Activin A upregulates PTGS2 expression and increases PGE2 production in human granulosa-lutein cells

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Abstract

Activin A is one of the members of transforming growth factor- β superfamily that is expressed in human large luteal cells, and may act in an autocrine/paracrine manner to regulate luteal function. Prostaglandin-endoperoxide synthase 2 (PTGS2) enzyme and its derivative, prostaglandin E2 (PGE2), play significant roles in the regulation of corpus luteum formation and maintenance. To date, whether activin A can induce the expression of PTGS2 and the production of PGE2 in human granulosa-lutein cells is largely unknown. The aim of this study was to examine the effects of activin A on the regulation of PTGS2 expression and PGE2 production in human granulosa-lutein cells, and to investigate the underlying signal transduction mechanisms. In this study, the immortalized (SVOG cells) and primary human granulosa-lutein cells were used as the cell models. A TGF- β /activin type I receptor inhibitor, SB431542 and small interfering RNAs were used to investigate the activin A-induced downstream signaling pathway. We have demonstrated that activin A upregulated the expression of PTGS2 and increased the production of PGE2 via an ACVR1B-mediated SMAD2/3–SMAD4 signaling pathway. Our results suggest that activin A may be involved in the modulation of human corpus luteum formation via the induction of PTGS2 expression and PGE2 production.

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Introduction

After ovulation, the corpus luteum that is developed from the granulosa and theca cells of the residual follicle plays an essential role in the establishment of embryo implantation and pregnancy (Niswender *et al.* 2000). In most mammals, the cyclic change of corpus luteum is featured by luteal development, maintenance and regression modulated by the interactions of several luteotrophic and luteolytic mediators (Arosh *et al.* 2004, Devoto *et al.* 2009). Among these mediators, prostaglandin (PG) E2 is considered as a luteotropin, whereas PGE2 α is a luteolysin (Horton & Poyser 1976). In a variety of species, PGE2 has been shown to act as a luteoprotective or luteotropic factor during the luteal phase (Magness *et al.* 1981). In ruminants, administration of PGE2 protects the corpus luteum function from induced or spontaneous luteolysis (Henderson *et al.* 1977). In domestic animals, exogenous PGE2 prevents spontaneous or induced luteolysis and maintains luteal function during early pregnancy (Pate 2003, Weems *et al.* 2013, 2014). Similar anti-luteolytic or luteotropic effects of PGE2 have been shown in primates (Zelinski-Wooten & Stouffer 1990, Bogan *et al.* 2008). In humans, PGE2 can stimulate the cAMP-mediated progesterone

secretion in the luteal phase (Marsh & LeMaire 1974, Hahlin *et al.* 1988, Wang *et al.* 2012). In ewes, administration of PGE2 antagonist indomethacin inhibits luteal PGE2 production and progesterone secretion (Kim *et al.* 2001). Furthermore, there is a positive correlation between the expression of PGE2 and progesterone in the human luteal tissue (Vijayakumar & Walters 1983). A clinical study has confirmed the contraceptive effect of a prostaglandin inhibitor due to its disruption on the luteal progesterone levels (Edelman *et al.* 2010). Collectively, all these studies indicate a critical role of PGE2 in the development and maintenance of a normal corpus luteum.

Prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase (COX), is the key enzyme responsible for the rate-limiting step of prostaglandin biosynthesis that has been recognized as the primary mediator of luteal function (Arosh *et al.* 2004). PTGS1 (COX-1) and PTGS2 (COX-2) are well-characterized PTGS enzymes that have similar activities (Sirois *et al.* 2004). PTGS1 is constitutively expressed in most cells and is involved in the production of prostaglandins in relation to various homeostatic functions. Under normal physiological conditions, PTGS2 is undetectable in most

tissues but can be induced by various inflammatory, carcinogenic and pathological processes (Stouffer *et al.* 2007, Liu *et al.* 2015). Studies have shown that the PG components, including PTGS1, PTGS2, PGE2 and PGE2 signaling (PGE2 receptor 3) are highly expressed in the large luteal cells of primate corpus luteum, indicating that the PGE2 biosynthesis and bioactivity are selectively activated during the luteal development and maintenance (Arosh *et al.* 2004, Bogan *et al.* 2008). Notably, gene targeting studies have shown that PTGS2 is one of the important mediators of corpus luteum formation, implantation, decidualization and early pregnancy (Lim *et al.* 1999, Reese *et al.* 2001).

