/-}$ cumulus cells compared with the WT cumulus cells [supporting information (SI) Table S1]. Among the 7 genes down-regulated in the *Ptger2*$^{-/-}$ cumulus cells, the *Ptger2* gene showed the greatest reduction, indicating that the expression analysis was working accurately. Among the 16 genes up-regulated in the *Ptger2*$^{-/-}$ cumulus cells, we found 4 genes for chemokine ligands, *Ccl2* (MCP-1), *Ccl9* (MIP-1γ), *Ccl7* (MCP-3), and *Cxcl1* (Groα), along with 2 genes for chemokine receptors, *Ccr5* (CCR5) and *Ccr1* (CCR1). We also detected significantly enhanced expression of *Ccr2* (CCR2) (Table S1). Because a role for chemokines in fertilization has not been well established, we focused on the three aforementioned C-C chemokines (CCL2, CCL7, and CCL9) and explored the possibility that these chemokines may regulate fertilization in an autocrine manner.

We first addressed the time course of gene expression of the chemokines and CCRs in both WT and *Ptger2*$^{-/-}$ cumuli. Cumuli from five animals were pooled for each time point and subjected to real-time reverse transcription–polymerase chain reaction (RT-PCR) (Fig. 1*A*). Before ovulation (at 3 and 9 h after hCG injection), the expression levels of *Ccl9* mRNA were as low as the background levels in both genotypes; however, *Ccl9* expression in the WT cumuli was increased by fivefold at 14 h, when the COCs moved to the oviduct. Moreover, the induction of *Ccl9* expression on ovulation was augmented significantly in *EP2*$^{-/-}$ cumuli. Similar results were obtained for the expression patterns of *Ccl2*, *Ccl7*, *Ccr1*, *Ccr2*, and *Ccr5* in the cumulus cells. In contrast, no expression of any chemokine genes was detected in the WT and *Ptger2*$^{-/-}$ oocytes (data not shown). These results indicate that chemokine genes are induced in the cumuli on ovulation, and that this induction is enhanced in *Ptger2*$^{-/-}$ cumuli. Indeed, a statistically significant

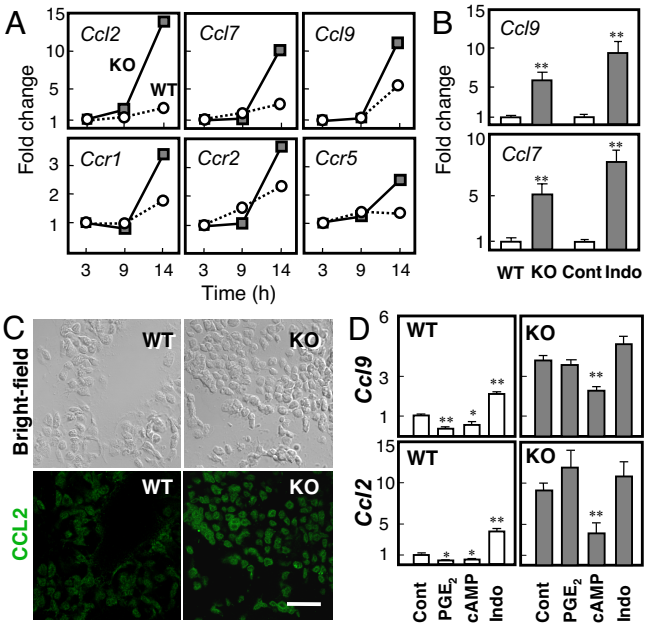

Fig. 1. Expression of chemokine genes in ovulated cumuli and its enhancement by EP2 deficiency. (*A*) Time course of chemokine gene expression in WT (open circles) and *Ptger2*$^{-/-}$ (KO; gray squares) cumulus cells. Expression levels are presented as fold change of the values at 3 h. (*B*) Increased expression of *Ccl9* and *Ccl7* in the cumuli isolated from *Ptger2*$^{-/-}$ and indomethacin-treated mice. The cumuli were collected individually from WT, *Ptger2*$^{-/-}$ (KO), vehicle-treated (Cont), and indomethacin-treated mice (Indo) at 14 h, and the RNA from each mouse was subjected to real-time RT-PCR analysis. Values are presented as mean ± standard error of the mean (SEM) (n = 3–5). **, $P < 0.01$. (*C*) Enhanced expression of CCL2 protein in oviductal cumuli in *Ptger2*$^{-/-}$ mice. Bright-field and immunofluorescence images showing signals for CCL2 protein in the cumuli from WT and *Ptger2*$^{-/-}$ (KO) mice. Scale bar: 20 μm. (*D*) PGE_2 attenuates and indomethacin augments *ccl* gene expression in WT, but not in *Ptger2*$^{-/-}$ COCs. COCs collected from WT and *Ptger2*$^{-/-}$ (KO) mice were incubated with 1 μM PGE_2, 2 mM dibutyryl cAMP (cAMP), 10 μM indomethacin (Indo), or vehicle (Cont) for 3 h. Total RNA was extracted and subjected to real-time RT-PCR analysis. Expression levels are shown as fold change of the level of WT control. Values are presented as mean ± SEM (n = 3). *, $P < 0.05$; **, $P < 0.01$. Representative results of two (*A* and *D*) or three (*B* and *C*) independent experiments are shown.

