Molecular and Cellular Endocrinology 505 (2020) 110731

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

ALK4-SMAD3/4 mediates the effects of activin A on the upregulation of PAI-1 in human granulosa lutein cells

Beili Chen[a,b], Hsun-Ming Chang[b], Zhiguo Zhang[a,c,d], Yunxia Cao[a,c,d,**], Peter C.K. Leung[b,*]

[a] Reproductive Medicine Center, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Anhui Medical University, No 218 Jixi Road, Hefei, 230022, Anhui, China

[b] Department of Obstetrics and Gynaecology, BC Children's Hospital Research Institute, University of British Columbia, Vancouver, British Columbia, Canada

[c] NHC Key Laboratory of Study on Abnormal Gametes and Reproductive Tract (Anhui Medical University), No 81 Meishan Road, Hefei, 230032, Anhui, China

[d] Key Laboratory of Population Health Across Life Cycle (Anhui Medical University), Ministry of Education of the People's Republic of China, No 81 Meishan Road, Hefei, 230032, Anhui, China

ARTICLE INFO

Keywords:
Activin a
PAI-1
ALK4
SMAD signaling
Human granulosa cells

ABSTRACT

In the mammalian ovary, the proteolysis of the extracellular matrix is dynamically regulated by plasminogen activator and plasminogen activator inhibitor (PAI), and it is a critical event that influences various physiological and pathological processes. Activin A is a member of the transforming growth factor-β superfamily and is expressed at a high level in human luteal cells that play an essential role in the regulation of the luteal function. At present, it is not known whether activin A can regulate the expression and production of PAI in human granulosa lutein (hGL) cells. The present study aimed to examine the effects of activin A on the expression and production of intraovarian PAI-1 and the underlying molecular mechanisms. Using primary and immortalized hGL cells as the cell model, we demonstrated that activin A upregulated the expression of PAI-1 and increased the production of PAI-1 in an autocrine/paracrine manner. Additionally, using a dual inhibition approach (molecular inhibitors and siRNA-mediated knockdown), we showed that this biological function is mediated by the ALK4-mediated SMAD3-SMAD4-dependent signaling pathway. Our findings suggest that activin A may be involved in the regulation of luteal function via the induction of PAI-1 expression and an increase in PAI-1 production.

1. Introduction

In the female reproductive cycle, the ovary produces and releases mature oocytes with the subsequent formation of the corpus luteum. These repeated processes include a series of sequential steps that involve dramatic structural changes of the ovarian matrix and stroma tissues. The accommodation of these physiological processes primarily depends on the functional degradation of extracellular matrix and is controlled by a tightly regulated and targeted extracellular proteolytic activity (Liu, 2004). The extracellular matrix proteolysis is catalyzed by plasminogen activators (PAs) and is tightly regulated by plasminogen activator inhibitors (PAIs) (Saksela and Rifkin, 1988). Accumulating evidence suggests that the precise multilevel regulation of the finetuned expression of the PA and PAI systems provides a properly controlled proteolytic activity in the mammalian ovary (Ny et al., 1985). Studies using kinetic analysis have identified four distinct PAIs; PAI-1 and PAI-2 are the only physiologically relevant plasminogen activator inhibitors (van Mourik et al., 1984). PAI-1, also known as serpine1, belongs to the serpine protease inhibitor superfamily that acts to inhibit plasminogen activation by suppressing the activity of tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) (Liu et al., 2013). Previous animal studies have demonstrated that the interaction and regulation of PAs and PAI-1 in mammalian ovaries is critical in the maintenance of normal follicular function and ovulation (Liu, 2004). Specifically, coordinated expression of PAI-1 and t-PA in the ovary is essential for the induction of successful ovulation in various mammals including rats, rhesus monkeys and cattle (Dow et al., 2002). Additionally, the intraovarian proteolytic activities that are spatiotemporally regulated by PAs and PAI-1 are crucial for the formation and regression of the corpus luteum in rats (Liu et al., 1996). In addition to the physiological role of PAI-1 in female reproductive function, the data obtained from clinical studies have shown that PAI-1 is overexpressed

* Corresponding author. Department of Obstetrics and Gynecology, Child & Family Research Institute, University of British Columbia, Room 317, 950 west 28th Ave, Vancouver, British Columbia, V5Z 4H4, Canada.

** Corresponding author. Reproductive Medicine Center, Department of Obstetrics and Gynecology, First Affiliated Hospital of Anhui Medical University, No 218 Jixi Road, Hefei, 230022, Anhui, China.

E-mail addresses: caoyunxia6@126.com (Y. Cao), peter.leung@ubc.ca (P.C.K. Leung).

https://doi.org/10.1016/j.mce.2020.110731
Received 24 September 2019; Received in revised form 3 January 2020; Accepted 20 January 2020
Available online 23 January 2020

in the ovarian tissues of women with polycystic ovary syndrome (PCOS), indicating that PAI-1 may be involved in the pathogenesis of PCOS (Atiomo et al., 1998, Lindholm et al., 2010; Orio et al., 2004).

Activin A is one of the members of the pleiotropic transforming growth factor-β (TGF-β) superfamily and is a homodimer of the inhibin βA subunit; activin A has broad physiological functions in various tissues, including the ovary (Mather et al., 1997). In humans, activin A is produced in the growing follicles; follicular fluid obtained from the antral follicles contains a mature form of activin A (Evans et al., 1997; Sidis et al., 1998). Previous studies have shown that activin A is a critical regulator of various ovarian functions, including the development of primordial follicles, folliculogenesis, steroidogenesis, oocyte maturation, ovulation and luteal formation and regression (Bayne et al., 2015; Chang et al., 2014a, 2015a, 2016a; Knight et al., 2012; Liu et al., 2016). Additionally, activin A is a principal luteinization inhibitor because of its suppressive effect on the production of progesterone in human granulosa-lutein (hGL) cells (Chang et al., 2014a, 2015a). Moreover, our recent studies have shown that activin A is involved in the modulation of extracellular matrix remodeling by the suppression of the expression of pentraxin 3 in human granulosa cells (Liu et al., 2019).

In a mouse study, activin A upregulated the expression of microphthalmia-associated transcription factor (MITF, a tissue-specific transcription factor expressed in melanocytes), which further increased the PAI-1 mRNA level in melanocytes, indicating that the production of PAI-1 can be regulated by activin A in in vitro system (Murakami et al., 2006). Considering the importance of PAI-1 for extracellular matrix proteolysis and subsequent ovulation and luteal function, a number of studies have investigated the regulation of PAI-1 in the ovarian follicle. In the corpus luteum of rats and monkeys, the PAI-1 mRNA reaches a peak level at the time of initiation of luteolysis (Liu et al., 2003). The peak expression level of PAI-1 mRNA dramatically declines in association with an increased expression level of t-PA, indicating that the PAI-1-mediated t-PA activity is critical for the initiation of luteolysis in the mammalian corpus luteum (Liu et al., 2003; Liu, 2004). Interestingly, inhibin βA (functional unit of activin A) is highly expressed in the corpus luteum, and the secreted level of the bioactive form of activin A protein reaches a peak at the mid-luteal phase of the female menstrual cycle, suggesting that activin A is a potent luteolytic factor (Muttukrishna et al., 1994). The coordinated spatiotemporal expression patterns of inhibin βA and PAI-1 in the corpus luteum may suggest that activin A serve as a luteolytic initiator to induce the expression and production of PAI-1 in hGL cells. Therefore, we designed an in vitro study to test this hypothesis and sought to examine the effect of activin A on the expression and production of PAI-1 and the underlying molecular mechanisms in hGL cells.

