TGF-β1 Induces COX-2 Expression and PGE2 Production in Human Granulosa Cells Through Smad Signaling Pathways

Lanlan Fang, Hsun-Ming Chang, Jung-Chien Cheng, Peter C. K. Leung, and Ying-Pu Sun

Reproductive Medical Center (L.F., Y.-P.S.), The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China 450052; and Department of Obstetrics and Gynaecology (L.F., H.-M.C., J.-C.C., P.C.K.L.), Child and Family Research Institute, University of British Columbia, Vancouver, British Columbia, Canada V5Z 4H4

Context: Cyclooxygenase-2 (COX-2) expression and prostaglandin E2 (PGE2) production have been shown to play key roles in the regulation of ovulation. The TGF-β superfamily members are important molecules that regulate many ovarian functions under normal physiological and pathological conditions. TGF-β1 and its receptors are expressed in human granulosa cells. However, to date, whether TGF-β1 can regulate COX-2 expression and PGE2 production, which in turn contribute to the process of ovulation, remains unknown.

Objective: The objective of the study was to investigate the effects of TGF-β1 on COX-2 expression and PGE2 production in human granulosa cells.

Design: SVOG cells are human granulosa cells that were obtained from women undergoing in vitro fertilization and immortalized with Simian virus 40 large T antigen. SVOG cells were used to investigate the effect of TGF-β1 on COX-2 expression and PGE2 production.

Setting: The study was conducted at an academic research center.

Main Outcome Measures: mRNA and protein levels were examined by RT-quantitative real-time PCR and Western blotting, respectively. The concentrations of PGE2 in the culture medium were measured by an ELISA.

Results: TGF-β1 treatment induced COX-2 expression and PGE2 production. The inductive effects of TGF-β1 on COX-2 and PGE2 were abolished by the inhibition of TGF-β type I receptor (TβRI). In addition, treatment with TGF-β1 activated phosphorylated mothers against decapentaplegic (Smad)-2 and Smad3 signaling pathways. Inhibition of the Smad signaling pathways by small interfering RNA-mediated approaches attenuated the TGF-β1-induced COX-2 expression and PGE2 production.

Conclusion: TGF-β1 induced PGE2 production by inducing the COX-2 expression through a Smad-dependent signaling pathway in human granulosa cells. **(*J Clin Endocrinol Metab* 99: E1217–E1226, 2014)**

In mammals, the release of the female ovum from the ovary is fundamental to the successful establishment of pregnancy. Ovulation disorder is the most common cause of infertility in women. It has been well characterized that ovulation is triggered by an LH surge, which can regulate expression of numerous ovulation-related genes and induce multiple biochemical and biophysical changes, such as oocyte maturation and cumulus expansion (1, 2). Cy-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Received November 13, 2013. Accepted April 1, 2014.
First Published Online April 8, 2014

Abbreviations: AREG, amphiregulin; BMP, bone morphogenetic protein; 8-Br-cAMP, 8-bromoadenosine-cAMP; BTC, betacellulin; COX, cyclooxygenase; Ct, cycle threshold; EGF, epithelial growth factor; EREG, epiregulin; GDF, growth and differentiation factor; hCG, human chorionic gonadotropin; IVF, in vitro fertilization; PGE2, prostaglandin E2; RT-qPCR, RT-quantitative real-time PCR; Smad, mothers against decapentaplegic; siRNA, small interfering RNA; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor.

clooxygenase (COX) enzyme is the key enzyme that controls the rate-limiting step of prostaglandin synthesis, which has been recognized as a key mediator of ovulation (3–5). To date, two COX enzymes with similar activity have been well characterized (4). COX-1 is recognized as a constitutive enzyme that is required for various homeostatic functions, whereas COX-2 is undetectable in most tissues under normal physiological conditions and can be rapidly induced by various stimuli such as hormones, growth factors, and cytokines (4). In the ovary, prostaglandins are mainly secreted from granulosa cells. It has been shown that the LH surge rapidly induces expression of COX-2 and its derivative, prostaglandin E2 (PGE2), in granulosa cells (5, 6). Many animal studies have revealed that exogenous PGE2 treatment reverses the pharmacological inhibition of COX-2-induced ovulation failure (7–10). Moreover, gene targeting experiments in mice further confirm the role of COX-2 in ovulation (11). Mice deficient in COX-1 are fertile. In contrast, COX-2-deficient mice are mostly infertile because of severely impaired ovulation (11). Overall, these studies strongly indicate that COX-2 and its derivative PGE2 are important mediators of ovulation.

The TGF-β superfamily is composed of TGF-βs, activins/inhibins, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), and other proteins that have been shown to play important roles in the regulation of follicular development (12). It has been characterized that the spatiotemporal expression patterns of TGF-β isoforms and their receptors in the ovary, TGF-β type I (TβRI) and type II (TβRII) receptors, are species dependent (12). In humans, the expression of TGF-βs has been detected in follicular fluid that was obtained from women undergoing in vitro fertilization (IVF) (13, 14). Immunohistochemical studies on human ovarian tissue indicate that TGF-β1 is expressed in both the granulosa and theca cells, whereas TGF-β2 is only localized in the theca cells (15, 16). Moreover, both TβRI and TβRII are expressed in both human granulosa and theca cells (17). Many animal studies have shown that TGF-β1 can regulate steroidogenesis, granulosa cell proliferation, and differentiation (12, 18). However, although the expression of TGF-βs and TGF-β receptors have been identified in the human ovary, to date, only a handful of studies have investigated the functions TGF-β1 in human granulosa cells. TGF-β1 treatment of primary cultures of human granulosa cells obtained from women undergoing IVF causes up-regulation of inhibin βB subunit mRNA levels but does not affect the mRNA levels of inhibin α- and βA subunits (19). Moreover, it has been shown that TGF-β1 suppresses spontaneous apoptosis in primary cultures of human granulosa cells (20).

The induction of COX-2 expression and PGE2 production is necessary for successful ovulation. In granulosa cells, it has been shown that COX-2 can be induced by gonadotropins as well as many other factors (2). In mouse granulosa cells, two oocyte-derived TGF-β superfamily members, GDF9 and BMP15, are able to induce COX-2 expression (21, 22). Furthermore, TGF-β1 has been shown to induce COX-2 expression in many tumor cells (23, 24). However, to the best of our knowledge, the effects of TGF-β1 on human ovulation-related functions are almost completely unknown. In the current study, we aimed to examine the effects of TGF-β1 on COX-2 expression and PGE2 production in human granulosa cells. Our results indicate that treatment with TGF-β1 induces COX-2 expression and PGE2 production. The stimulatory effects of TGF-β1 on COX-2 expression and PGE2 production are abolished by the inhibition of TβRI. In addition, treatment with TGF-β1 activates the phosphorylated mothers against decapentaplegic (Smad)-2 and Smad3 signaling pathways. Moreover, knockdown of Smad2, Smad3, and Smad4 attenuates TGF-β1-induced COX-2 expression and PGE2 production.