difference in *Ccl9* and *Ccl7* gene expression was detected between WT and *Ptger2*$^{-/-}$ cumuli at 14 h, when the cumulus RNA isolated from each animal was individually subjected to real-time RT-PCR (Fig. 1*B*).

To examine the protein expression of chemokines in the cumuli, we prepared ovary sections (at 3 and 9 h) and oviductal ampulla sections (at 14 h) from WT and *Ptger2*$^{-/-}$ animals and subjected them to immunofluorescence analysis for CCL2, CCL7, and CCL9. Whereas immunofluorescence signals for these chemokines were at background levels in both WT and *Ptger2*$^{-/-}$ ovarian tissues before ovulation (data not shown), weak but specific signals for CCL2 were detected in the WT cumuli after ovulation (i.e, at 14 h) (Fig. 1*C*). Notably, these CCL2 signals were stronger in the *Ptger2*$^{-/-}$ cumuli than in the WT cumuli. Similar results were obtained for CCL7 and CCL9 (data not shown).

To verify that a loss of PG signaling around the time of ovulation enhances the induction of chemokines in cumulus cells, we examined the effect of indomethacin (an inhibitor of PG biosynthesis) on chemokine induction. Indomethacin was administered to mice at the time of hCG injection, COCs were isolated at 14 h, and chemokine expression was examined by real-time RT-PCR and immunofluorescence analysis. Significant increases in the level of *Ccl* expression and higher fluorescence signal intensities for CCL2 were detected in the cumuli isolated from indomethacin-treated

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WT mice than those from control animals (Fig. 1*B* and data not shown), suggesting that PG production *in vivo* is linked to the suppression of chemokine expression in cumuli.

We then examined whether exogenously added PGE_2 can suppress *Ccl* gene expression in the cumulus cells. WT and *Ptger2*$^{-/-}$ COCs were collected from oviducts and incubated *in vitro* in media containing 1 μM PGE_2, 2 mM dbcAMP (a membrane-permeable cAMP analog), or 10 μM indomethacin for 3 h. The mRNA levels of the chemokines were subsequently quantified by real time RT-PCR (Fig. 1*D*). PGE_2 and dbcAMP suppressed the expression of *Ccl9* in WT COCs, whereas indomethacin augmented the expression by twofold, suggesting that the endogenous PGE_2-cAMP system participates in the negative regulation of *Ccl9* expression. T$^{-/-}$he basal expression level of *Ccl9* mRNA was about fourfold higher in *Ptger2*$^{-/-}$ COCs than in WT COCs. Whereas dbcAMP decreased the expression level of *Ccl9*, both PGE_2 and indomethacin failed to alter the expression levels, indicating that the suppression of *Ccl9* expression by PGE_2 is mediated through the EP2 receptor. Similar results were obtained in the gene expression analysis for *Ccl2* (Fig. 1*D*, lower panels) and *Ccl7* (data not shown). These findings indicate that *Ccl* expression is induced in the cumuli after ovulation, but its expression level is negatively regulated by the autocrine action of PGE_2 through the EP2-cAMP system.