2. Materials and methods

2.1. Culture of the immortalized human granulosa lutein cells (SVOG cells)

In the present study, we used a nontumorigenic immortalized human granulosa cell line, SVOG cells as a model to investigate the effect of activin A on the activity of PAI-1 in human cells. This cell line was produced by transfecting primary human granulosa-lutein (hGL) cells with the simian virus 40 large T antigen (Chang, Qiao et al., 2016c; Lie et al., 1996). Biological responses of these immortalized cells to various hormone or growth factor treatments are similar to those of hGL cells because this cell line was generated from primary hGL cells (Bai et al., 2017a, 2018; Wu et al., 2017; Zhang et al., 2018). SVOG cells were cultured in Dulbecco's Modified Eagle's Medium/Ham's F-12 nutrient mixture (DMEM/F-12; Sigma-Aldrich Corp., Oakville, ON) supplemented with 10% charcoal/dextran-treated fetal bovine serum (FBS, HyClone, Logan, UT), 100 U/ml penicillin (Invitrogen, Life Technologies, Carlsbad, CA), 100 μg/ml streptomycin sulfate, (Invitrogen), and 1 × GlutaMAX (Invitrogen) in a humidified atmosphere containing 5% CO2 at 37 °C. The cells were counted with a hemocytometer using 0.04% Trypan blue to assess cell viability and seeded (2–4 × 10^5 cells/ml) in 6-well plates. Culture medium was changed every 48 h in all experiments. Prior to specific treatments, these cells were cultured overnight in serum-free medium. To prepare the conditioned medium, the cultures were changed to DMEM/F-12 without serum (2 ml per well in 6-well plates) and this medium was left on the cultures after specific treatment. The serum-free conditioned medium was collected with micropipette under a microscope to ensure that the aspirate was free of cells. Finally, the collected conditioned medium was centrifuged at 10,000 × *g* for 10 min at 4 °C to remove debris and stored at −20 °C until assay.

2.2. Preparation and culture of primary human granulosa-lutein cells

Primary hGL cells were obtained from patients undergoing an in vitro fertilization (IVF) procedure; these cells express the steroidogenesis-related genes (*CYP19A1*, *StAR*, *CYP11A1* and *HSD3B*) and several growth factors, including members of the TGF-β superfamily (Chang et al., 2013a, 2014b; Chang et al., 2014a, 2016c; Chang, Fang et al., 2016b; Chang, Klausen et al., 2013b; Chang et al., 2019). The study was approved by the Research Ethics Board of the University of British Columbia, and all participants signed a written informed consent form. Follicular aspirates were obtained at the time of oocyte retrieval from IVF patients as previously described (Chang et al., 2016b). Briefly, hGL cells were seeded in 12-well plates (2 × 10^5 cells per well) and cultured in DMEM/F-12 (Sigma-Aldrich Corp) supplemented with 10% charcoal/dextran-treated FBS (HyClone Laboratories, Inc.), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (Life Technologies, Inc.), and 1 × GlutaMAX (Life Technologies, Inc.). Cells were cultured in a humidified atmosphere with 95% air and 5% CO_2 at 37 °C, and the culture medium was changed every other day in all experiments. Before specific treatments, the cells were cultured in medium with 0.5% charcoal/dextran-treated FBS.

2.3. Antibodies and reagents

Polyclonal rabbit anti-phospho-SMAD2 (Ser465/467, 138D4, diluted 1:1000), anti-phospho-SMAD3 (Ser423/425, C25A9, diluted 1:1000), anti-SMAD3 (C67H9, diluted 1:1000) and anti-SMAD4 (D3M6U, diluted 1:1000) antibodies were obtained from Cell Signaling Technology (Beverly, MA). These antibodies do not cross-react with other SMAD-related proteins. Monoclonal mouse anti-SMAD2 antibody (L16D3, diluted 1:1000) was obtained from Cell Signaling Technology. Monoclonal mouse anti-PAI-1 (C-9, sc-5297, diluted 1:1000) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (G-9; sc-365062, diluted 1:2000) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were obtained from Bio-Rad and Santa Cruz Biotechnology, respectively. Recombinant human activin A from R&D Systems (Minneapolis, MN) was expressed in Chinese hamster ovary cells and was > 95% pure based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining. Activin A was lyophilized from a 0.2 μm filtered solution composed of 4 mM HCl with 0.1% BSA as a carrier protein. Dorsomorphin dihydrochloride (dorsomorphin, 3093/10) and SB431542 (301836-41-9) were obtained from R&D Systems and Sigma-Aldrich Corp., respectively.

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

The cells were washed with cold PBS, and total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A total of 2 μg of RNA was reverse-transcribed into the first-strand cDNA with random primers and MMLV reverse transcriptase (Promega, Madison, WI). RT-PCR was performed on an Applied

Biosystem 7300 real-time PCR system in 96-well optical reaction plates. The total reaction volume of 20 μl included 10 μl of 2X SYBR Green PCR master mix (Applied Biosystems), 20 ng of cDNA, and 250 nM of each specific primer mix. The following primers were used in this study: *PAI-1 (SERPINE1)*: 5′-GAGAAACCCAGCAGCAGATT-3' (sense) and 5′-TGG TGCTGATCTCATCCTTG -3' (antisense); *SMAD2*: 5′-GCCTTTACAGCTT CTCTGAACAA-3' (sense) and 5′- ATGTGGCAATCCTTTTCGAT-3' (antisense); *SMAD3*: 5′- CCCCAGCACATAATAACTTGG-3' (sense) and 5′-AGGAGATGGAGCACCAGAAG-3' (antisense); *SMAD4*: 5′-TGGCCCA GGATCAGTAGGT-3′(sense) and 5′-CATCAACACCAATTCCA GCA-3′(antisense); and *GAPDH*: 5′-ATGGAAATCCCATCACCATCTT-3' (sense) and 5′-CGCCCCACTTGATTTTGG-3' (antisense). Alternatively, TaqMan gene expression assays were used to examine *ACVR1B* (*ALK4*), *TGFBR1* (*ALK5*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression (Hs00244715_m1, Hs00610320_m1, and Hs02758991_g1, respectively; Applied Biosystems) in triplicate using the corresponding cDNA samples. The total reaction volume of 20 μl included 4 μl of cDNA, 5 μl of RNase-free water, 10 μl of 2X TaqMan gene expression master mix (Applied Biosystems) and 1 μl of 20 × TaqMan gene expression assay mix. RT-qPCR was performed on an Applied Biosystems 7300 real-time PCR system in 96-well optical reaction plates. The PCR parameters used for the reaction were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Assay performance was validated by evaluating amplification efficiencies using the calibration curves while ensuring that the plot of log input amount vs. ΔCq (also known as ΔCt) has a slope < 0.1. Three separate experiments were performed for different cultures, and each sample was assayed in triplicate. A mean value was used to determine the mRNA levels using the comparative Cq method according to equation $2^{-\Delta\Delta Cq}$ ($2^{-\Delta\Delta Ct}$). GAPDH was used as the reference gene.