Materials and Methods

Cell culture

A nontumorigenic immortalized human granulosa cell line (SVOG) that had been produced by Simian virus 40 large T antigen transfection of early luteal phase of human granulosa cells obtained from women undergoing IVF was used (25). SVOG cells retain steroidogenic functions such as basal and 8-bromoadenosine-cAMP (8-Br-cAMP)- or human chorionic gonadotropin (hCG)-stimulated progesterone secretion (25). Cells were grown in DMEM/F12 medium (Sigma-Aldrich) supplemented with 10% charcoal/dextran-treated fetal bovine serum (Hyclone Laboratories Inc). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Antibodies and reagents

Polyclonal anti-COX-2 antibody was obtained from Abcam. Polyclonal anti-Smad4, anti-phospho-Smad2, anti-TGF-β receptor I, monoclonal anti-Smad2, anti-phospho-Smad3, and anti-Smad3 antibodies were obtained from Cell Signaling. Polyclonal antiactin antibody was obtained from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated goat antimouse and goat antirabbit IgG were obtained from Bio-Rad Laboratories. Horseradish peroxidase-conjugated donkey antigoat IgG was obtained from Santa Cruz Biotechnology. Recombinant human TGF-β1, amphiregulin, betacellulin, and epiregulin were obtained from R&D Systems. LH, 8-Br-cAMP, and SB431542 were obtained from Sigma.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies) in accordance with the manufacturer's in-

structions. Reverse transcription was performed with 3 μg RNA, random primers, and Moloney murine leukemia virus reverse transcriptase (Promega). The primers used for SYBR Green RT-qPCR were the following: COX-2, 5′-CCC TTG GGT GTC AAA GGT AA-3′ (sense) and 5′-GCC CTC GCT TAT GAT CTG TC-3′ (antisense); TGF-βRI, 5′-GTT AAG GCC AAA TAT CCC AAA CA-3′ (sense) and 5′-ATA ATT TTA GCC ATT ACT CTC AAG G-3′ (antisense); Smad2, 5′-GCC TTT ACA GCT TCT CTG AAC AA-3′ (sense) and 5′-ATG TGG CAA TCC TTT TCG AT-3′ (antisense); Smad3, 5′-CCC CAG CAC ATA ATA ACT TGG-3′ (sense) and 5′-AGG AGA TGG AGC ACC AGA AG-3′ (antisense); Smad4, 5′-TGG CCC AGG ATC AGT AGG T-3′ (sense) and 5′-CAT CAA CAC CAA TTC CAG CA-3′ (antisense); and glyceraldehyde-3-phosphate dehydrogenase, 5′-GAG TCA ACG GAT TTG GTC GT-3′ (sense) and 5′-GAC AAG CTT CCC GTT CTC AG-3′ (antisense).

The mRNA levels of TGF-β1 were examined by TaqMan gene expression assays (Applied Biosystems). RT-qPCR was performed using the Applied Biosystems 7300 real-time PCR system equipped with a 96-well optical reaction plate. The specificity of each assay was validated by melting curve analysis and agarose gel electrophoresis of PCR products. All of the RT-qPCR experiments were run in triplicate, and a mean value was used to determine the mRNA levels. Water and mRNA without reverse transcriptase were used as negative controls. Relative quantification of the mRNA levels was performed using the comparative cycle threshold (Ct) method with glyceraldehyde-3-phosphate dehydrogenase as the reference gene and the formula $2^{-\Delta\Delta Ct}$. In RT-qPCR, to make the $\Delta\Delta$Ct calculation be valid, the amplification efficiencies of the target and reference must be approximately equal. A sensitive method for assessing if two amplicons have the same efficiency is to look at how ΔCt varies with template dilution (26). Based on the Applied Biosystems real-time PCR systems guideline (part number 4348358), the absolute value of the slope of log input amount vs ΔCt should be less than 0.1. All primers used in this study passed the validation test.

Western blotting

Cells were lysed in cell lysis buffer (Cell Signaling). Equal amounts (50 μg) of protein were separated by SDS-PAGE and transferred onto polyvinyl difluoride membranes. After 1 hour of blocking with 5% nonfat dry milk in Tris-buffered saline, the membranes were incubated overnight at 4°C with primary antibodies, which were diluted in 5% nonfat milk/Tris-buffered saline. After primary antibody incubation, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected using an enhanced chemiluminescent substrate. Membranes were stripped with stripping buffer (62.5 mM Tris; 10 mM dithiothreitol; 2% sodium dodecyl sulfate, pH 6.7) at 50°C for 30 minutes and reprobed with antiactin as a loading control.

Small interfering RNA (siRNA) transfection

To knock down endogenous TβRI, Smad2, Smad3, and Smad4, cells were transfected with 50 nM ON-TARGET*plus* SMART*pool* siRNA that targeting specific gene (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen, Life Technologies). The siCONTROL Non-Targeting *pool* siRNA (Dharmacon) was used as the transfection control. The knockdown efficiency was examined using RT-qPCR or Western blotting.

Prostaglandin E2 ELISA

Cells were cultured in a six-well plate with 2 mL medium. After treatments, the culture media and cell lysates were collected, and the PGE2 levels in the culture media were measured by an ELISA. A human PGE2-specific ELISA kit was used in accordance with the manufacturer's protocol (Cayman Chemical). PGE2 values were represented as picograms per milliliter per microgram total protein.

Statistical analysis

The results are presented as the mean ± SEM of at least three independent experiments. Multiple comparisons were analyzed first by one-way ANOVA followed by Tukey's multiple comparison tests. A significant difference was defined as $P < .05$.

Results

TGF-β1 induces COX-2 expression in human granulosa cells

SVOG cells were established previously by our group by Simian virus 40 large T antigen transfection of human granulosa cells obtained from women undergoing IVF (25). Depending on the stage of the menstrual cycle during which the human granulosa cells are collected, SVOG cells provide an ideal in vitro model for studying hormonal effects on granulosa cell-mediated ovulation-related functions in human cells. Using SVOG cells, our recent study demonstrates that treatment with epithelial growth factor (EGF)-like growth factors induces COX-2 expression and PGE2 production, which indicates the importance of EGF-like growth factors in human ovulation (27). In the current study, we continued to use this well-established human granulosa cell model to investigate the effect of TGF-β1 on COX-2 expression. To examine whether TGF-β1 can induce COX-2 expression, SVOG cells were treated with 5 ng/mL TGF-β1 at different time points. As shown in Figure 1A, treatment with TGF-β1 induced an increase in COX-2 mRNA expression, with the maximal effect observed 3 hours after TGF-β1 treatment. The induced COX-2 mRNA expression declined after 12 hours of TGF-β1 treatment. Treatment with 1 ng/mL TGF-β1 for 3 hours did not induce expression of COX-2 mRNA, whereas treatment with 5 and 10 ng/mL TGF-β1 for 3 hours exhibited comparable effects on the induction of COX-2 mRNA expression (Figure 1B). Western blotting analyses showed that COX-2 protein expression was induced after 3 and 6 hours of TGF-β1 treatment (Figure 1C). Although TGF-β1 is expressed in human granulosa cells, to the best of our knowledge, whether TGF-β1 can be regulated by LH remains unknown. Therefore, we tested whether LH treatment could up-regulate TGF-β1 expression in SVOG cells. As shown in Figure 1D, in