Chemokines Facilitate Sperm Migration to Cumuli, but Their Excessive Signaling on Cumuli Suppresses Fertilization. As reported previously, *Ptger2*$^{-/-}$ COCs demonstrated a lower efficiency of *in vitro* fertilization (IVF) compared with WT COCs. Considering the enhanced chemokine production in the *Ptger2*$^{-/-}$ COCs, we next explored whether the addition of C-C chemokines would affect the IVF processes. When we isolated WT COCs and incubated them with sperm for 8 h, 75–80% of the oocytes were fertilized. We next added 100 ng/ml of CCL2, CCL9, or CCL7 to the medium containing COCs 30 min before sperm addition and examined the effect of these chemokines on the fertilization rates. CCL9 and CCL7 are ligands for CCR1, and CCL2 and CCL7 are ligands for CCR2 (20). The addition of CCL2 and CCL9 decreased the fertilization rate significantly, to 48.4 ± 3.2% and 46.3 ± 2.5%, respectively. The addition of CCL7 decreased the fertilization rate further to 31.1 ± 6.0%; this inhibitory effect of CCL7 was abolished by the addition of an antagonist for CCR1/CCR2, vMIP-II (21) (Fig. 2*A*). In contrast, CCL22, a ligand for CCR4, which was not detected in the cumuli by RT-PCR analysis, did not affect the fertilization rate. In addition, CCL2, CCL7, and CCL9 did not affect the fertilization rates of cumulus-free oocytes. These findings demonstrate that CCR1/CCR2 signaling suppresses fertilization by acting on cumulus cells and raise the hypothesis that the impaired fertilization in the *Ptger2*$^{-/-}$ mice is due to the enhanced actions of these chemokines. To test this hypothesis, we added vMIP-II to the medium containing WT and *Ptger2*$^{-/-}$ COCs 30 min before sperm addition and examined the effect of the antagonist on the fertilization rate of *Ptger2*$^{-/-}$ COCs. vMIP-II significantly enhanced the fertilization rate of *Ptger2*$^{-/-}$ COCs but had no effect on the fertilization rate of WT COCs (Fig. 2*B*).

We also examined the effect of *in vivo* indomethacin administration on fertilization and the role of CCR1/CCR2 signaling in the fertilization rate of indomethacin-treated COCs. Indomethacin administration at the time of hCG injection significantly decreased the fertilization rate both *in vivo* and *in vitro* (*in vivo*: control, 87.5% ± 1.7% vs. indomethacin, 46.7% ± 4.4%; *in vitro*: control, 79.7% ± 1.7% vs. indomethacin, 45.0% ± 2.1%). The addition of vMIP-II partially but significantly improved the IVF rate of indomethacin-treated COCs (Fig. 2*B*). These findings suggest that the impaired fertilization in *Ptger2*$^{-/-}$ and indomethacin-treated mice is due, at least in part, to the increased production of chemokines.

Although *Ptger2* deficiency enhances the production of chemokines by cumuli, these chemokines are produced at significant concentrations by WT cumuli with intact EP2 signaling (Fig. 1*A*).