2.5. Western blot analysis

After the treatments, the cells were washed with cold PBS and lysed in lysis buffer (Cell Signaling) 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 a DC protein assay (Bio-Rad). Equal amounts of protein were separated by 10% SDS–PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 h in TBS containing 0.05% Tween 20 and 5% nonfat dried milk and incubated overnight at 4 °C with the relevant primary antibodies. The membranes were washed and incubated with a peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h. Immunoreactive bands were detected using enhanced chemiluminescence reagents or a SuperSignal West Femto chemiluminescence substrate (Pierce, Rockford, IL) and exposed to a CL-XPosure film (Thermo Fisher, Waltham, MA). The 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 reprobed with goat anti-actin antibodies as a loading control. Films were scanned and quantified by densitometry using the Scion imaging software (Scion Corp., Frederick, MD, USA).

2.6. Small interfering RNA (siRNA) transfection

Transient knockdown assays were performed using an ON-TARGETplus nontargeting control pool or separate ON-TARGETplus SMARTpools siRNA reagents targeting ALK4, ALK5, SAMD2, SMAD3 or SMAD4 (Thermo Fisher Scientific, Lafayette, CO) as described previously (Bai, Chang et al., 2017b; Chang et al., 2013b). Briefly, the cells were cultured to 50% confluence in antibiotic-free DMEM/F12 containing 10% charcoal/dextran-treated FBS and transfected with 25 nM siRNA using Lipofectamine RNAiMAX (Life Technologies) for 48 h. The knockdown efficiency of each target was confirmed by RT-qPCR or Western blot analysis as previously described (Li et al., 2018).

2.7. Measurement of PAI-1 concentration

After treatment with activin A for 24 h, the concentration of PAI-1 in the conditioned medium was measured using a quantitative sandwich enzyme immunoassay Quantikine kit (DSE100, R&D Systems) according to the manufacturer's instructions. After the removal of the particulates by centrifugation, cell culture supernatants were collected and immediately assayed. The inter- and intra-assay coefficients of variation for this assay were less than 9%, and the minimum detectable dose of PAI-1 ranged from 0.014 to 0.142 ng/ml with a mean of 0.059 ng/ml. Each sample was measured in triplicate, and the secreted PAI-1 concentration was normalized to the total cellular protein content of each cell lysate. The normalized PAI-1 concentration for each treated sample is displayed as fold changes of the normalized control levels.

2.8. Statistical analysis

The data are presented as the mean ± standard error of the mean of at least three independent experiments. PRISM software (GraphPad Software Inc, San Diego, CA) was used to perform one-way analysis of variance followed by Duncan's test for multiple comparisons of the means. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Activin A induced the expression of PAI-1 in immortalized and primary hGL cells

Initially, we examined the effect of activin A on PAI-1 expression in hGL cells. Immortalized SVOG cells were treated with vehicle control and various concentrations (1, 10 or 100 ng/ml) of recombinant human activin A (activin A) for 3 h, and the mRNA levels were examined using RT-qPCR. The results showed that activin A treatment significantly increased the mRNA levels of PAI-1 in a concentration-dependent manner (Fig. 1A). Similar to the mRNA results, Western blot analysis showed that activin A treatment (1, 10 or 100 ng/ml) significantly increased the protein levels of PAI-1 in SVOG cells (Fig. 1B). The concentrations of activin A used in this study varied from 1 ng/ml to 100 ng/ml and were within the range of the average concentrations of activin A in follicular fluid and serum (1110–1210 ng/ml and 30–40 ng/ml, respectively) (Harada et al., 1996; Lau et al., 1999). Therefore, 30 ng/ml activin A was used as the treatment in subsequent experiments. The time-course studies showed that the stimulatory effects of activin A (30 ng/ml) on the mRNA levels of PAI-1 were detected after 1 h incubation and persisted until 24 h (Fig. 1C). The protein levels of PAI-1 started to increase at 6 h and remained elevated 24 h after activin A treatment (Fig. 1D). To confirm the stimulatory effect of activin A on PAI-1 expression, primary hGL cells were used to investigate the effect of activin A on PAI-1 expression. Consistent with the results obtained in SVOG cells, activin A (1, 10 or 100 ng/ml) treatment for 3 h or 12 h significantly increased the mRNA and protein levels of PAI-1 in primary hGL cells in a concentration-dependent manner (Fig. 1E and F).

3.2. Activin A induced the phosphorylation of SMAD2 and SMAD3 and upregulation of PAI-1 in SVOG cells in a SB431542-dependent manner

The SMAD-dependent pathway is activated by activin A in various human cells, including granulosa cells (Chang et al., 2014a; Ding et al., 2016). To determine whether activin A activates the SMAD signaling pathways in hGL cells, SVOG cells were treated with activin A (30 ng/ml) for 30, 45 or 60 min, and the levels of phosphorylated SMAD2 and SMAD3 were examined using Western blot analysis. As shown in Fig. 2A and B, activin A significantly increased the levels of phosphorylated SMAD2 (Fig. 2A) and SMAD3 (Fig. 2B) at all examined time points.