Activins belong to the transforming growth factor- β (TGF- β) superfamily and are widely expressed in many tissues, where they regulate a variety of physiological processes, including reproduction (Knight & Glistler 2001, 2006). In the ovaries, activin A is expressed in granulosa cells and may act in an autocrine/paracrine manner to regulate ovarian function, including steroidogenesis, granulosa cell proliferation, follicular development, oocyte maturation and corpus luteum function (Findlay 1993, Knight & Glistler 2001). In rodents, the LH surge significantly downregulates the expression of INHA (inhibin α) and INHBA (inhibin β A, the precursor monomer of active A) which leads to little activin or inhibin production or secretion by the corpus luteum (Woodruff *et al.* 1988). However, in some ruminants and primate ovaries, inhibin β A, activin receptors and its binding protein follistatin are all expressed in the corpus luteum (Fraser *et al.* 1993, Silva *et al.* 2004). In the human corpus luteum, inhibin β A and follistatin mRNA and protein are expressed in the luteinized granulosa cells (large luteal cells), but not in the luteinized theca cells (small luteal cells), indicating a potential role of activin–follistatin system in the regulation of human luteal activity (Eramaa *et al.* 1993, Roberts *et al.* 1993). In view of the critical roles of PTGS2 and PGE2 in the corpus luteum formation and the subsequent implantation and early pregnancy, the regulation of PTGS2 expression and PGE2 production has been an area of interest for research. It has been reported that TGF- β 1 and nitric oxide are able to induce the expression of PTGS2 and the synthesis of PGE2 in human granulosa-lutein cells (Fang *et al.* 2014, 2015). To date, whether activin A can induce the expression of PTGS2 and PGE2 production in human granulosa-lutein cells remains to be elucidated. Given that activin A, PTGS2 and PGE2 are all essential factors for corpus luteum function, we hypothesized that activin A may regulate the expression of PTGS2 and the production of PGE2 in human granulosa-lutein cells to support the normal luteal function in the human ovary.

Materials and methods

Preparation and culture of primary human granulosa-lutein (hGL) cells

Primary hGL cells were obtained from patients who had provided informed consent after approval from the University of British Columbia Research Ethics Board. The controlled ovarian stimulation protocol for IVF patients consisted of the downregulation of either the luteal phase with nafarelin acetate (Synarel, Pfizer) or of the follicular phase with a GnRH antagonist (Ganirelix; Merck, Frosst). Gonadotropin stimulation with human menopausal gonadotropin (hMG; Menopur, Ferring) and recombinant FSH (Puregon, Merck) began on day 2 of the menstrual cycle and was followed by hCG (Pregnyl, Merck) administration 34–36 h before oocyte retrieval, based on follicle size. The collected hGL cells were purified via density centrifugation from the follicular aspirates collected from women undergoing oocyte retrieval as described previously (Chang *et al.* 2014a, 2016a). Individual primary cell cultures were composed of cells from one individual patient. Purified hGL cells were seeded (2×10^5 cells/well in 12 well plates) and cultured at 37°C in a humidified atmosphere with 5% CO₂ in 95% air in Dulbecco's Modified Eagle Medium/nutrient mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich Corp) supplemented with 10% charcoal/dextran-treated fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/mL penicillin (Life Technologies, Inc/BRL), 100 µg/mL streptomycin sulfate (Life Technologies, Inc/BRL), and 1× GlutaMAX (Invitrogen, Life Technologies). The culture medium was changed every other day in all experiments. Before being treated with growth factors, hGL cells were cultured in serum-free medium for 24 h.

Simian virus 40 large T antigen-immortalized human granulosa cell (SVOG) culture

A non-tumorigenic immortalized hGL cell line, SVOG, which was previously produced by the Simian virus 40 large T antigen transfection of early luteal phase human granulosa cells obtained from patients undergoing IVF (Lie *et al.* 1996), was used in this study. Because the immortalized SVOG cells were generated from primary hGL cells, they display biological responses to many different treatments that are similar to the responses of hGL cells (Chang *et al.* 2014b, 2015a,c, 2016b, Chen *et al.* 2015). SVOG cells were counted with a hemocytometer, and cell viability was assessed by 0.04% Trypan blue exclusion. The cells were seeded ($2-4 \times 10^5$ cells/mL in 6-well plates) and cultured in DMEM/F-12 medium supplemented with 10% charcoal/dextran-treated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate and 1× GlutaMAX. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. The culture medium was changed every other day in all experiments. Before being treated with growth factors, SVOG cells were cultured in serum-free medium for 24 h.