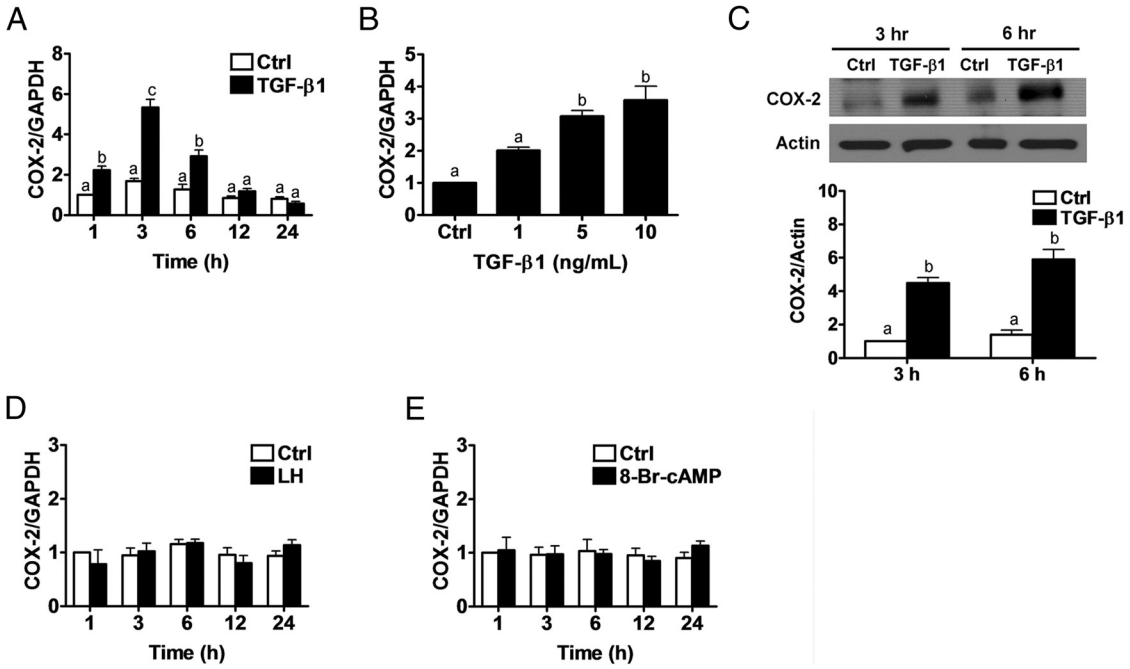

Figure 1. TGF-β1 induces COX-2 expression in SVOG cells. A, SVOG cells were treated with 5 ng/mL TGF-β1, and the mRNA levels of COX-2 were analyzed at different time points by RT-qPCR. Ctrl, control. B, SVOG cells were treated with 1, 5, and 10 ng/mL TGF-β1 for 3 hours, and the mRNA levels of COX-2 were examined by RT-qPCR. C, SVOG cells were treated with 5 ng/mL TGF-β1 for 3 and 6 hours, and the protein levels of COX-2 were examined by Western blotting. D, SVOG cells were treated with 100 ng/mL LH, and the mRNA levels of TGF-β1 were analyzed at different time points using RT-qPCR. E, SVOG cells were treated with 1 mM 8-Br-cAMP, and the mRNA levels of TGF-β1 were analyzed at different time points using RT-qPCR. The results are expressed as the means ± SEM of at least three independent experiments. Values without a common letter were significantly different ($P < .05$).

SVOG cells, treatment with 100 ng/mL LH did not affect TGF-β1 mRNA expression. Similarly, TGF-β1 mRNA expression was not affected by treatment with 1 mM 8-Br-cAMP (Figure 1E).

TGF-β receptor is required for TGF-β1-induced COX-2 expression

TGF-β1 activates downstream signaling pathways by binding to the transmembrane receptors TβRI and TβRII (28). SB431542 is a potent and specific TβRI inhibitor (29). Treatment with SB431542 abolished the TGF-β1-induced COX-2 mRNA expression (Figure 2A). Similarly, the induction expression of COX-2 protein was abolished by treatment with SB431542 (Figure 2B). To avoid off-target effects of the pharmacological inhibitor and further confirm the requirement of the TGF-β receptor in the TGF-β1-induced COX-2 expression, TβRI siRNA was used to knock down endogenous TβRI expression. As shown in Figure 2C, TβRI siRNA significantly down-regulated TβRI mRNA expression in SVOG cells. Moreover, the TGF-β1-induced COX-2 mRNA expression was abolished by the siRNA-mediated knockdown of TβRI (Figure 2C). Western blotting analyses further confirmed the blocking effect of TβRI knockdown on TGF-β1-induced COX-2 protein expression (Figure 2D).

TGF-β1 enhances AREG, BTC, and EREG-induced COX-2 expression in human granulosa cells

Our recent study has shown that treatment with EGF-like growth factors, amphiregulin (AREG), betacellulin (BTC), and epiregulin (EREG) up-regulates COX-2 expression and PGE2 production in SVOG cells (27). To examine whether TGF-β1 can affect AREG-, BTC-, and EREG-induced COX-2 expression, cells were treated with 50 ng/mL AREG, BTC, or EREG alone or in combination with 5 ng/mL TGF-β1. As shown in Figure 3A, similar to our previous study, treatment with AREG, BTC, or EREG induced COX-2 mRNA expression. Interestingly, treatment with TGF-β1 enhanced AREG-, BTC-, and EREG-induced COX-2 mRNA expression. Western blotting showed the similar results (Figure 3B).

Smad signaling is required for TGF-β1-induced COX-2 expression

Upon TGF-β1 binding, the activated TGF-β receptors induce phosphorylation of the receptor-regulated Smad proteins, Smad2 and Smad3. Phosphorylated Smad2 and Smad3 then bind to the common mediator Smad, Smad4, which translocates to the nucleus to mediate TGF-β1-regulated gene expression (30). To examine the involvement of Smad signaling in TGF-β1-induced COX-2 expression,