To confirm that the stimulatory effect of activin A on the activation

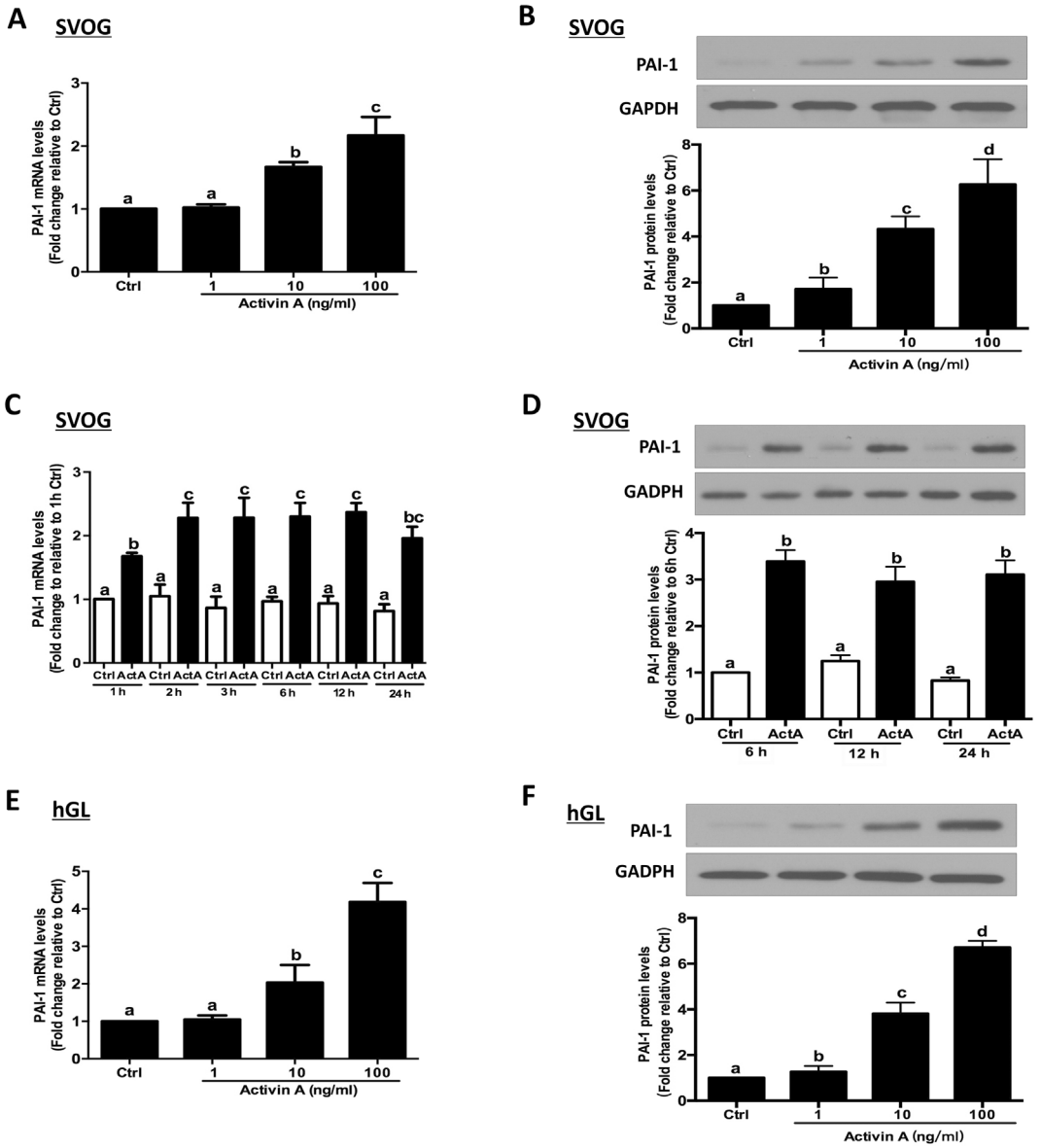

Fig. 1. Activin A upregulates the expression of PAI in primary and immortalized human granulosa lutein cells. (A and B) SVOG cells (n = 5) were treated with vehicle control or various concentrations (1, 10, or 100 ng/ml) of activin A for 3 h (A) or 12 h (B), and the mRNA and protein levels of PAI-1 were examined using RT-qPCR (A) and Western blot analysis (B), respectively. (C and D) SVOG cells (n = 6) were treated with 30 ng/ml activin A for 1, 2, 3, 6, 12, or 24 h, and the mRNA and protein levels of PAI-1 were examined using RT-qPCR (C) and Western blot analysis (D), respectively. (E and F) Primary human granulosa lutein cells (hGL, n = 6) were treated with vehicle control or various concentrations (1, 10, or 100 ng/ml) of activin A for 3 h (E) or 12 h (F), and the mRNA and protein levels of PAI-1 were examined using RT-qPCR (E) and Western blot analysis (F), respectively. The results are expressed as the mean ± SEM of six independent experiments, and values labeled with different letters are significantly different ($P < 0.05$). Ctrl, control; ActA, activin A treatment.

of the SMAD signaling pathway is mediated by the conventional activin receptor, we used two potent TGF-β type I receptor inhibitors, SB431542 and dorsomorphin. SB431542 is a specific inhibitor of ALK4/5/7, and dorsomorphin is a specific inhibitor of ALK2/3/6 (Inman et al., 2002; Yu et al., 2008). The results obtained by the inhibition approach showed that pretreatment of SVOG cells with 10 μM SB431542 for 60 min completely abolished the activin-A-induced increases in the phosphorylated protein levels of SMAD2 (Fig. 2C) and SMAD3 (Fig. 2D). However, pretreatment of SVOG cells with 10 μM dorsomorphin for 60 min did not influence the activin A-induced increase in the levels of phosphorylated SMAD2 (Fig. 2C) and SMAD3 (Fig. 2D). Similarly, pretreatment with 10 μM SB431542, but not with 10 μM dorsomorphin, for 60 min completely abolished the activin A-induced increase in mRNA (Fig. 2E) and protein (Fig. 2F) levels of PAI-1 in SVOG cells.

3.3. ALK4 is the activin type I receptor that mediates the activin A-induced phosphorylation of SMAD2/3 and the upregulation of PAI-1 in SVOG cells

To determine which type I receptor mediates the activin A-induced effects in hGL cells, we used an siRNA-mediated knockdown approach. We examined the knockdown efficiency of siRNAs targeting two TGF-β type I receptors, ALK4 and ALK5. As shown in Fig. 3A, knocking down ALK4 or ALK5 using siRNAs targeting ALK4 (siALK4) or ALK5 (siALK5) for 24 h or 48 h specifically decreased the mRNA levels of ALK4 and ALK5, respectively, by up to 75%–85%. Additionally, knocking down ALK4 but not ALK5 for 48 h significantly abolished the activin A-induced phosphorylation of SMAD2 and SMAD3 in SVOG cells (Fig. 3B). Notably, knocking down ALK4 for 48 h significantly abolished the activin A-induced increase in the mRNA and protein levels of PAI-1 in SVOG cells (Fig. 3C). However, knocking down ALK5 for 48 h had no