Antibodies and reagents

Recombinant human activin A was obtained from R&D Systems and was composed of a Chinese hamster ovary cell-derived recombinant human protein that was >95% pure based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining. It was supplied in a lyophilized form from a 0.2 µm filtered solution of 4 mM HCL with 0.1% BSA as a carrier protein. The TGF-β type I receptor inhibitor SB431542 and PI3K inhibitor LY290042 were purchased from Sigma-Aldrich. The MAPK (ERK1/MEK2) inhibitor U0126 was obtained from Calbiochem. The polyclonal anti-PTGS2 antibody was obtained from Abcam. Rabbit anti-PTGS1 and goat anti-actin antibodies were obtained from Santa Cruz Biotechnology. Monoclonal mouse anti-phospho-ERK1/2 and polyclonal rabbit anti-phospho-Akt, anti-ERK1/2, anti-Akt, anti-phospho-c-jun, anti-c-jun, anti-phospho-SMAD2, anti-phospho-SMAD3, anti-SMAD3, and anti-SMAD4 and monoclonal mouse anti-SMAD2 antibodies were obtained from Cell Signaling Technology. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were obtained from Bio-Rad Laboratories. Horseradish peroxidase-conjugated donkey anti-goat IgG was obtained from Santa Cruz Biotechnology.

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

Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. Reverse transcription into first-strand cDNA was performed with 2 µg RNA, random primers, and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). Each 20 µL qPCR reaction contained 1× SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA and 250 nM of each specific primer. The following primers were used: *PTGS2*, 5'-CCC TTG GGT GTC AAA GGT AA-3' (sense) and 5'-GCC CTC GCT TAT GAT CTG TC-3' (antisense); *PTGS1*, 5'-TGC CCA GCT CCT GGC CCG CCG CTT-3' (sense); 5'-GTG CAT CAA CAC AGG CGC CTC TTC-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 (*GAPDH*), 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 activin receptor type I (*ACVR1*) and activin receptor type IB (*ACVR1B*) (also known as activin receptor-like kinase (ALK)2 and ALK4) were examined using TaqMan gene expression assays (Applied Biosystems). RT-qPCR was performed using an Applied Biosystems 7300 real-time PCR system equipped with a 96-well optical reaction plate. The specificity of each assay was validated based on a dissociation curve analysis and agarose gel electrophoresis of the PCR products. Three independent experiments were performed on different cultures, and each sample of the RT-qPCR experiment was assayed in triplicate. The mRNA levels were determined by taking the mean value of the triplicate measurements.

Relative quantification of the mRNA levels was performed using the comparative cycle threshold (Ct) method and the $2^{-\Delta\Delta C_t}$ formula, with *GAPDH* as the reference gene.

Western blot analysis

After treatment, cells were lysed in a lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µM aprotinin, 1 µM leupeptin and 1 mM PMSF) (Cell Signaling Technology) containing a protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000 g for 15 min at 4°C to remove cellular debris, and protein concentrations were quantified using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts (50 µg) of protein were separated via 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 for 1 h and incubated overnight at 4°C with primary antibodies. After washing the membranes with 1× Tris-buffered saline, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. Immunoreactive bands were detected via an enhanced chemiluminescent substrate or a SuperSignal West Femto Chemiluminescence Substrate (Pierce). Membranes were stripped with stripping buffer (50 mM Tris-HCl (pH 7.6), 10 mmol/L β-mercaptoethanol and 1% SDS) at 50°C for 30 min and re-probed with anti-SMAD2 or anti-SMAD3.

Small interfering RNA (siRNA) transfection

To knock down endogenous *ACVR1*, *ACVR1B*, *SMAD2*, *SMAD3* or *SMAD4* expression, cells were transfected with 25 nM ON-TARGET *plus* SMART *pool* siRNA targeting specific genes, *ACVR1* (L-004924-00-0005), *ACVR1B* (L-004925-00-0005), *SMAD2* (L-003561-00-0005), *SMAD3* (L-0020067-00-0005) or *SMAD4* (L-003902-00-0005) (Dharmacon Technologies, Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Invitrogen, Life Technologies). siCONTROL Non-Targeting *pool* siRNA (D-001810-10-20) (Dharmacon) was used as the transfection control. The knockdown efficiency was confirmed via RT-PCR or western blot analysis.