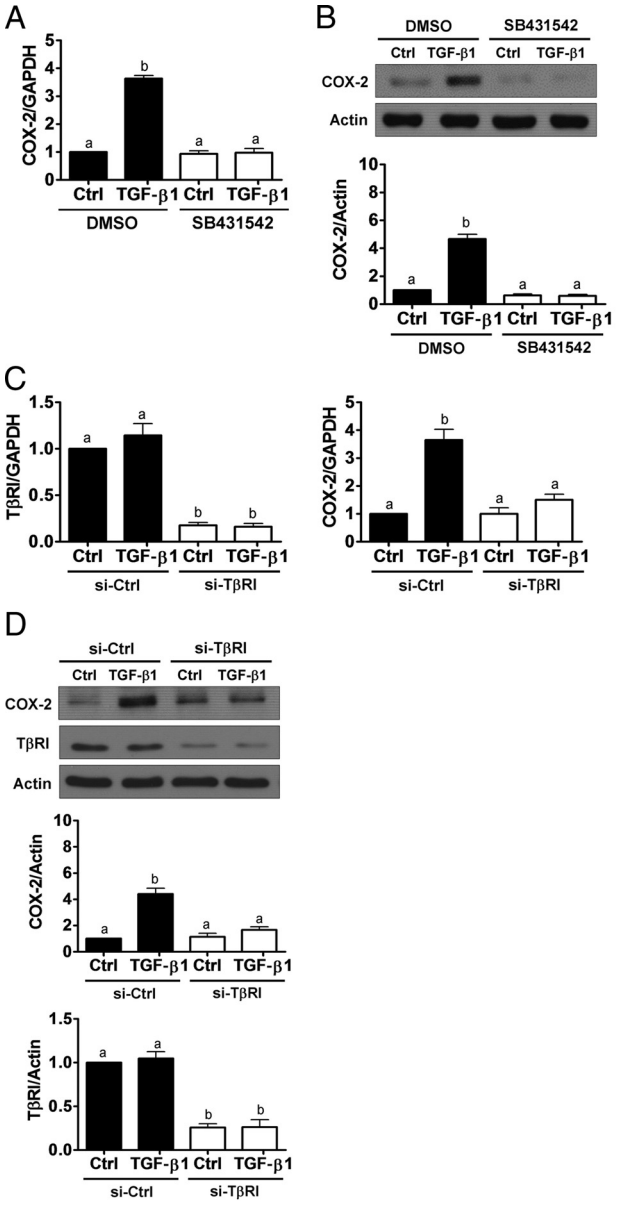

Figure 2. The TGF-β type I receptor is required for TGF-β1-induced COX-2 expression in SVOG cells. A and B, SVOG cells were treated with 5 ng/mL TGF-β1 in combination with SB431542 (10 μM) for 3 hours. The mRNA (A) and protein (B) levels of COX-2 were examined by RT-qPCR and Western blotting, respectively. Ctrl, control. C and D, SVOG cells were transfected with a 50-nM control siRNA (si-Ctrl) or a TβRI siRNA (si-TβRI) for 48 hours and then treated with 5 ng/mL TGF-β1 for 3 hours. The mRNA (C) and protein (D) levels of COX-2 and TβRI were examined by RT-qPCR and Western blotting, respectively. The results are expressed as the means ± SEM of at least three independent experiments. Values without a common letter were significantly different ($P < .05$).

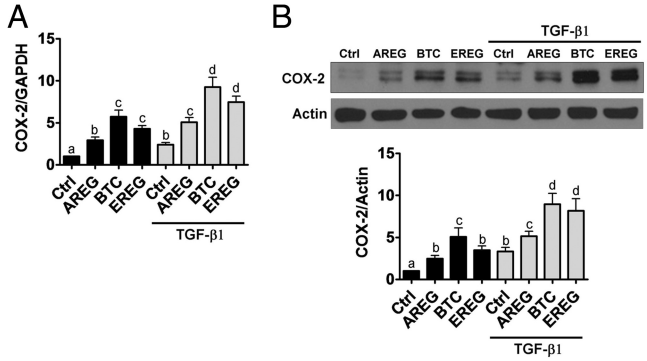

Figure 3. TGF-β1 enhances AREG-, BTC-, and EREG-induced COX-2 expression in SVOG cells. A, SVOG cells were treated with 50 ng/mL AREG, BTC, or EREG alone or in combination with 5 ng/mL TGF-β1 for 1 hour. The mRNA levels of COX-2 were examined by RT-qPCR. Ctrl, control. B, SVOG cells were treated with 50 ng/mL AREG, BTC, or EREG alone or in combination with 5 ng/mL TGF-β1 for 3 hours. The protein levels of COX-2 were examined by Western blotting. The results are expressed as the means ± SEM of at least three independent experiments. Values without a common letter were significantly different ($P < .05$).

we first tested the effect of TGF-β1 on the phosphorylation of Smad2 and Smad3 in SVOG cells. Western blotting analyses showed that treatment with 5 ng/mL TGF-β1 for 10 minutes induced significant phosphorylation of Smad2 and Smad3 (Figure 4A). A similar induction of Smad2 and Smad3 phosphorylation was observed 30 and 60 minutes after TGF-β1 treatment (Figure 4A). To examine the requirement of Smad signaling for TGF-β1-induced COX-2 expression, the siRNA knockdown approach was used to knock down Smad4. As shown in Figure 4B, Smad4 siRNA significantly knocked down the endogenous Smad4 mRNA expression. In addition, the TGF-β1-induced COX-2 mRNA expression was attenuated by the knockdown of Smad4 (Figure 4B). Similarly, Western blotting analyses showed that the knockdown of Smad4 attenuated TGF-β1-induced COX-2 protein expression in SVOG cells (Figure 4C).

Smad2 and Smad3 signaling pathways are required for TGF-β1-induced COX-2 expression

To further examine the involvement of Smad2 and Smad3 in TGF-β1-induced COX-2 expression, Smad2 and Smad3 were knocked down by using specific siRNAs. As shown in Figure 5A, the knockdown of Smad2 or Smad3 alone attenuated TGF-β1-induced COX-2 mRNA expression. In addition, knockdown of Smad2 or Smad3 alone could attenuate TGF-β1-induced COX-2 protein expression (Figure 5B). Knockdown of both Smad2 and Smad3 exhibited more attenuation in TGF-β1-induced expression of COX-2 mRNA and protein than knockdown of Smad2 or Smad3 alone (Figure 5, C and D). These results indicated that Smad2 and Smad3 were both required for TGF-β1-induced COX-2 expression and suggested that Smad2 and Smad3 acted differentially and independently in mediating TGF-β1-induced COX-2 expression in human granulosa cells.

Smad signaling is required for TGF-β1-induced PGE2 production

PGE2 production has been shown to play a key role in the regulation of ovulation (7, 31). Thus, it is important to