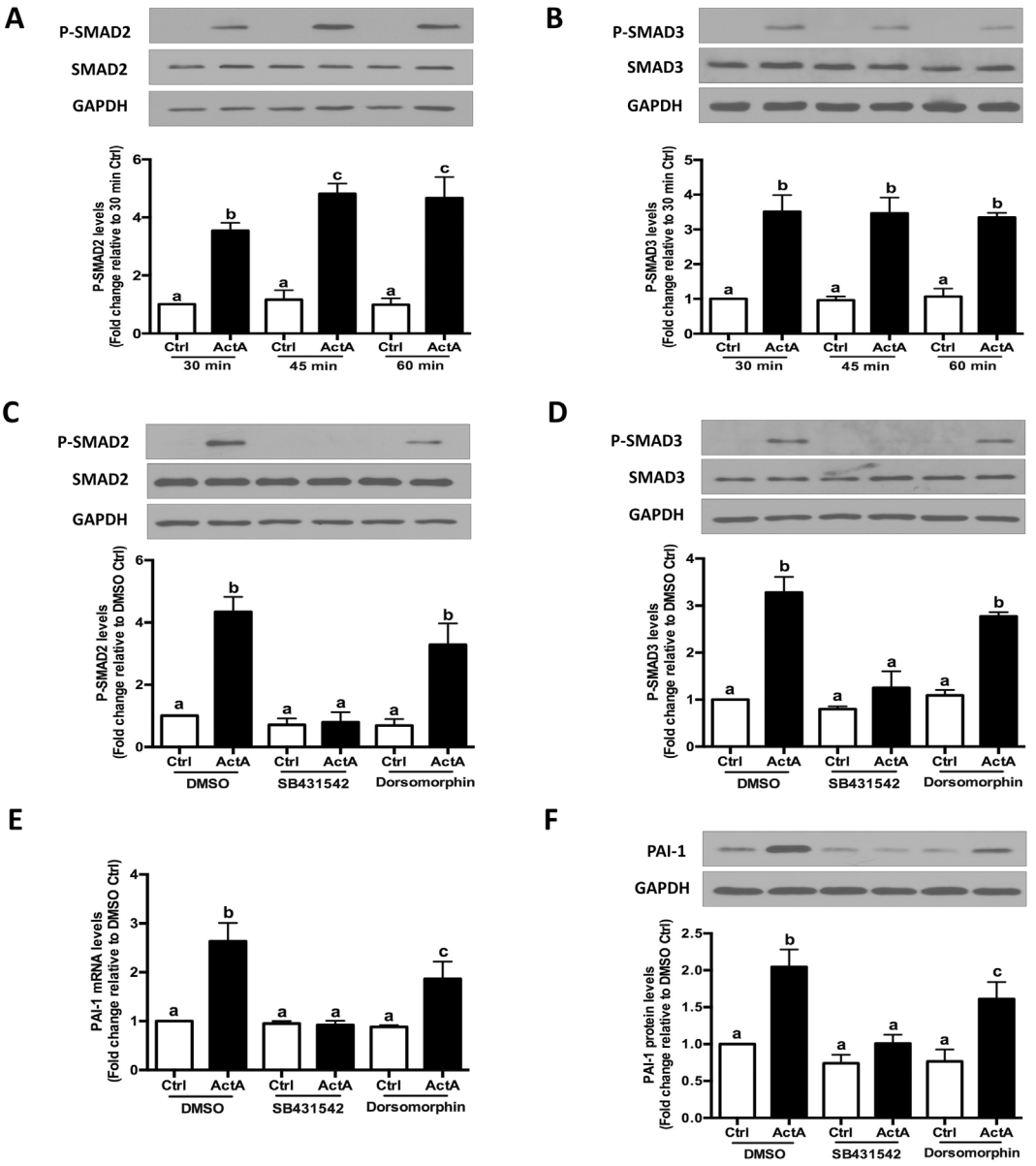

Fig. 2. TGF-β receptor inhibitor SB431542 but not dorsomorphin attenuates the activin A-induced phosphorylation of SMAD2/3 and upregulation of PAI-1 in SVOG cells. (A and B) SVOG cells (n = 6) were treated with 30 ng/ml activin A for 30, 45 or 60 min, and the levels of phosphorylated SMAD2 (A) or SMAD3 (B) were examined using Western blot analysis. (C and D) SVOG cells (n = 5) were pretreated with dimethyl sulfoxide (DMSO) or TGF-β receptor inhibitors, SB431542 (10 μM) or dorsomorphin (10 μM) for 1 h and then treated with 30 ng/ml activin A for an additional 30 min. The levels of phosphorylated SMAD2 (C) and SMAD3 (D) were examined using Western blot analysis. (E and F) SVOG cells (n = 4) were pretreated with DMSO or TGF-β receptor inhibitors, SB431542 (10 μM) or dorsomorphin (10 μM), for 1 h and then treated with 30 ng/ml activin A for an additional 3 h (E) or 12 h (F). The mRNA (E) and protein (F) levels of PAI-1 were examined using RT-qPCR (E) and Western blot analysis (F), respectively. The results are expressed as the mean ± SEM of six independent experiments, and values labeled with different letters are significantly different ($P < 0.05$). Ctrl, control; ActA, activin A treatment.

effect on the activin A-induced increase in the mRNA and protein levels of PAI-1 in SVOG cells (Fig. 3D).

3.4. SMAD3-SMAD4-mediated SMAD signaling pathway is required for the activin A-induced upregulation of PAI-1 in SVOG cells

To determine whether the SMAD-dependent pathway mediates the activin A-induced effects in hGL cells, we used the siRNA-mediated inhibition approach that targeted specific SMADs. As shown in Fig. 4A, knocking down SMAD2, SMAD3 or SMAD4 for 24 h or 48 h using siRNAs targeting SMAD2 (siSMAD2), SMAD3 (siSMAD3) or SMAD4 (siSMAD4) specifically decreased the mRNA levels of SMAD2, SMAD3 and SMAD4, respectively, by up to 70%–80%. Notably, knocking down SMAD3 but not SMAD2 for 48 h completely abolished the activin A-induced increases in the mRNA (Fig. 4B) and protein (Fig. 4C) levels of PAI-1 in SVOG cells. Similarly, knocking down SMAD4 for 48 h completely abolished the activin A-induced increase in the mRNA (Fig. 4D) and protein (Fig. 4E) levels of PAI-1 in SVOG cells.

3.5. Activin A increased the production of PAI-1 in an SB431542-dependent manner in SVOG cells

To investigate whether activin A increases the production of PAI-1 in hGL cells, we used an enzyme immunoassay to examine the PAI-1 concentration in the conditioned medium after activin A treatment. As shown in Fig. 5A, treatment with various concentrations (1, 10 or