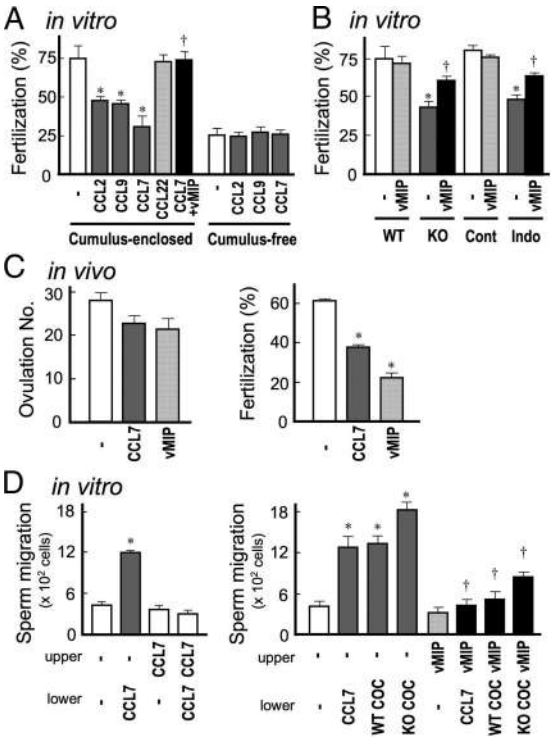

Fig. 2. Chemokines facilitated sperm migration to cumuli, but their excessive signaling on cumuli suppressed fertilization. (*A*) CCL2, CCL7, and CCL9, but not CCL22, suppressed fertilization of WT COCs (cumulus-enclosed). The inhibition by CCL7 was abolished by addition of the CCR1/CCR2 antagonist, vMIP-II (vMIP). Such inhibitory effects were not observed in the absence of cumulus cells (cumulus-free). *, $P < 0.01$ (vs. control); †, $P < 0.01$ (vs. CCL7 only). (*B*) Restoration of the fertilization rate by vMIP-II in EP2 signal–depleted COCs. vMIP-II (vMIP) itself did not alter the fertilization rate of control COCs (WT, Cont), but significantly restored the rates of COCs from *Ptger2*$^{-/-}$ (KO) or indomethacin-treated mice (Indo). Values are presented as mean ± SEM (18–25 eggs for each well; $n = 6$). *, $P < 0.01$ (vs. control of WT or vehicle-treated mice); †, $P < 0.01$ (vs. without vMIP-II). (*C*) Effects of CCL7 and vMIP-II on ovulation and fertilization *in vivo*. WT female mice were injected i.p. with vehicle (-), CCL7 (50 μg/kg), or vMIP-II (250 μg/kg) at 8 h after hCG injection and then mated with males overnight. Values are presented as mean ± SEM ($n = 6$–7). *, $P < 0.05$ (vs. vehicle). (*D*) CCL7 and COC facilitated sperm migration in a vMIP-II-sensitive manner. Sperm were subjected to a Boyden chamber assay. The upper or lower wells were filled with medium only (−) or medium containing CCL7 (100 ng/ml). Sperm (1×10^5 cells/ml) were added into the upper wells; 1 h later, the number of sperm that migrated into the lower wells was counted. Alternatively, the lower wells were filled with medium only (-), medium containing CCL7, or WT or *Ptger2*$^{-/-}$ (KO) COCs, and sperm were added into the upper wells in the presence or absence of vMIP-II (1 μg/ml). Values are presented as mean ± SEM (18–24 COCs for each well; $n = 4$). *, $P < 0.001$ (vs. control); †, $P < 0.001$ (vs. without vMIP-II). Representative results of two (*A*–*C*) or three (*D*) independent experiments are shown.

Consequently, we next addressed the physiological significance of chemokine signaling in the cumulus. CCL7 or vMIP-II was administered intraperitoneally (i.p.) to WT mice at 8 h after hCG administration (just before ovulation), and their effects on ovulation and fertilization were examined *in vivo*. Although neither compound significantly altered the ovulation number (Fig. 2*C*), CCL7 decreased the fertilization rate, and, interestingly, vMIP-II significantly affected *in vivo* fertilization, although this compound exhibited no significant effects on the IVF rate of WT COCs. These findings indicate that endogenous CCR1/CCR2 signaling not only negatively regulates fertilization, but also is required for proper fertilization. Given that CCR1 signaling has chemoattractant activity for sperm *in vitro* (6), we suspected that chemokine signaling could facilitate fertilization through its chemoattractant activity for sperm. To test this possibility, we used a Boyden chamber assay to

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evaluate whether ovulated COCs facilitate sperm migration toward COCs themselves. We used CCL7 as a control and found that CCL7 significantly stimulated sperm chemotaxis without affecting sperm chemokinesis, and that both WT and *Ptger2*$^{-/-}$ COCs facilitated sperm migration toward COCs under the same conditions (Fig. 2*D*). Moreover, the stimulating effects of COCs were suppressed by the addition of vMIP-II. These finding suggest that endogenous CCR1/CCR2 signaling facilitates *in vivo* fertilization by stimulating sperm migration toward cumuli.

Integrin-Dependent Hyaluronidase Resistance Causes Fertilization Failure in *Ptger2*$^{-/-}$ COCs. Why does excessive CCR1/CCR2 signaling on cumuli suppress fertilization? One difference between WT and *Ptger2*$^{-/-}$ COCs is in the viscosity of ECMs. During our IVF experiments, we noted that *Ptger2*$^{-/-}$ COCs were more sticky and more resistant to hyaluronidase treatment compared with WT COCs. Hyaluronan is a major component of the cumulus ECM, and production of hyaluronan induces cumulus expansion (22). Sperm use their own hyaluronidase to penetrate the cumulus ECM layer. If excessive chemokine signaling indeed induced the resistance of cumulus ECM to hyaluronidase, this could interfere with sperm penetration. Consequently, we examined the hyaluronidase sensitivity of *Ptger2*$^{-/-}$ COCs. The COCs were exposed to 0.01% hyaluronidase for 15 min, and the number of nondenuded eggs was counted. Both the WT and *Ptger2*$^{-/-}$ COCs responded to hyaluronidase treatment, but the *Ptger2*$^{-/-}$ COCs were significantly more resistant to hyaluronidase than the WT COCs; 38% of the *Ptger2*$^{-/-}$ COCs retained several layers of cumulus cells, whereas only 10% of the WT COCs were nondenuded under this condition (Fig. 3*A*). We next evaluated the total hyaluronan content in WT and *Ptger2*$^{-/-}$ COCs. No significant difference in hyaluronan content was found between the WT and *Ptger2*$^{-/-}$ COCs (data not shown). Based on these findings, we hypothesized that the organization of ECM components other than hyaluronan is altered in *Ptger2*$^{-/-}$ COCs and may cause the retention of cumulus cells around the oocyte.