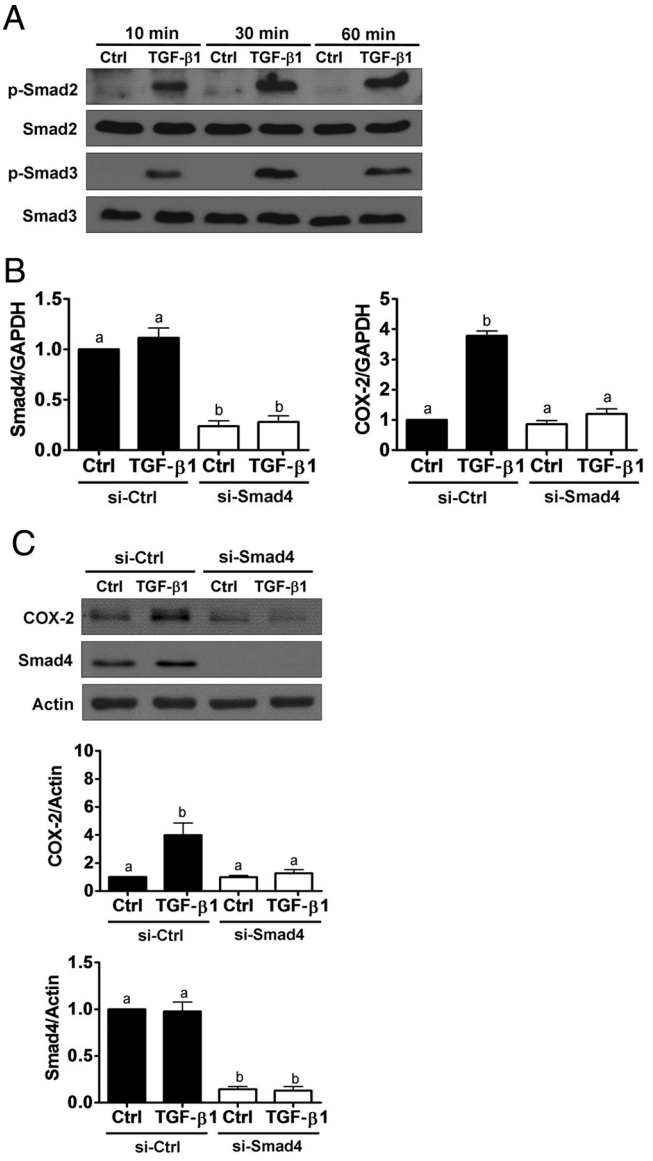

Figure 4. Smad signaling pathways are required for TGF-β1-induced COX-2 expression in SVOG cells. A, SVOG cells were treated with 5 ng/mL TGF-β1 for 10, 30, and 60 minutes. Phosphorylation of Smad2 and Smad3 were determined by Western blotting using specific antibodies for phosphorylated (activated) forms of Smad2 (p-Smad2) and Smad3 (p-Smad3). Membranes were stripped and reprobed with antibodies to total Smad2 and Smad3. Ctrl, control. B and C, SVOG cells were transfected with a 50 nM control siRNA (si-Ctrl) or a Smad4 siRNA (si-Smad4) for 48 hours and then treated with 5 ng/mL TGF-β1 for 3 hours. The mRNA (B) and protein (C) levels of COX-2 and Smad4 were examined by RT-qPCR and Western blotting, respectively. The results are expressed as the means ± SEM of at least three independent experiments. Values without a common letter were significantly different ($P < .05$).

confirm whether TGF-β1-induced COX-2 expression can contribute to the PGE2 production in SVOG cells. To examine this, SVOG cells were treated with 5 ng/mL TGF-β1 for 3 and 6 hours, and the levels of PGE2 in the culture medium were examined. As shown in Figure 6A, treatment with TGF-β1 for 3 and 6 hours induced PGE2 production. In addition, treatment with TGF-β1 enhanced AREG-, BTC-, and EREG-induced PGE2 production (Figure 6B). Moreover, treatment with SB431542 abolished TGF-β1-induced PGE2 production (Figure 6C). Knockdown of Smad2 or Smad3 attenuated TGF-β1-induced PGE2 production in SVOG cells (Figure 6D). To further confirm the COX-2 is required for TGF-β1-induced PGE2 production, COX-2 siRNA was used to knock down the endogenous COX-2. As shown in Figure 6E, COX-2 siRNA not only down-regulated the basal levels of COX-2 but also abolished the TGF-β1-induced COX-2 protein expression. In addition, TGF-β1-induced PGE2 productions were attenuated by the knockdown of COX-2 (Figure 6F). Taken together, these results indicated that Smad signaling is required for TGF-β1-induced PGE2 production in human granulosa cells.

Discussion

Although it has been well characterized that TGF-β superfamily members are expressed in the ovary and regulate many important ovarian functions, the direct involvement of TGF-β superfamily members in the regulation of ovulation itself in animals and humans remains largely unknown (12). Studies in a variety of animal species have demonstrated the key roles of COX-2 and its major derivative PGE2 in the regulation of ovulation (5). However, unlike animal studies, a model for studying the regulation of ovulation in humans is unavailable. In the present study, we used cultured immortalized human granulosa cells to examine the effect of TGF-β1 on COX-2 expression and PGE2 production. Our results demonstrated that TGF-β1 treatment induced COX-2 expression and PGE2 production. Treatment with TGF-β1 activated Smad2 and Smad3 signaling pathways. Moreover, by using siRNA-mediated knockdown approaches, our results showed that the activation of Smad2 and Smad3 signaling pathways was required for TGF-β1-induced COX-2 expression and PGE2 production. These results strongly implicate that TGF-β1 may act as an important mediator in the regulation of human ovulation.

Given the increasing evidence that there is cross talk between the different endocrine and intraovarian signaling systems, it is apparent that all aspects of ovarian function, from steroidogenesis to follicle development, ovulation, corpus luteum formation, and regression, are regulated by multifactorial mechanisms. Our recent study demonstrates that treatment with EGF-like growth factors, AREG, BTC, and EREG, induce COX-2 expression and PGE2 production in human granulosa cells (27). In the present study, our results showed that treatment with TGF-β1 enhanced AREG-, BTC-, and EREG-induced