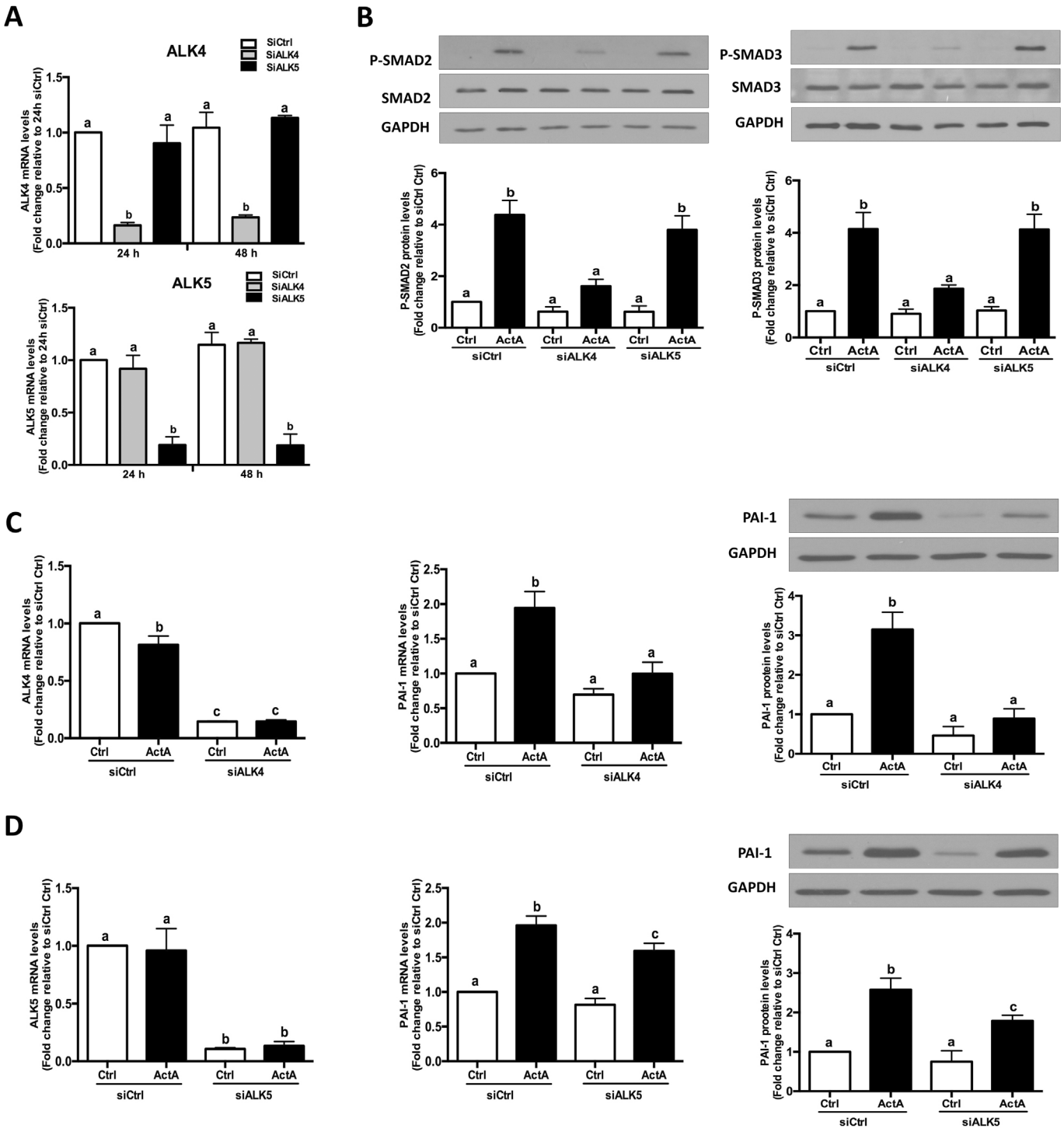

Fig. 3. The TGF-β type I receptor ALK4, but not ALK5, mediates the activin A-induced phosphorylation of SMAD2/3 and upregulation of PAI-1 in SVOG cells. (A) SVOG cells (n = 4) were transfected with 25 nM siCtrl, siALK4 or siALK5 for 24 h or 48 h. The mRNA levels of ALK4 and ALK5 were examined using RT-qPCR. (B) SVOG cells (n = 4) were transfected with siCtrl, siALK4 or siALK5 for 48 h, and then treated with 30 ng/ml activin A for an additional 60 min. The protein levels of phosphorylated SMAD2 and SMAD3 were examined using Western blot analysis. (C) SVOG cells (n = 5) were transfected with 25 nM siCtrl, siALK4 or siALK5 for 48 h, and then treated with 30 ng/ml activin A for an additional 3 h. The mRNA levels of PAI-1 were examined using RT-qPCR. (D) SVOG cells (n = 5) were transfected with 25 nM siCtrl, siALK4 or siALK5 for 48 h and then treated with 30 ng/ml activin A for an additional 12 h. The protein levels of PAI-1 were examined using Western blot analysis. The results are expressed as the mean ± SEM of 5 independent experiments, and values labeled with different letters are significantly different ($P < 0.05$). siCtrl, siControl; Ctrl, control; ActA, activin A treatment.

100 ng/ml) of activin A significantly increased the production of PAI-1 in SVOG cells in a concentration-dependent manner. Notably, pre-treatment of SVOG cells with 10 μM SB431542 for 60 min completely abolished the activin-A-induced increases in the production of PAI-1 (Fig. 5B).

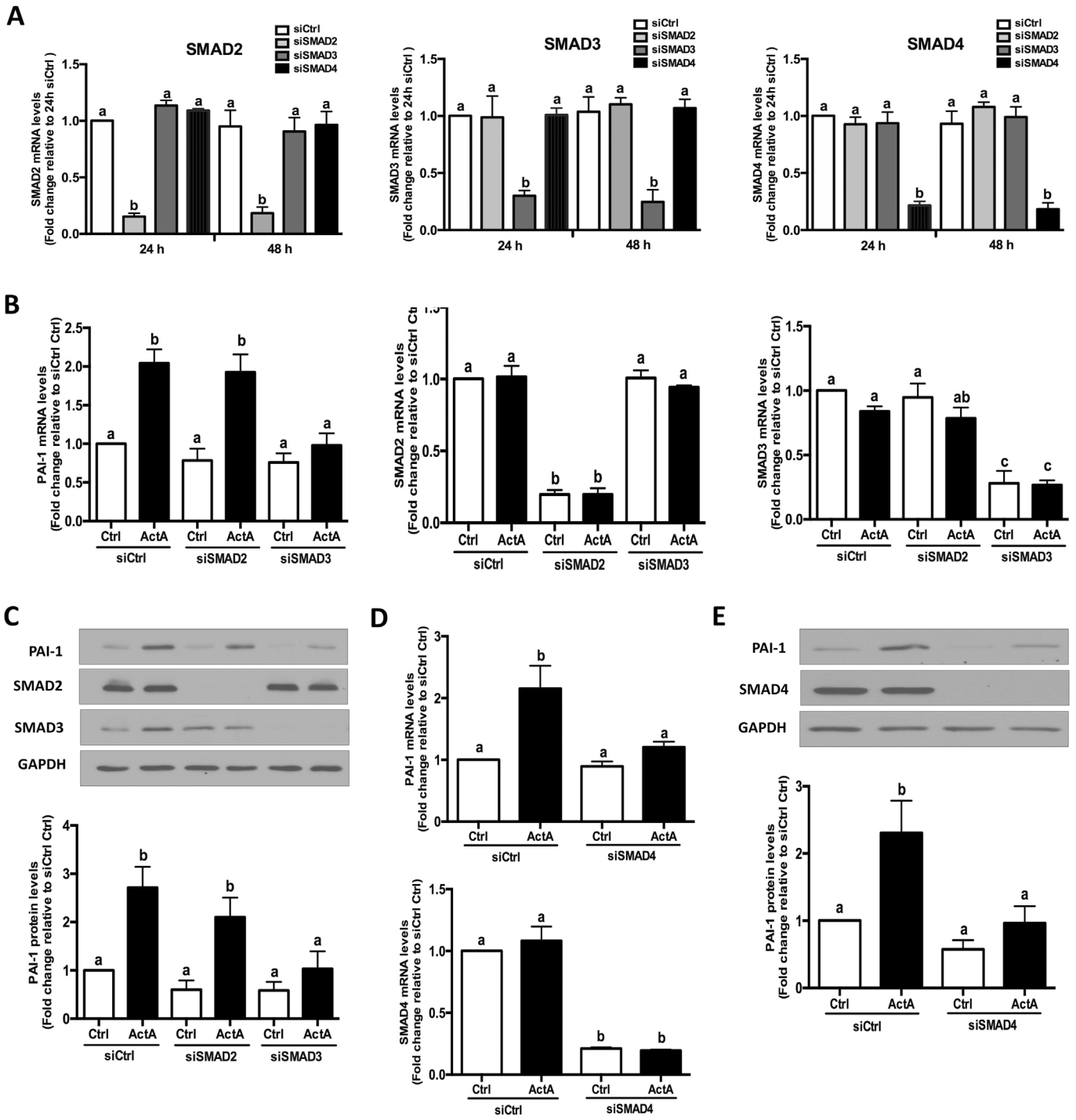

Fig. 4. SMAD3 and SMAD4, but not SMAD2, mediate the activin A-induced upregulation of PAI-1 in SVOG cells. (A) SVOG cells (n = 5) were transfected with 25 nM control siRNA (siControl), SMAD2 siRNA (siSMAD2), SMAD3 siRNA (siSMAD3) or SMAD4 siRNA (siSMAD4) for 48 h. The mRNA levels of SMAD2, SMAD3 and SMAD4 were examined using RT-qPCR. (B and C) SVOG cells (n = 4) were transfected with 25 nM control siRNA (siControl), SMAD2 siRNA (siSMAD2) or SMAD3 siRNA (siSMAD3) for 48 h and then treated with 30 ng/ml activin A for an additional 3 h (B) or 12 h (C). The mRNA (B) and protein (C) levels of SMAD2, SMAD3 and PAI-1 were examined using RT-qPCR (B) and Western blot analysis (C), respectively. (D and E) SVOG cells (n = 5) were transfected with 25 nM control siRNA (siControl) or SMAD4 siRNA (siSMAD4) for 48 h and then treated with 30 ng/ml activin A for an additional 3 h (D) or 12 h (E). The mRNA (D) and protein (E) levels of PAI-1 were examined using RT-qPCR (D) and Western blot analysis (E), respectively. The results are expressed as the mean ± SEM of 5 independent experiments, and values labeled with different letters are significantly different ($P < 0.05$). siCtrl, siControl; Ctrl, control; ActA, activin A treatment.