Key molecules in the regulation of cell-to-ECM organization are integrins, which bind ECM components, such as fibronectin and laminin, in a divalent cation-dependent manner (23). To investigate the possible involvement of integrins in the cumulus cell adhesion, we examined the effect of EDTA on ECM resistance to hyaluronidase in *Ptger2*$^{-/-}$ COCs. We found that although EDTA itself did not induce the dissociation of cumulus cells in the absence of hyaluronidase, the addition of EDTA resulted in the complete disappearance of nondenuded COCs in the *Ptger2*$^{-/-}$ group (Fig. 3*A*), suggesting that molecules mediating divalent cation-dependent adhesion, such as integrins, are involved in the resistance to hyaluronidase in *Ptger2*$^{-/-}$ COCs. Resistance to hyaluronidase also was found in CCL7-treated WT COCs, and this effect of CCL7 was blocked by vMIP-II. The effect of CCL7 also was abolished by an integrin-inhibitor peptide, GRGDS, but not by a control peptide (Fig. 3*B*). These findings suggest that the integrins and their binding to RGD-containing ECM proteins are involved in ECM resistance to hyaluronidase found in the *Ptger2*$^{-/-}$ COCs and in WT COCs treated with CCL7. Indeed, when we stained the *Ptger2*$^{-/-}$ COCs with an anti-integrin β1 antibody in a nonpermeabilized condition, we detected strong signals on the surface of the cumulus cells. We failed to detect such strong signals on the surface of cumulus cells of WT COCs, however (Fig. 3*C*). We found similar results for the surface accumulation of integrin αv in the cumulus cells (Fig. S1). We detected no significant changes in the gene expression levels of integrin β1 (*Itgb1*) or αv (*Itgav*) between WT and *Ptger2*$^{-/-}$ cumuli (data not shown). We also found that *in vitro* CCL7 treatment stimulated the accumulation of integrins β1 and αv on the cumulus cell surface (Fig. 3*C* and data not shown). These findings demonstrate that the loss of EP2 signaling, and an input of chemokine signaling, in cumulus cells led to integrin clustering on the cell surface. Moreover, fibronectin densely accumulated in the ECM of