PGE2 enzyme-linked immunosorbent assay (ELISA)

A human PGE2-specific ELISA was performed in accordance with the manufacturer's instructions (Cayman Chemical). SVOG cells were cultured in a six well plate with 2 mL DMEM/F-12 medium. After the treatments has been applied, the culture media and cell lysates were collected, and the concentrations of PGE2 in the culture media were measured using an ELISA and were normalized to the protein concentrations in the cell lysate. The normalized PGE2 values are presented as values relative to those of the control group.

Statistical analysis

The results are presented as the mean ± S.E.M. of at least three independent experiments. Multiple comparisons were

analyzed via one-way analysis of variance followed by Tukey's multiple comparison tests using PRISM software (GraphPad Software). Statistical significance was defined as $P < 0.05$.

Results

Activin A upregulates the expression of *PTGS2* but not *PTGS1* in hGL cells

To investigate the effect of activin A on the expression of *PTGS2* in hGL cells, we treated SVOG cells with different concentrations (1, 10 or 100 ng/mL) of recombinant human activin A (activin A). The results in

Figure 1 Activin A upregulates the expression of *PTGS2* in human granulosa-lutein cells. (A and B) SVOG cells were treated with vehicle control or different concentrations (1, 10 or 100 ng/mL) of activin A for 3 h or 6 h, and the levels of *PTGS2* mRNA (3 h) and protein (6 h) were examined via RT-qPCR and western blot analysis, respectively. (C and D) SVOG cells were treated with 30 ng/mL of activin A for 1, 3, 6 or 12 h and the levels of *PTGS2* mRNA (1, 3, 6 or 12 h) and protein (6 or 12 h) were examined via RT-qPCR and western blot analysis, respectively. (E and F) Primary hGL cells were treated with vehicle control or different concentrations (1, 10 or 100 ng/mL) of activin A for 3 h or 6 h, and the levels of *PTGS2* mRNA (3 h) and protein (6 h) were examined via RT-qPCR and western blot analysis, respectively. The results are expressed as the mean $\pm$ S.E.M. of at least three independent experiments. Values marked with different letters are significantly different ($P < 0.05$). Ctrl, control; hGL, human granulosa-lutein.

Figure 2 Activin A does not alter the expression of *PTGS1* in SVOG cells. (A and C) SVOG cells were treated with vehicle control or different concentrations (1, 10 or 100 ng/mL) of activin A, and the levels of *PTGS1* mRNA (A) and protein (C) were examined via RT-qPCR and western blot analysis, respectively. (B and D) SVOG cells were treated with 30 ng/mL of activin A for 3, 6, 12 or 24 h, and the levels of *PTGS1* mRNA (B) and protein (D) were examined via RT-qPCR and western blot analysis, respectively. The results are expressed as the mean $\pm$ S.E.M. of at least three independent experiments. Ctrl, control.

Fig. 1 reveal that treatment with activin A for 3 and 6 h significantly increased the *PTGS2* mRNA (Fig. 1A) and protein (Fig. 1B) levels in a concentration-dependent manner. In addition, the treatment of SVOG cells with 30 ng/mL of activin A induced an increase in *PTGS2* mRNA, with the maximal effect observed 3 h after treatment, and the mRNA levels declined thereafter (Fig. 1C). *PTGS2* protein expression was increased at 6 and 12 h after treatment (Fig. 1D). Next, we used primary hGL cells obtained from IVF patients to further confirm the regulatory effects of activin A on *PTGS2* expression. As shown in Fig. 1E and F, activin A treatment increased *PTGS2* mRNA and protein levels in a concentration-dependent manner in primary hGL cells. Because *PTGS1* and *PTGS2* enzymes have similar activities in many cellular functions, we next investigated the effect of activin A on the expression of *PTGS1* in SVOG cells. As shown in Fig. 2, treatment with activin A did not alter *PTGS1* mRNA (Fig. 2A and B) or protein levels (Fig. 2C and D) at the different concentrations or time points examined.