4. Discussion

In the developing follicles of rats and mice, the t-PA activity in the oocyte is mainly controlled by inhibins produced in granulosa cells (Liu, 2004). The expression level of inhibin subunits in granulosa cells has been demonstrated to be negatively correlated with the activity of t-PA in oocytes (Liu, 2004). The present study is the first to demonstrate that activin A increased the production of PAI-1 by upregulating the expression of PAI-1 in hGL cells. Because inhibins are the antagonists of activins and PAI-1 suppresses the activity of t-PA, we speculate that the

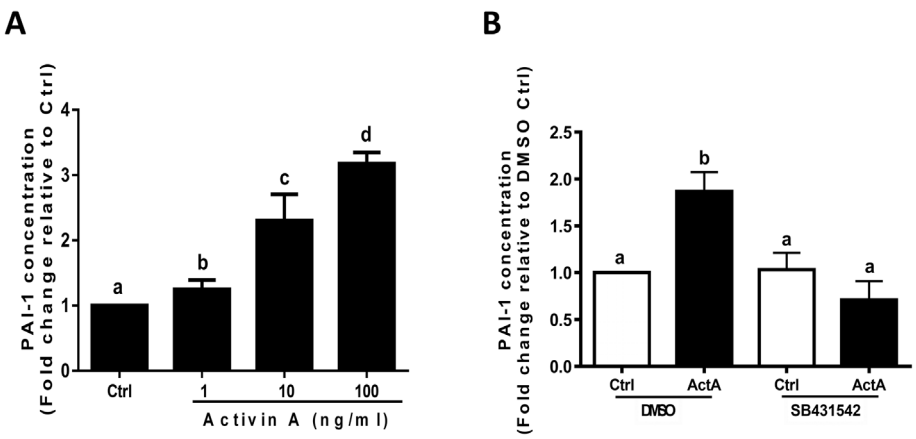

Fig. 5. TGF-β receptor inhibitor SB431542 abolishes the activin A-induced increase in PAI-1 production in SVOG cells. (A) SVOG cells (n = 5) were treated with vehicle control or various concentrations (1, 10, or 100 ng/ml) of activin A for 24 h, and the concentration of PAI-1 was examined using an enzyme immunoassay (ELISA). (B) SVOG cells (n = 5) were pretreated with dimethyl sulfoxide (DMSO) or a TGF-β receptor inhibitor SB431542 (10 μM) for 1 h and then treated with 30 ng/ml activin A for an additional 24 h. The concentration of PAI-1 was examined using an enzyme immunoassay (ELISA). The results are expressed as the mean ± SEM of 5 independent experiments, and values labeled with different letters are significantly different ($P < 0.05$). siCtrl, siControl; Ctrl, control; ActA, activin A treatment.

increased level of inhibins produced by granulosa cells inhibits the action of intraovarian activins and, in turn, decreases the production of PAI-1 and increases the activity of t-PA in the developing follicles in the ovary. After ovulation, the human corpus luteum has a glandular structure and primarily secretes progesterone to maintain early pregnancy (Stocco et al., 2007). This temporary endocrine tissue is derived from a ruptured ovarian follicle that contains granulosa cells, theca cells, capillaries and fibroblasts. The development of the corpus luteum is modulated by the interactions among multiple luteotropic and luteolytic factors and is characterized by a complex process of a series of tissue remodeling processes, including luteal formation, maintenance and regression (Stocco et al., 2007). The formation of corpus luteum involves dramatic morphological and biochemical processes that are tightly and dynamically controlled by the matrix-degrading proteases (Findlay, 1986; Pate, 1994). However, if no conception occurs, the functional maintenance of corpus luteum is terminated, followed by the regression of the corpus luteum. This process involves two changes: a rapid decline in progesterone production (functional luteolysis) and the degradation of the luteal tissue (structural luteolysis) (Liu, 2004). Studies have shown that the luteal expression level of steroidogenic regulatory protein (StAR) is highly correlated with the production of progesterone and is thus a key regulator of the corpus luteum function (Chen et al., 2003). In the corpus luteum of rats and monkeys, an increased activity of PAI-1 is closely related to a dramatic decrease in the production of StAR/progesterone in the late luteal phase, indicating that the coordinated expression levels of PAI-1 and StAR in the corpus luteum are involved in the process of luteal regression (Chen et al., 1999; Liu et al., 2003). Previous studies have shown that activins (including activin A, activin B and activin AB) are potent luteinization inhibitors because activins suppress basal and FSH-, LH- and human chorionic gonadotropin-induced StAR/progesterone production in hGL cells (Chang et al., 2014a; Rabinovici et al., 1990). Moreover, the expression levels of inhibin βA and PAI-1 reach their peak levels at the time of initiation of luteolysis (Liu et al., 2003; Liu et al., 1995; Muttukrishna et al., 1994). In combination with the results of the previous studies, our findings suggest that activin A acts as a luteolytic factor that initiates luteolysis by promoting the expression and activity of PAI-1 in human corpus luteum. In this regard, activin A is a principal luteinization inhibitor or a luteolytic factor in the corpus luyeum. Indeed, previous studies have shown that activins suppress the progesterone production induced by human chorionic gonadotropin (hCG)-, FSH-, or LH in hGL cells (Chang et al., 2014a; Rabinovici et al., 1990). The source of activin A during the luteolytic period is mainly from granulosa-lutein cells and the production of bioactive form of activin A is regulated by bone morphogenetic protein (BMP)4 and BMP7, two theca cell-derived growth factors (Chang, Cheng et al., 2015b). Taken together, these three intraovarian factors (activin A, BMP4 and BMP7) coordinately inhibit luteiolization by suppressing progesterone production in the human ovary (Chang et al., 2014a; Zhang et al., 2015). Moreover, in cultured hGL cells, treatment with hCG up-regulated the expression of follistatin (an activin-binding protein), which further antagonized the cellular activities of endogenous activin A (Tuuri et al., 1994). Intriguingly, both inhibin α and inhibin βA (the components of mature inhibin A) are expressed at a high level in the corpus luteum, and the bioactive mature inhibin A reaches a peak level at the mid-luteal phase of the menstrual cycle, indicating that activin A is a critical luteolytic factor (Muttukrishna et al., 1994).