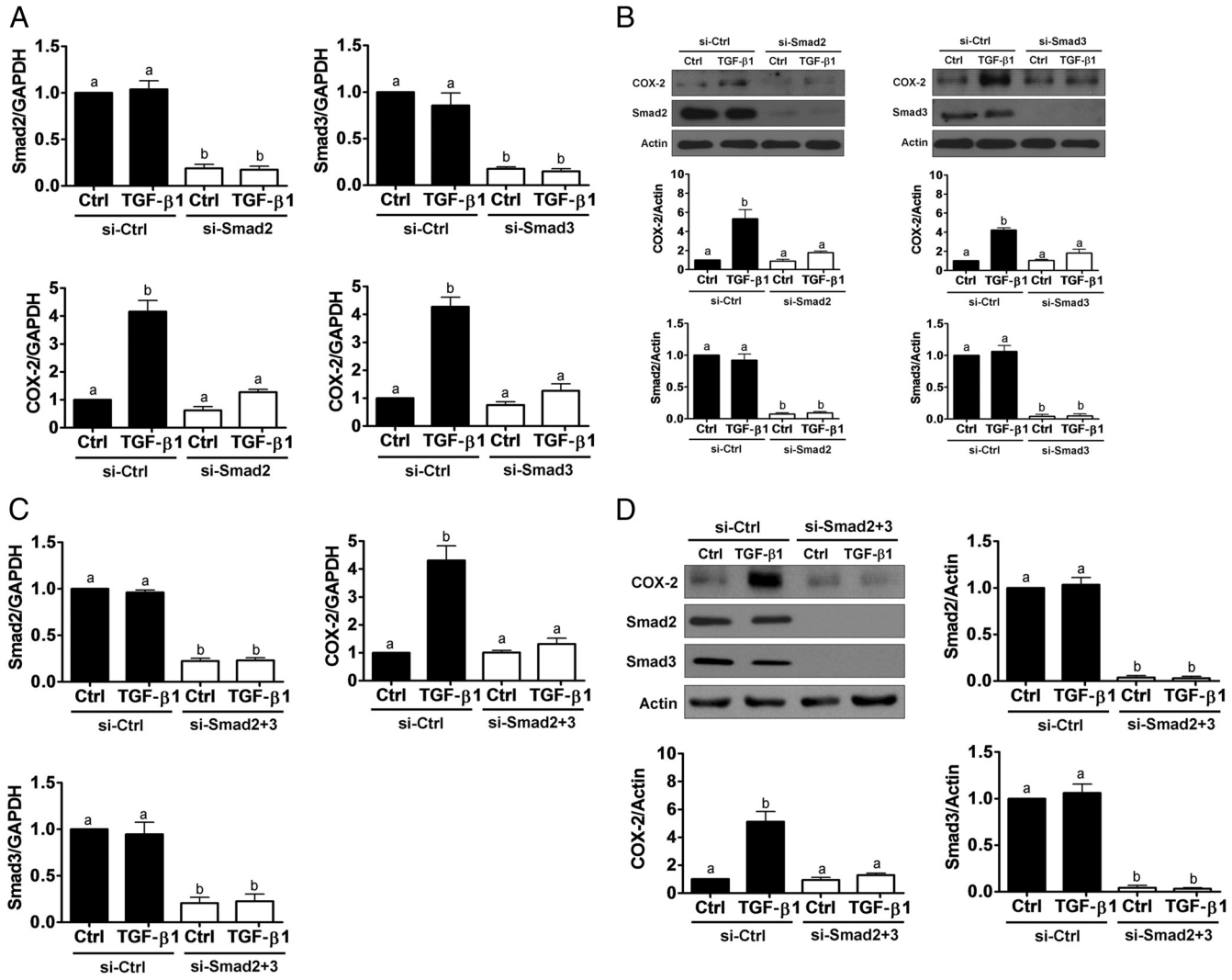

Figure 5. Smad2 and Smad3 are required for TGF-β1-induced COX-2 expression in SVOG cells. A and B, SVOG cells were transfected with a 50-nM control siRNA (si-Ctrl), a Smad2 siRNA (si-Smad2), or a Smad3 siRNA (si-Smad3) for 48 hours and then treated with 5 ng/mL TGF-β1 for 3 hours. The mRNA levels (A) and protein levels (B) of COX-2, Smad2, and Smad3 were examined by RT-qPCR and Western blotting, respectively. Ctrl, control. C and D, SVOG cells were transfected with a 50-nM control siRNA (si-Ctrl) or Smad2 plus Smad3 siRNA (si-Smad2+3) for 48 hours and then treated with 5 ng/mL TGF-β1 for 3 hours. The mRNA levels (C) and protein levels (D) of COX-2, Smad2, and Smad3 were examined by RT-qPCR and Western blotting, respectively. The results are expressed as the means ± SEM of at least three independent experiments. Values without a common letter were significantly different ($P < .05$).

COX-2 expression and PGE2 production. Taken together, our studies indicate that cooperation between the EGF-like growth factor and TGF-β1 may be an important mechanism that regulates ovulation.

Immunohistochemical studies in the mouse ovary have demonstrated that TGF-β1 can be detected in granulosa and theca cells after treatment with pregnant mare serum gonadotrophin plus hCG (32). The effects of TGF-β1 on ovulation-related functions have been reported by a few animal studies. In the rat, TGF-β1 stimulates oocyte maturation in follicle-enclosed oocytes and cumulus-oocyte complexes but not in denuded oocytes (33). In mouse granulosa cells, TGF-β1 induces the expression of hyaluronic acid, which has been shown to be involved in cumulus expansion (34). Intrabursal administration of TGF-β1 inhibits follicle rupture in gonadotropin-primed mice (35). Overall, these studies indicate that TGF-β1 may be involved in the regulation of ovulation through autocrine/paracrine signaling. In granulosa cells, COX-2 can be regulated by many hormones, growth factors, and cytokines (5). Treatment with hCG to mimic the LH surge induces follicular COX-2 expression prior to ovulation (36). In addition, GnRH has been shown to induce COX-2 expression in rat granulosa cells of preovulatory follicles (37). Moreover, IL-1 is able to induce COX-2 expression in both cultured rat and human granulosa cells (38, 39). In hen granulosa cells, COX-2 expression and prostaglandin production are induced by TGF-α but suppressed by TGF-β1 (40). Interestingly, two oocyte-derived TGF-β superfamily members, GDF9 and BMP15, have been shown to induce COX-2 ex-

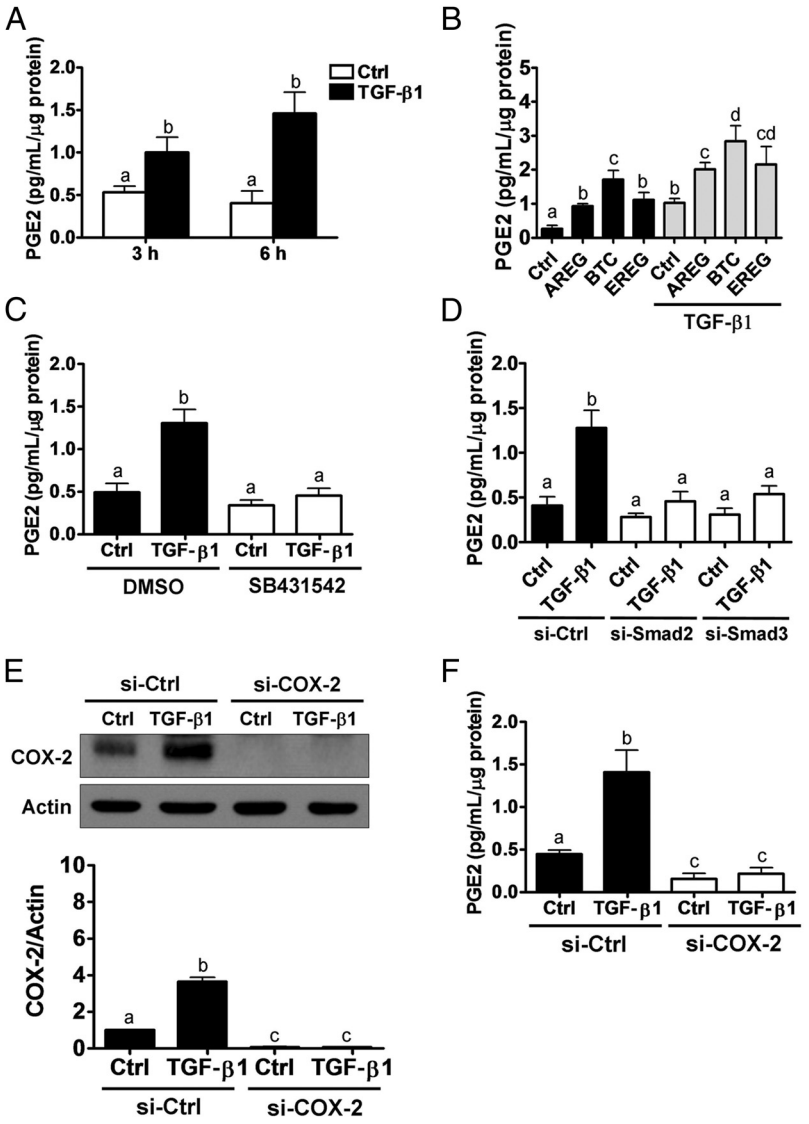

Figure 6. TGF-β1 induces PGE2 production in SVOG cells. A–D, SVOG cells were treated with 5 ng/mL TGF-β1 for 3 and 6 hours (A). SVOG cells were treated with 50 ng/mL AREG, BTC, or EREG alone or in combination with 5 ng/mL TGF-β1 for 3 hours (B). SVOG cells were treated with 5 ng/mL TGF-β1 in combination with SB431542 (10 μM) for 6 hours (C). SVOG cells were transfected with a 50-nM control siRNA (si-Ctrl), a Smad2 siRNA (si-Smad2), or a Smad3 siRNA (si-Smad3) for 48 hours and then treated with 5 ng/mL TGF-β1 for 6 hours (D). The levels of PGE2 in culture media were examined by ELISA. Ctrl, control. E, SVOG cells were transfected with a 50-nM control siRNA (si-Ctrl) or a COX-2 siRNA (si-COX-2) for 48 hours and then treated with 5 ng/mL TGF-β1 for 3 hours. The protein levels of COX-2 were examined by Western blotting. F, SVOG cells were transfected with a 50-nM control siRNA (si-Ctrl) or a COX-2 siRNA (si-COX-2) for 48 hours and then treated with 5 ng/mL TGF-β1 for 6 hours. The levels of PGE2 in culture media were examined by ELISA. The results are expressed as the means ± SEM of at least three independent experiments. Values without a common letter were significantly different ($P < .05$).