Activin A does not induce the phosphorylation of *ERK1/2*, *PI3K/Akt* and *c-jun* signaling in SVOG cells

Apart from the SMAD-dependent signaling pathway, convincing evidence has indicated that the TGF- β superfamily members can activate SMAD-independent

Figure 3 MAPK signaling pathways do not involve the activin A-induced upregulation of PTGS2 expression in SVOG cells. (A) SVOG cells were treated with vehicle control, 30 or 100 ng/mL of activin A or 10 ng/mL of EGF for 15 min, and the levels of phospho-ERK1/2, phospho-PI3K/Akt or phospho-c-jun were examined via western blot analysis. (B) SVOG cells were treated with 30 ng/mL of activin A for 3 h or 6 h in the absence (DMSO) or presence of 10 μ M U0126 and the levels of PTGS2 mRNA (3 h) and protein (6 h) were examined via RT-qPCR and western blot analysis, respectively. (C) SVOG cells were treated with 30 ng/mL of activin A for 3 h or 6 h in the absence (DMSO) or presence of 10 μ M LY294002 and the levels of PTGS2 mRNA (3 h) and protein (6 h) were examined via RT-qPCR and western blot analysis, respectively. The results are expressed as the mean $\pm$ s.e.m. of at least three independent experiments. Values marked with different letters are significantly different ($P < 0.05$). Act A, activin A; Ctrl, control; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase.

signaling pathway (Derynck & Zhang 2003). We sought to investigate whether activin A can act through several intracellular signal transduction pathways, such as mitogen-activated protein kinase (MAPK) pathways in SVOG cells. In contrast to the effects of epidermal growth factor (EGF, 10 ng/mL for 15 min), an activator of MAPK pathways, activin A (30 or 100 ng/mL) did not alter the phosphorylated levels of ERK1/2, phosphoinositide 3-kinase (PI3K)/Akt and c-jun (Fig. 3A).

MAPK pathways do not involve in the activin A-induced upregulation of PTGS2 expression in SVOG cells

To further determine the involvement of ERK1/2 or PI3K/Akt signaling in the activin A-induced upregulation of

PTGS2 expression, an ERK1/2 inhibitor U0126 was used. As shown in Fig. 3B, pre-treatment of U0126 did not alter the activin A-induced increases in PTGS2 mRNA and protein levels in SVOG cells. Likewise, pre-treatment of a PI3K/Akt inhibitor LY294002 did not affect the A-induced increases in PTGS2 mRNA and protein levels in SVOG cells (Fig. 3C).

Activin A induces the phosphorylation of SMAD2/3 proteins in SVOG cells

To investigate the underlying mechanism by which activin A regulates the expression of PTGS2, we used western blot analysis to examine the levels of phosphorylated SMAD2/3 after exposure to activin A in SVOG cells. The results reveal that treatment with 30 ng/mL activin A for 30 and 60 min significantly increased the abundance of the phosphorylated forms of the SMAD2 and SMAD3 proteins (Fig. 4A and B).

Figure 4 Effects of a TGF- β /activin type I receptor inhibitor (SB431542) on the activin A-induced increases in phosphorylated SMAD2/3 levels and the upregulation of PTGS2 expression in SVOG cells. (A and B) SVOG cells were treated with 30 ng/mL of activin A for 30 min or 60 min, and the levels of phosphorylated SMAD2 (A) and SMAD3 (B) were examined via western blot analysis. (C and D) SVOG cells were treated with 30 ng/mL activin A for 1 h in the absence (DMSO) or presence of 5 μ M SB431542 (an inhibitor of the TGF- β type I receptor), and phosphorylated SMAD2 (C) and SMAD3 (D) levels were examined via western blot analysis. (E and F) SVOG cells were treated with 30 ng/mL activin A for 3 h or 6 h in the absence (DMSO) or presence of 5 μ M SB431542 and the levels of PTGS2 mRNA (3 h) and protein (6 h) were examined via RT-qPCR and western blot analysis, respectively. The results are expressed as the mean $\pm$ s.e.m. of at least three independent experiments. Values marked with different letters are significantly different ($P < 0.05$). Ctrl, control.

TGF- β type I receptor is required for the activin A-induced phosphorylation of SMAD2/3 and the upregulation of PTGS2 expression

Activin A initiates a signal transduction pathway by binding to transmembrane serine/threonine receptors (TGF- β type II and type I receptors) (Miyazawa *et al.* 2002). SB431542 is a potent and specific inhibitor of TGF- β type I activin receptor-like kinase receptors (Inman *et al.* 2002). To investigate the involvement of TGF- β type I receptors in activin A-induced cellular functions, we pre-treated SVOG cells with SB431542 for 60 min before adding activin A. As shown in Fig. 4C and D, the activin A-induced increases in phosphorylated SMAD2 (Fig. 4C) and SMAD3 (Fig. 4D) levels were entirely abolished by the pre-treatment of SVOG cells with 5 μ M SB431542. Notably, the pretreatment of SVOG cells with 5 μ M SB431542 also totally abolished the activin A-induced upregulation of PTGS2 mRNA (Fig. 4E) and protein (Fig. 4F) expression.