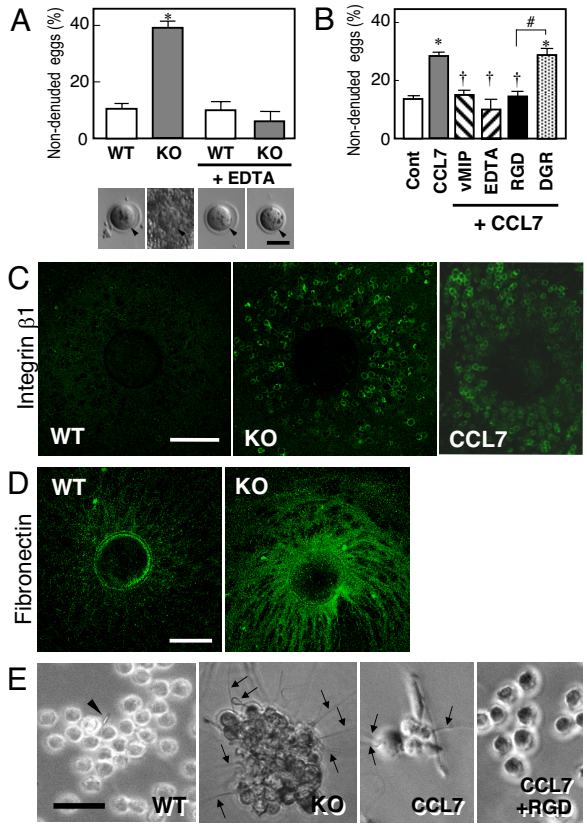

Fig. 3. *Ptger2* deficiency and CCL7 enhanced cumulus ECM assembly in an integrin-dependent manner. (*A*) The ECM of *Ptger2*$^{-/-}$ COCs showed divalent cation-dependent hyaluronidase resistance. WT and *Ptger2*$^{-/-}$ COCs (KO) were incubated with 0.01% hyaluronidase in the presence or absence of 1 mM EDTA for 15 min. The number of oocytes surrounded by the cumulus layer was counted. A typical view of an oocyte for each treatment is shown. Arrowhead, egg surface. *, $P < .01$ (vs. WT). (*B*) CCL7-induced hyaluronidase resistance in the cumulus ECM. COCs were incubated with CCL7 in combination with vMIP-II (vMIP), EDTA, an integrin inhibitor (RGD), or a control peptide (DGR) for 3 h and then subjected to treatment with 0.01% hyaluronidase. Values are presented as mean ± SEM (20–26 COCs for each well; $n = 4$). *, $P < .01$ (vs. Cont); †, $P < .01$ (vs. CCL7 only); #$P < .05$. (*C*) Surface accumulation of integrin β1 in the *Ptger2*$^{-/-}$ and CCL7-treated cumulus cells. WT, *Ptger2*$^{-/-}$ COCs (KO), or WT COCs treated with CCL7 were stained with a fluorescein isothiocyanate (FITC)-labeled anti-mouse integrin β1 antibody in a nonpermeabilized condition. (*D*) Enhanced accumulation of fibronectin in the *Ptger2*$^{-/-}$ cumulus ECM. (*E*) *Ptger2* deficiency resulted in incomplete cumulus ECM disassembly and sperm capture by cumuli during IVF. In the WT COCs, individual cumulus cells were scattered, and sperm passed between the cells (arrowhead). In the *Ptger2*$^{-/-}$ COCs (KO), cumulus cells remained in clusters, and some sperm (arrows) were captured by cumulus cells. CCL7 treatment mimicked the effect of *Ptger2* deficiency, but an integrin inhibitor (RGD) suppressed the effect of CCL7. (Scale bars: *A*, *C*, and *D*, 100 μm; *E*, 20 μm.) Representative results of three (*A* and *B*) or five (*C*–*E*) independent experiments are shown.

Ptger2$^{-/-}$ COCs, whereas low levels of fibronectin were detected in the ECM of WT COCs (Fig. 3*D*). Based on these results, we conclude that excessive chemokine signaling in the cumulus cells facilitates the surface accumulation of integrins, leading to the excessive formation and assembly of fibronectin fibrils and conferring the cumulus ECM with resistance to hyaluronidase in *Ptger2*$^{-/-}$ COCs.

To evaluate whether this integrin-mediated resistance of the ECM to hyaluronidase is sufficient to affect the disassembly of the cumulus ECM by sperm, we examined the appearance of cumuli after incubation of WT and *Ptger2*$^{-/-}$ COCs with sperm for 1 h. In the WT COCs, sperm disassembled the cumulus ECM, and the

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cumulus cells were stripped from the eggs and individually scattered apart from one another. The sperm often came in contact with the dispersed cumulus cells but were able to pass between the cumulus cells (Fig. 3*E*). In contrast, in the *Ptger2*$^{-/-}$ COCs, most of the cumulus cells were incompletely dispersed, still forming clusters, indicating resistance of the cumulus ECM to sperm hyaluronidase. In addition, the cumulus cells captured some motile sperm, with the heads of the sperm adhering to the cumulus surface. This adhesion appeared to be tight and irreversible, because captured sperm were never released (Fig. 3*E*). Prominent surface expression of integrin β1 and αv was still detected on the cell surface of such cumulus masses (data not shown). Both cumulus cluster formation (i.e., ECM resistance to hyaluronidase) and sperm capture were observed in a similar analysis of indomethacin-pretreated WT COCs and CCL7-treated WT COCs (Fig. 3*E*, Movies S1 and S2), in which surface accumulation of integrin was enhanced. An integrin-inhibitor peptide, and EDTA, reversed CCL7-induced cumulus cluster formation and sperm capture (Fig. 3*E* and data not shown), suggesting that integrins are involved not only in ECM resistance to hyaluronidase, but also in sperm capture.

Taken together, our findings highlight the two distinct roles of chemokines in fertilization; chemokine signaling strengthens the assembly of the cumulus ECM during transition into the ampulla facilitates sperm migration toward COCs. Our findings further demonstrate that if these chemokine actions persist without down-regulation, they interfere with the disintegration of the cumulus ECM by sperm, and that PGE_2-EP2 signaling suppresses this chemokine signaling and facilitates disintegration of the cumulus ECM by sperm for successful fertilization (Fig. 4).