pression in mouse granulosa cells (21, 22). Given the importance of COX-2 and PGE2 in ovulation, we aimed to investigate the involvement of TGF-β1 on human ovulation by examining its effect on COX-2 expression and PGE2 production. Our results showed that TGF-β1 treatment induced COX-2 expression and PGE2 production in cultured human granulosa cells. These results indicate that TGF-β1 may play an important role in the regulation of human ovulation. Moreover, the results from our work and previous studies indicate that the effect of TGF-β1 on COX-2 expression is species dependent.

Smads are well-defined signaling molecules that mediate functions induced by TGF-β superfamily members (28). It has been shown that the specific knockout of Smad4 in the mouse ovary leads to multiple defects in ovulation (41, 42). These animal studies indicate the important roles of Smad signaling pathways and the involvement of the TGF-β superfamily members in the regulation of ovulation. In the present study, our results showed that knockdown of Smad4 attenuated TGF-β1-induced COX-2 expression in human granulosa cells. These results demonstrated that Smad signaling pathways were required for TGF-β1-induced COX-2 expression and further confirmed the key roles of Smad signaling pathways in the regulation of ovulation-related functions. In granulosa cells, Smad2 and Smad3 have been shown to function redundantly (43). The knockdown of Smad2 and Smad3 attenuated TGF-β1-induced COX-2 expression and PGE2 production. These results indicated that Smad2 and Smad3 both mediated TGF-β1-induced COX-2 expression and PGE2 production in human granulosa cells.

In addition to the canonical pathways that TGF-β superfamily members activate, Smad-dependent signaling pathways, several non-Smad signaling cascades such as ERK1/2 and phosphatidylinositol 3-kinase/Akt can also be activated by TGF-β superfamily members (44). It has been shown that ovulation failure can be observed in *Erk1/2*-deficient mice (45). In human granulosa cells, inhibition of ERK1/2 signaling abolishes hCG-induced COX-2 expression (46). EGF-like growth factors induce COX-2 expression and PGE2 production by activating the ERK1/2 signaling pathway in human granulosa cells (27). In the current study, it should be noted that the inhibition of Smad signaling did not totally

abolish TGF-β1-induced COX-2 expression and PGE2 production. Thus, future studies are required to examine the involvement of the non-Smad signaling pathways in TGF-β1-induced COX-2 expression and PGE2 production in human granulosa cells.

In summary, our study demonstrated that TGF-β1 induced COX-2 expression and PGE2 production in cultured human granulosa cells. Treatment with TGF-β1 activated Smad2 and Smad3 signaling pathways. Moreover, the inhibition of Smad signaling pathways attenuated TGF-β1-induced COX-2 expression and PGE2 production. These results indicate that TGF-β1 plays important roles in the regulation of human ovulation and that it may act through the induction of COX-2 and PGE2. Our results provide important insights into the molecular mechanisms that mediate TGF-β1-regulated ovulation functions in humans.

Acknowledgments

L.F. is the recipient of a scholarship from the China Scholarship Council.

Address all correspondence and requests for reprints to: Peter C. K. Leung, PhD, FCAHS, FRSC, Department of Obstetrics and Gynaecology, Child and Family Research Institute, University of British Columbia, Room 317, 950 West 28th Avenue, Vancouver, British Columbia, Canada V5Z 4H4. E-mail: peter.leung@ubc.ca; or Ying-Pu Sun, MD, PhD, Center for Reproductive Medicine, The First Affiliated Hospital of Zhengzhou University, 40 Daxue Road, Zhengzhou, Henan, China 450052. E-mail: syp2008@vip.sina.com.

This work was supported by an operating grant from the Canadian Institutes of Health Research (to P.C.K.L.) and National Natural Science Foundation of China Grant 31271605 (to Y.P.S.).

Disclosure Summary: The authors have nothing to disclose.