ACVR1B is required for the activin A-induced increases in phosphorylated SMAD2/3 and the upregulation of PTGS2 expression in SVOG cells

To further investigate which TGF- β type I receptor mediate activin A-induced intracellular actions, specific targeted siRNAs were used to knock down endogenous ACVR1 or ACVR1B expression in SVOG cells. The knockdown efficiency was first confirmed via RT-qPCR to show that the targeted mRNAs were attenuated by the transfection of the specific siRNAs (Fig. 5A and B). Notably, only the knockdown of ACVR1B abolished the activin A-induced increases in phosphorylated SMAD2/3 levels, whereas the knockdown of ACVR1 had no effect (Fig. 5C and D). Similarly, only the knockdown of ACVR1B abolished the activin A-induced upregulation of PTGS2 expression (mRNA and protein), whereas the knockdown of ACVR1 had no effect (Fig. 5E and F). These results indicate that the ACVR1B type I receptor is required for the increases in phosphorylated SMAD2/3 levels and the upregulation of PTGS2 expression induced by activin A in SVOG cells.

SMAD2/3-SMAD4 signaling pathways are required for the activin A-induced upregulation of PTGS2 expression in SVOG cells

After the binding of activin A to TGF- β receptors, the receptors are activated to induce the phosphorylation of the receptor-regulatory SMADs (R-SMADs), SMAD2 and SMAD3. Phosphorylated R-SMADs then bind to the co-mediator SMAD (Co-SMAD) SMAD4, which forms a heteromeric complex and translocates into the nucleus for target gene expression (Shi & Massague 2003). To further investigate the involvement of SMAD2 and/or SMAD3 in the activin A-induced

upregulation of PTGS2 expression, we knocked down the downstream mediators SMADs using specific siRNA. The knockdown efficiency of SMAD2 or SMAD3 was confirmed via RT-qPCR (Fig. 6A and B). As shown in Fig. 6C and D, the knockdown of either SMAD2 or SMAD3 abolished the activin A-induced upregulation of PTGS2 mRNA and protein expression. To further confirm the role of SMAD signaling in the activin A-induced upregulation of PTGS2 expression, SVOG cells were treated with activin A after the knockdown of the Co-SMAD SMAD4 (knockdown efficiency was confirmed via RT-qPCR and western blot analysis, Fig. 6E and F). Notably, the knockdown of SMAD4 abolished the activin A-induced increases in PTGS2 mRNA (Fig. 6E) and protein (Fig. 6F) expression in SVOG cells.

Figure 5 ACVR1B mediates the stimulatory effects of activin A on SMAD2/3 phosphorylation and PTGS2 expression in SVOG cells. (A) Cells were transfected with 25 nM siCtrl or siACVR1 for 48 h, and then treated with 30 ng/mL activin A. The ACVR1 mRNA levels were examined via RT-qPCR. (B) Cells were transfected with 25 nM siCtrl or siACVR1B for 48 h and then treated with 30 ng/mL activin A. The ACVR1B mRNA levels were examined via RT-qPCR. (C and D) Cells were transfected with 25 nM siCtrl, siACVR1 or siACVR1B for 48 h and then treated with 30 ng/mL activin A for 1 h. The levels of phosphorylated SMAD2 (C) and SMAD3 (D) were examined via western blot analysis. (E and F) Cells were transfected with 25 nM siCtrl, siACVR1 or siACVR1B for 48 h and then treated with 30 ng/mL activin A for 3 h (E) or 6 h (F). The levels of PTGS2 mRNA (E) and protein (F) were examined via RT-qPCR and western blot analysis, respectively. The results are expressed as the mean $\pm$ s.e.m. of at least three independent experiments. Values marked with different letters are significantly different ($P < 0.05$). Ctrl, control.