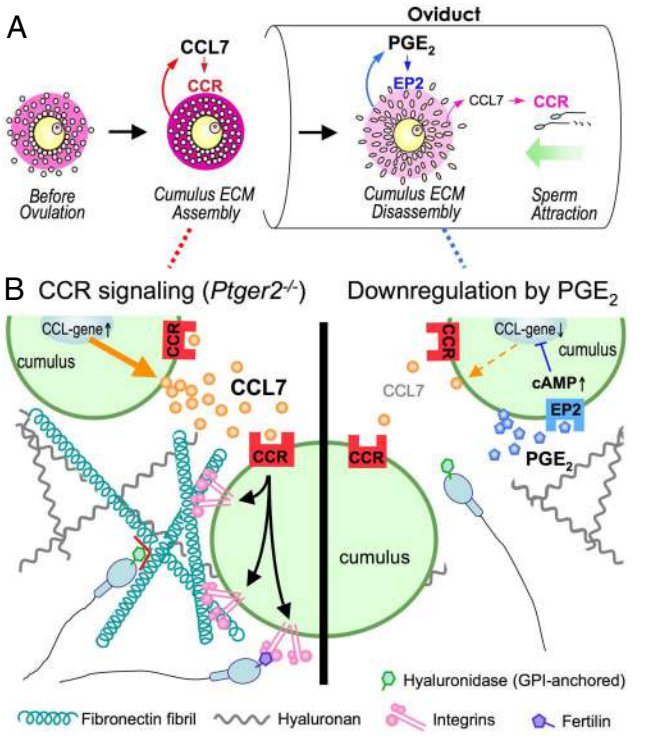

Fig. 4. Schematic model of the interactions of chemokines and PGE_2 signaling in cumulus ECM disassembly and successful fertilization. (*A*) Ovulation-associated signals induced gene expression and production of chemokines, such as CCL7, in cumulus cells. CCL7 stimulated cumulus ECM protein assembly to protect the oocyte in an autocrine manner and facilitated sperm migration to COCs. (*B*) In cumulus cells, CCL7 stimulated the surface accumulation of integrins, thereby enhancing fibronectin fibril formation (left). Once the COCs reached the fertilization site, PGE_2/EP2/cAMP signaling down-regulated these actions of chemokines on the cumuli (right); however, in *Ptger2*$^{-/-}$ cumuli (left), chemokine signaling persisted and interfered with sperm penetration because of its hyaluronidase resistance and the direct binding between integrins (cumulus) and fertilin (sperm).

Discussion

Integrin–Fibronectin Assembly in the Maintenance of ECM Status. Accumulating evidence has suggested that the orchestrated organization of the oocyte, cumulus, and ECM is essential for successful fertilization. In the cumulus ECM, hyaluronan is known to be the major component responsible for the viscoelastic properties and expansion of COCs. Recent studies have shown that several proteins, including inter-α-trypsin inhibitor, act as "nodes" to cross-link multiple hyaluronan chains, and that this hyaluronan network is essential for *in vivo* fertilization (11–13). In the present study, we have demonstrated that the chemokine/PGE_2 system regulates the assembly of a different cumulus ECM component, an integrin-mediated fibronectin assembly, and that this pathway plays an important role in the fertilization process. Fibronectin is a polymorphic adhesive glycoprotein present in biological fluids (soluble form) and the ECM (insoluble form), with its fibril formation controlled by its binding to integrins on neighboring cells (24). Fibronectin is produced by cumulus cells located in the innermost layer, the corona radiata cells, which functionally interact with the oocyte even after ovulation (25, 26). Indeed, fibronectin is present most abundantly around the innermost layer in the WT cumulus ECM (Fig. 3*D*), and the cumulus ECM, showing resistance to hyaluronidase, is the innermost layer in *Ptger2*$^{-/-}$ COCs (Fig. 3*A*). These properties indicate that fibronectin assembly is dynamic and presumably regulated by chemokine production. Once excessive fibronectin fibrils are formed by chronic exposure of cumulus cells to chemokines, these fibrils may serve as a barrier, providing resistance to hyaluronidase and disturbing sperm penetration through the cumulus ECM layer. The balance between chemokines and PGE_2 controls the cumulus surface expression of integrins, maintaining an ECM status suitable for fertilization.

Capture of Sperm by the Cumulus. Capture of sperm by the cumulus cells may be another mechanism underlying impaired fertilization in the *Ptger2*$^{-/-}$ COCs. Integrin $\alpha6\beta1$ on the oocyte has been suspected to be a potential receptor for fertilin β and cyritestin, two members of the ADAM family of proteins present on the sperm surface (27). The current study demonstrates that accumulation of integrins β1 and αv is augmented on the surface of EP2 signal–depleted cumulus cells (Fig. S1), that some motile sperm are bound to a cluster of the cumulus cells (Fig. 3*E*). Moreover, CCL7 induces sperm capture, which is reversed by an integrin inhibitor peptide and by EDTA (Fig. 3*E* and Table S2), indicating that capture of sperm by the cumulus also is due to augmented surface accumulation of integrins. Thus, sperm may be captured by cumulus cells through the binding of integrins (cumulus) and ADAM proteins (sperm). Indeed, an anti-ADAM2 antibody was found to effectively suppress sperm capture without affecting cumulus clusters (data not shown).