References

1. **Richards JS, Russell DL, Ochsner S, Espey LL.** Ovulation: new dimensions and new regulators of the inflammatory-like response. *Annu Rev Physiol*. 2002;64:69–92.
2. **Russell DL, Robker RL.** Molecular mechanisms of ovulation: coordination through the cumulus complex. *Hum Reprod Update*. 2007;13:289–312.
3. **Armstrong DT, Grinwich DL.** Blockade of spontaneous and LH-induced ovulation in rats by indomethacin, an inhibitor of prostaglandin biosynthesis. I. *Prostaglandins*. 1972;1:21–28.
4. **Smith WL, DeWitt DL, Garavito RM.** Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem*. 2000;69:145–182.
5. **Sirois J, Sayasith K, Brown KA, Stock AE, Bouchard N, Dore M.** Cyclooxygenase-2 and its role in ovulation: a 2004 account. *Hum Reprod Update*. 2004;10:373–385.
6. **Duffy DM, Stouffer RL.** The ovulatory gonadotrophin surge stimulates cyclooxygenase expression and prostaglandin production by the monkey follicle. *Mol Hum Reprod*. 2001;7:731–739.
7. **Duffy DM, Stouffer RL.** Follicular administration of a cyclooxygenase inhibitor can prevent oocyte release without alteration of normal luteal function in rhesus monkeys. *Hum Reprod*. 2002;17:2825–2831.
8. **Hester KE, Harper MJ, Duffy DM.** Oral administration of the cyclooxygenase-2 (COX-2) inhibitor meloxicam blocks ovulation in non-human primates when administered to simulate emergency contraception. *Hum Reprod*. 2010;25:360–367.
9. **Mikuni M, Pall M, Peterson CM, et al.** The selective prostaglandin endoperoxide synthase-2 inhibitor, NS-398, reduces prostaglandin production and ovulation in vivo and in vitro in the rat. *Biol Reprod*. 1998;59:1077–1083.
10. **Sogn JH, Curry TE Jr, Brannstrom M, et al.** Inhibition of follicle-stimulating hormone-induced ovulation by indomethacin in the perfused rat ovary. *Biol Reprod*. 1987;36:536–542.
11. **Lim H, Paria BC, Das SK, et al.** Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell*. 1997;91:197–208.
12. **Knight PG, Glister C.** TGF-β superfamily members and ovarian follicle development. *Reproduction*. 2006;132:191–206.
13. **Fried G, Wramsby H.** Increase in transforming growth factor β1 in ovarian follicular fluid following ovarian stimulation and in vitro fertilization correlates to pregnancy. *Hum Reprod*. 1998;13:656–659.
14. **Fried G, Wramsby H, Tally M.** Transforming growth factor-β1, insulin-like growth factors, and insulin-like growth factor binding proteins in ovarian follicular fluid are differentially regulated by the type of ovarian hyperstimulation used for in vitro fertilization. *Fertil Steril*. 1998;70:129–134.
15. **Chegini N, Flanders KC.** Presence of transforming growth factor-β and their selective cellular localization in human ovarian tissue of various reproductive stages. *Endocrinology*. 1992;130:1707–1715.
16. **Chegini N, Williams RS.** Immunocytochemical localization of transforming growth factors (TGFs) TGF-α and TGF-β in human ovarian tissues. *J Clin Endocrinol Metab*. 1992;74:973–980.
17. **Roy SK, Kole AR.** Ovarian transforming growth factor-β (TGF-β) receptors: in vitro effects of follicle stimulating hormone, epidermal growth factor and TGF-β on receptor expression in human preantral follicles. *Mol Hum Reprod*. 1998;4:207–214.
18. **Knight PG, Glister C.** Local roles of TGF-β superfamily members in the control of ovarian follicle development. *Anim Reprod Sci*. 2003;78:165–183.
19. **Eramaa M, Ritvos O.** Transforming growth factor-β1 and -β2 induce inhibin and activin βB-subunit messenger ribonucleic acid levels in cultured human granulosa-luteal cells. *Fertil Steril*. 1996;65:954–960.
20. **Matsubara H, Ikuta K, Ozaki Y, et al.** Gonadotropins and cytokines affect luteal function through control of apoptosis in human luteinized granulosa cells. *J Clin Endocrinol Metab*. 2000;85:1620–1626.
21. **Elvin JA, Clark AT, Wang P, Wolfman NM, Matzuk MM.** Paracrine actions of growth differentiation factor-9 in the mammalian ovary. *Mol Endocrinol*. 1999;13:1035–1048.
22. **Yoshino O, McMahon HE, Sharma S, Shimasaki S.** A unique preovulatory expression pattern plays a key role in the physiological functions of BMP-15 in the mouse. *Proc Natl Acad Sci USA*. 2006;103:10678–10683.
23. **Fosslien E.** Biochemistry of cyclooxygenase (COX)-2 inhibitors and molecular pathology of COX-2 in neoplasia. *Crit Rev Clin Lab Sci*. 2000;37:431–502.
24. **Fosslien E.** Review: molecular pathology of cyclooxygenase-2 in cancer-induced angiogenesis. *Ann Clin Lab Sci*. 2001;31:325–348.
25. **Lie BL, Leung E, Leung PC, Auersperg N.** Long-term growth and steroidogenic potential of human granulosa-lutein cells immortalized with SV40 large T antigen. *Mol Cell Endocrinol*. 1996;120:169–176.
26. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data

using real-time quantitative PCR and the 2[-δ δ C(T)] method. *Methods*. 2001;25:402–408.
27. **Fang L, Cheng JC, Chang HM, Sun YP, Leung PC.** EGF-like growth factors induce COX-2-derived PGE2 production through ERK1/2 in human granulosa cells. *J Clin Endocrinol Metab*. 2013;98:4932–4941.
28. **Shi Y, Massague J.** Mechanisms of TGF-β signaling from cell membrane to the nucleus. *Cell*. 2003;113:685–700.
29. **Inman GJ, Nicolas FJ, Callahan JF, et al.** SB-431542 is a potent and specific inhibitor of transforming growth factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol*. 2002;62:65–74.
30. **Heldin CH, Miyazono K, ten Dijke P.** TGF-β signalling from cell membrane to nucleus through SMAD proteins. *Nature*. 1997;390:465–471.
31. **Davis BJ, Lennard DE, Lee CA, et al.** Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1β. *Endocrinology*. 1999;140:2685–2695.
32. **Ghiglieri C, Khatchadourian C, Tabone E, Hendrick JC, Benahmed M, Menezo Y.** Immunolocalization of transforming growth factor-β1 and transforming growth factor-β2 in the mouse ovary during gonadotrophin-induced follicular maturation. *Hum Reprod*. 1995;10:2115–2119.
33. **Feng P, Catt KJ, Knecht M.** Transforming growth factor-β stimulates meiotic maturation of the rat oocyte. *Endocrinology*. 1988;122:181–186.
34. **Salustri A, Ulisse S, Yanagishita M, Hascall VC.** Hyaluronic acid synthesis by mural granulosa cells and cumulus cells in vitro is selectively stimulated by a factor produced by oocytes and by transforming growth factor-β. *J Biol Chem*. 1990;265:19517–19523.
35. **Juneja SC, Chegini N, Williams RS, Ksander GA.** Ovarian intrabursal administration of transforming growth factor β1 inhibits follicle rupture in gonadotropin-primed mice. *Biol Reprod*. 1996;55:1444–1451.
36. **Huslig RL, Malik A, Clark MR.** Human chorionic gonadotropin stimulation of immunoreactive prostaglandin synthase in the rat ovary. *Mol Cell Endocrinol*. 1987;50:237–246.
37. **Wong WY, Richards JS.** Induction of prostaglandin H synthase in rat preovulatory follicles by gonadotropin-releasing hormone. *Endocrinology*. 1992;130:3512–3521.
38. **Narko K, Ritvos O, Ristimaki A.** Induction of cyclooxygenase-2 and prostaglandin F2α receptor expression by interleukin-1β in cultured human granulosa-luteal cells. *Endocrinology*. 1997;138:3638–3644.
39. **Ando M, Kokia E, Ruutiainen-Altman K, et al.** Rat ovarian prostaglandin endoperoxide synthase-1 and -2: periovulatory expression of granulosa cell-based interleukin-1-dependent enzymes. *Endocrinology*. 1998;139:2501–2508.
40. **Li J, Simmons DL, Tsang BK.** Regulation of hen granulosa cell prostaglandin production by transforming growth factors during follicular development: involvement of cyclooxygenase II. *Endocrinology*. 1996;137:2522–2529.
41. **Yu C, Zhang YL, Fan HY.** Selective Smad4 knockout in ovarian preovulatory follicles results in multiple defects in ovulation. *Mol Endocrinol*. 2013;27:966–978.
42. **Pangas SA, Li X, Robertson EJ, Matzuk MM.** Premature luteinization and cumulus cell defects in ovarian-specific Smad4 knockout mice. *Mol Endocrinol*. 2006;20:1406–1422.
43. **Li Q, Pangas SA, Jorgez CJ, Graff JM, Weinstein M, Matzuk MM.** Redundant roles of SMAD2 and SMAD3 in ovarian granulosa cells in vivo. *Mol Cell Biol*. 2008;28:7001–7011.
44. **Wakefield LM, Roberts AB.** TGF-β signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev*. 2002;12:22–29.
45. **Fan HY, Liu Z, Shimada M, et al.** MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. *Science*. 2009;324:938–941.
46. **Tsai EM, Chan TF, Chen YH, Hsu SC, Chuang CY, Lee JN.** Mifepristone attenuates human chorionic gonadotropin-induced extracellular signal-regulated kinase 1/2 phosphorylation, cyclooxygenase-2, and prostaglandin E2 production in human granulosa luteal cells. *Fertil Steril*. 2008;89:1522–1529.