Figure 6 The SMAD2/3–SMAD4 signaling pathway is required for the activin A-induced upregulation of PTGS2 expression in SVOG cells. (A and B) Cells were transfected with 25 nM siCtrl, siSMAD2 or siSMAD3 for 48 h, and the mRNA levels of SMAD2 (A) or SMAD3 (B) were examined via RT-qPCR. (C and D) SVOG cells were transfected with a 25 nM siCtrl, siSMAD2, or siSMAD3 for 48 h and then treated with 30 ng/mL of activin A for 3 h (C) or 6 h (D). The levels of PTGS2 mRNA (C) and protein (D) were examined via RT-qPCR and western blot analysis, respectively. (E and F) SVOG cells were transfected with 25 nM siCtrl or siSMAD4 for 48 h and then treated with 30 ng/mL activin A for 3 h (E) or 6 h (F). The levels of PTGS2 mRNA (E) and protein (F) were examined via RT-qPCR and western blot analysis, respectively. The results are expressed as the mean $\pm$ S.E.M. of at least three independent experiments. Value marked with different letters are significantly different ($P < 0.05$). Ctrl, control.

Activin A increases the production of PGE2 in SVOG cells

PGE2 is the major product of PTGS enzymes and plays a key role in the regulation of corpus luteum formation (Arosh *et al.* 2004). To further investigate whether activin A-induced PTGS2 expression contributes to the increase in PGE2 production, we treated SVOG cells with different concentrations (1, 10 or 100 ng/mL) of activin A for 6 h and examined the PGE2 concentrations in the culture medium. As shown in Fig. 7A, activin A significantly increased PGE2 levels in a concentration-dependent manner. Moreover, pre-treatment with

Figure 7 Activin A increases the production of PGE2 in SVOG cells. (A) SVOG cells were treated with vehicle control or different concentrations (1, 10 or 100 ng/mL) of activin A for 6 h, and the levels of PGE2 in the medium were examined via ELISA. (B) SVOG cells were treated 30 ng/mL of activin A in the absence (DMSO) or presence of SB431542 (5 μ M) for 6 h, and the levels of PGE2 in the medium were examined via ELISA. The results are expressed as the mean $\pm$ S.E.M. of at least three independent experiments. Value marked with different letters are significantly different ($P < 0.05$). Ctrl, control.

SB431542 entirely abolished the activin A-induced increase in PGE2 levels in SVOG cells (Fig. 7B).

Discussion

Locally produced activins have been shown to play a critical role in regulating various ovarian functions, such as folliculogenesis, steroidogenesis and oocyte maturation (Ethier & Findlay 2001, Knight & Glister 2001). Increasing evidence has suggested that activin/inhibin system continues to modulate luteal activity after ovulation (Myers *et al.* 2007, Knight *et al.* 2012). In primate corpus luteum, the transcripts of INHA and INHBA, but not of INHBB remain highly expressed during luteal phase (Fraser *et al.* 1993). Notably, the human corpus luteum has been shown to be the major source of inhibin A or activin A during the mid-luteal stage (Muttukrishna *et al.* 1994). Indeed, inhibin A has been shown to positively regulate progesterone production in marmoset luteal cells (Webley *et al.* 1994). Conversely, previous studies have demonstrated the role of activin A in delaying luteinization because of its ability to inhibit basal, LH/hCG or forskolin-induced progesterone production by rat and human granulosa-lutein cells (Miro *et al.* 1991, Myers *et al.* 2008, Chang *et al.* 2014a). *In vitro* studies in human granulosa-lutein cells have suggested that activin A may also promote tissue remodeling and induce luteolysis during luteal regression (Myers *et al.* 2007). Furthermore, conditioned knockout of INHBA and INHBB in mice leads to a phenotype of progressive accumulation of corpus luteum and elevated serum progesterone, indicating a functional role of activin in the luteal regression (Pangas *et al.* 2007). Nonetheless, the present study demonstrated for the first time that activin A could upregulate the expression of PTGS2, which in turn induces the production of PGE2, indicating that activin A may be involved in the corpus

luteum development and maintenance. A previous *in vitro* study has shown that activin A promotes the proliferation of luteinized granulosa cells in humans (Rabinovici *et al.* 1990). The luteinized granulosa cells finally develop into large luteal cells that combined with small luteal cells (derived from luteinized theca cells) to give rise to two distinct types of luteal cells (Weber *et al.* 1987, Friden *et al.* 1999). The activin A-induced increase in PGE2 production further stimulates the progesterone secretion in luteal cells, which supports the normal corpus luteum function (Marsh & LeMaire 1974, Hahlin *et al.* 1988). Apart from the pituitary gland-derived LH, there is increasing evidence that different growth factors or chemicals can regulate the process of luteinization through various intra-ovarian signaling pathways. It has been reported by our group that EGF-like growth factors (amphiregulin, betacellulin and epiregulin), TGF- β 1, and nitric oxide can upregulate PTGS2 expression and PGE2 production in hGL cells (Fang *et al.* 2013, 2014, 2015). In this regard, all of these intra-ovarian factors might work together or interact with each other to upregulate the PTGS2 expression and increase the PGE2 secretion to achieve the goal of luteal phase support. In the present study, we did not demonstrate a role of activin A in the regulation of PTGS1 expression in hGL cells. Currently, the understanding of PTGS1 expression in the regulation of luteal function is limited, and the unveiling of its role will be a major goal in future studies.