Stimulants for *Ccl* Gene Expression. Which signals initiate chemokine gene expression after ovulation? The COCs are exposed to widely varying circumstances after ovulation. Ovulation and follicular rupture are characterized by inflammation-like processes, such as matrix metalloproteinase expression and cytokine production (28). Gene expression of interleukin-1β (IL-1β; *Il1b*) in the ovary has been shown to increase after ovulation (29). This cytokine is known to stimulate CCL2 production in various cell types (30, 31). Recently, it was reported that IL-1β stimulates CCL2 production in human granulosa cell culture (32). Thus, IL-1β is a strong candidate molecule for the stimulation of *Ccl* gene expression in the cumulus. In this respect, it is interesting that the expression *Il1b* was increased in *Ptger2*$^{-/-}$ cumuli (Table S1), and thus synergistic interactions in the gene expression of *Ccl* and *Il1b* may exist in the cumulus cells.

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Moreover, because IL-1β also has been shown to induce the expression of *Ptgs2* and *Ptges* (PGE synthase gene) in various cells (33, 34), PGE_2 synthesis in cumulus cells may be resumed by the action of IL-1β. If this mechanism were operating, then IL-1β could determine the timely interaction between PGE_2 and CCL actions in the cumulus ECM assembly.

In summary, we have demonstrated that chemokine signaling facilitates both sperm attraction to the COC and COC compaction by the integrin-mediated cumulus ECM assembly, that PGE_2-EP2 signaling acts as a negative regulator of chemokine action to sensitize the cumulus ECM to disassembly by sperm hyaluronidase. These findings contribute to our understanding of not only the mechanism of the promotion of fertilization by PGE_2, but also the importance of chemokine signaling in the regulation of the cumulus ECM, and sperm guidance, for successful fertilization.

Materials and Methods

Detailed methods are provided in the *SI Text*. An abbreviated summary is provided here.

Animals and Hormone Treatment. Follicular growth and ovulation were stimulated in WT and *Ptger2*$^{-/-}$ mice at 3 weeks of age using the following hormonal regimen: 5 IU of pregnant mare serum gonadotropin (PMSG) was injected i.p., followed 48 h later by 5 IU of hCG injected i.p. Alternatively, WT female mice were injected subcutaneously (s.c.) with indomethacin (5 mg/kg) simultaneously with the hCG injection.

Real-Time RT-PCR and Immmunofluorescence. Cumulus cells were collected from WT or *Ptger2*$^{-/-}$ mice at 3, 9, and 14 h after hCG injection. The COCs were incubated with vehicle, PGE_2, dbcAMP, or indomethacin. Cumulus RNA from five animals (Fig. 1*A*), or from each animal (Fig. 1*B*) and COC RNA (Fig. 1*D*), was subjected to real-time RT-PCR. Primer sequences and PCR conditions are given in Table S3. The specificity of the primers was confirmed by checking the product size through gel electrophoresis and the melting temperature. Oviductal sections containing COCs were fixed and incubated with an antibody specific for CCL2. For integrin β1 detection, isolated COCs were incubated with FITC-conjugated hamster anti-rat CD29 antibody in detergent-free conditions.

IVF and Hyaluronidase Resistance. IVF was performed as described in ref. 19. Briefly, COCs were incubated with sperm in IVF medium supplemented with a physiological concentration of chemokines (100 ng/ml; 7.7 nM each) or vMIP-II (1 μg/ml). After 12 h, fertilized eggs were identified by the formation of pronuclei. COCs were incubated in 0.01% hyaluronidase for 15 min, and the number of oocytes with several cumulus layers was counted. To examine the resistance of the ECM to sperm-derived hyaluronidase, COCs were incubated with sperm for 1 h, and stripped cumulus cells were examined.

Statistical Analyses. Data are expressed as mean $\pm$ SEM. Comparison of two groups was analyzed using the Student *t* test. For comparison of more than two groups with comparable variances, one-way ANOVA was performed first, then Tukey's test was used to evaluate the pairwise group difference.

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