To clearly understand the functions and underline mechanisms of the ovarian physiology, materials that are suitable for *in vitro* studies are required. During the terminal stage of folliculogenesis, granulosa cells proliferate and undergo differentiation and luteinization. In the present study, immortalized SVOG cells and primary hGL cells obtained from IVF patients (Lie *et al.* 1996) were used as cell models. These clinically obtained hGL cells are normally luteinized because of their stimulation of high dose of human chorionic gonadotropin (hCG) before cell isolation, which provides an attractive model for studying the related luteal function, especially the early luteal development.

In this study, we have showed that activin A induces the upregulation of PTGS2 expression and PGE2 production via an ACVR1B-mediated SMAD2/3–SMAD4-dependent pathway in human granulosa-lutein cells. It has been reported that the selective knockout of SMAD4 in the ovaries of mice results in multiple defects in folliculogenesis, decreasing fertility and premature ovarian failure (Pangas *et al.* 2006, Yu *et al.* 2013). In this study, our results show that the knockdown of SMAD4 abolishes the activin A-induced increase in PTGS2 expression in SVOG cells. According to the animal studies mentioned above and the results of our study, the SMAD signaling pathway plays an important role in the regulation of luteal function. Our data reveal that activin

A induces PTGS2 expression by activating the SMAD2/3 pathway in hGL cells. The knockdown of SMAD2 or SMAD3 abrogated the activin A-induced increase in PTGS2 expression. Our results demonstrate that both SMAD2 and SMAD3 are involved in mediating activin A-induced PTGS2 expression, which is consistent with a previous report by our group showing that TGF- β 1 upregulates PTGS2 expression via both the SMAD2 and SMAD3 signaling pathways (Fang *et al.* 2014). However, inconsistent with this study, one of our previous studies showed that only SMAD2 but not SMAD3 signaling mediates the activin A-induced downregulation of StAR expression in the SVOG cells (Chang *et al.* 2015b). Studies in knockout mice comparing the phenotypes of target deletion of either SMAD2 or SMAD3 suggested that two SMADs may have distinct roles in the mediation of TGF- β /activin ligands (Piek *et al.* 2001). Specifically, the activin A-induced specific responses being mediated by SMAD2 or SMAD3 are target gene-dependent. In addition to the canonical SMAD pathway, other non-SMAD signaling cascades, such as ERK1/2, can also mediate PTGS2 expression for the members of the TGF- β superfamily. In a previous animal study, ovarian failure and infertility were found in ERK1/2-deficient female mice (Fan *et al.* 2009). It has also been reported that human chorionic gonadotropin-induced PTGS2 expression and PGE2 production are attenuated by the inhibition of the ERK1/2 signaling pathway in hGL cells (Tsai *et al.* 2008). Previously, our group reported that EGF-like growth factors upregulated PTGS2 expression and PGE2 production through the ERK1/2 signaling pathway in hGL cells (Fang *et al.* 2013). In addition, we have demonstrated that nitric oxide may induce PTGS2 expression and PGE2 production by activating the CREB signaling pathway in the same cell model (Fang *et al.* 2015). All of these data suggest that PTGS2 expression and PGE2 production can be induced by non-SMAD signaling pathways. However, as shown in the present study, non-SMAD signaling pathways are not involved in the activin A-induced changes in PTGS2 expression and PGE2 production in hGL cells. Taken together, previous studies and our data increase our understanding of the role of activin A in regulating luteal function and might benefit to develop a pharmaceutical strategy for luteal dysfunction, implantation failure and early pregnancy loss.

In summary, this study indicates that activin A upregulates PTGS2 expression and PGE2 production in hGL cells. In addition, treatment with activin A activates the ACVR1B-mediated SMAD2/3 and SMAD4 signaling pathways. Furthermore, the inhibition of the type I receptor abolishes the activin A-induced increase in PTGS2 expression and PGE2 production. Our results suggest that activin A may play a critical role in the modulation of the human corpus luteum formation and maintenance via the induction of PTGS2 expression and PGE2 secretion.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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