The selection of a suitable material for functional in vitro studies is critical to comprehensively understand the cellular activities and the underlying molecular mechanisms of ovarian biology. In the mature ovarian follicle, granulosa cells undergo proliferation, differentiation and luteinization during the terminal stage of follicular development. In this study, we used primary and immortalized (SVOG cells) hGL cells as an in vitro model to investigate the effect of activin A on the expression and production of PAI-1. These cells were clinically derived from hGL cells that were obtained from patients undergoing in vitro fertilization procedure and luteinized because of stimulation with a pharmacological dose of human chorionic gonadotropin before cell isolation (Lie et al., 1996). Therefore, these hGL cells provide an attractive model for studying the cellular activities related to luteal function during luteal development. However, a limitation of this study is that the results obtained in an in vitro system may be not relevant to the in vivo microenvironment in the ovarian tissue, which contains multiple components. Future studies should be aimed at addressing the interactions of activin/inhibin and t-PA/PAI-1 systems in the developing follicle or corpus luteum using an in vivo animal model; such studies will be of great interest.

Because the dysregulation of the activin signaling pathway is associated with several ovarian pathologies, including PCOS (Roberts et al., 1994), a clear understanding of the detailed molecular determinants of the activin A-mediated cellular activities will be beneficial to develop therapeutic strategies for the ovarian diseases and fertility-related disorders. In the present study, we demonstrate that activin A increases the expression and production of PAI-1 via the ALK4 -mediated SMAD3/SMAD4-dependent signaling pathway in hGL cells. Knockout studies in mice have shown that selective depletion of SMAD4 leads to multiple defects in the mouse ovary, including impaired folliculogenesis, premature ovarian failure and decreased fertility (Pangas et al., 2006; Yu et al., 2013). The results obtained in our study showed that knocking down SMAD4 completely abolishes the activin A-induced upregulation of PAI-1 in hGL cells. Previous animal studies and our findings suggest that the SMAD-dependent signaling pathway plays a critical role in the regulation of follicular function. Using a dual inhibition approach (molecular inhibitors and siRNA-mediate knockdown), we demonstrated that activin A-induced cellular activities (phosphorylation of SMAD2/3 and increase in PAI-1 expression) in hGL cells are mediated

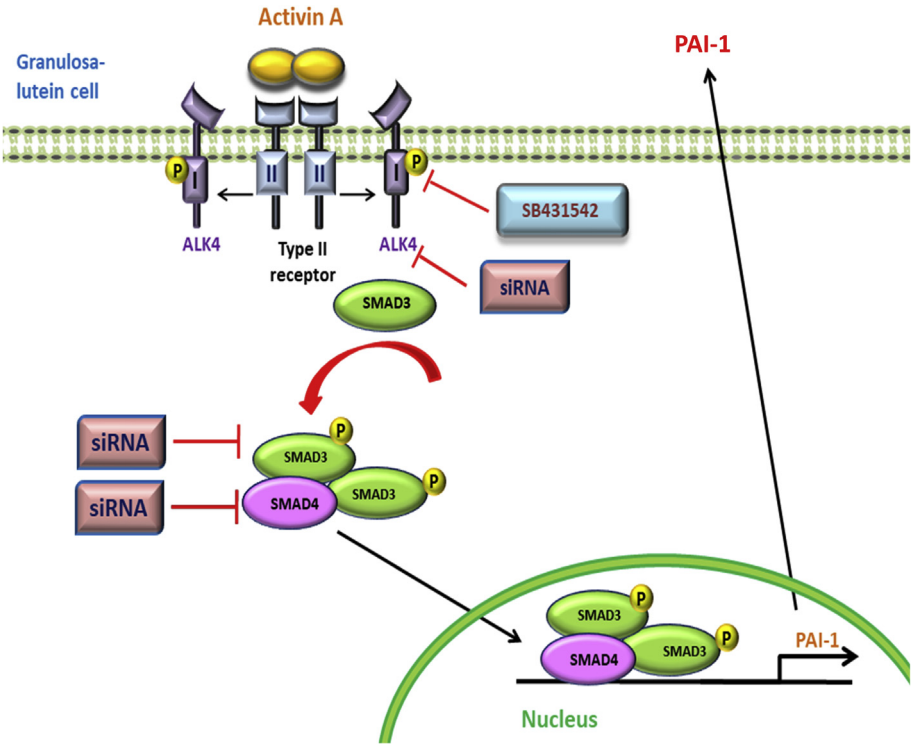

Fig. 6. Proposed model of the stimulatory effect of activin A on PAI-1 production in human granulosa lutein cells. Activin A binds to a heterotetrameric receptor complex comprised of type I (ALK4) and type II receptors. Ligand-induced activation of the receptor complex results in the phosphorylation and activation of ALK4, leading to the activation of receptor-regulated SMAD3. Phosphorylated SMAD3 forms a heterotrimeric complex with common SMAD4 that translocates into the nucleus where it binds the PAI-1 promoter and stimulates the transcription of PAI-1, which in turn increases the production of PAI-1.

by the TGF-β type I receptor ALK4 (ACVR1B) but not by ALK5 (TGFBR1). Indeed, recent studies have demonstrated the therapeutic perspective of several ALK inhibitors, including LY-2157299 and SB431542, in tumor progression and stem cell differentiation (Cui et al., 2019). Using a similar inhibition approach, we showed that activin A treatment induces the activation of SMAD2 and SMAD3 in hGL cells. However, the knockdown of SMAD3, but not SMAD2, completely reversed the activin A-induced increase in PAI-1 expression, indicating that only SMAD3 is required for the activin A-induced upregulation of PAI-1 in hGL cells (Fig. 6). The results of our previous studies are not consistent with the data of the present study and showed that only the SMAD2, but not the SMAD3, signaling pathway mediates the suppressive effect of activin A-induced regulation of StAR in SVOG cells (Chang et al., 2015a). Additionally, SMAD2 and SMAD3 are required for the activin A-induced upregulation of prostaglandin-endoperoxide synthase 2 (PTGS2) in the same cell model (Liu et al., 2016). Animal studies using knockout mouse models with targeted depletion of either SMAD2 or SMAD3 indicated that these two SMADs have distinct functional roles in mediating the action of activins (Piek et al., 2001). Therefore, previous studies suggest that the activin A-induced cellular responses mediated by SMAD2 or SMAD3 are dependent on the downstream target genes. Activin A can initiate the cellular activities via SMAD or non-SMAD signaling pathways in various cell types (Zhang, 2009); however, our results indicate that activin A upregulates the expression of PAI-1 via the SMAD-dependent signaling pathway in hGL cells.

In summary, we have demonstrated that activin A upregulates the expression of PAI-1 and increases the production of PAI-1 in hGL cells. Additionally, this biological function is mediated via the ALK4-mediated SMAD-dependent signaling pathway. Furthermore, the inhibition of activin type I receptor or knockdown of SMAD3 completely reverses the activin A-induced upregulation of PAI-1 expression or the increase in PAI-1 production (Fig. 6). Our findings suggest that activin A may play an important role in the regulation of luteal function via the induction of PAI-1 production in the human ovary.

Declaration of competing interest

The authors have nothing to disclose.

Acknowledgements

This research was supported by the CIHR Foundation Scheme Grant #143317 to P.C.K.L. This research was also supported by the Non-profit Central Research Institute Fund of Chinese Academy of Medical Sciences # 2019PT310002 to CYX.

References

Atiomo, W.U., Bates, S.A., Condon, J.E., Shaw, S., West, J.H., Prentice, A.G., 1998. The plasminogen activator system in women with polycystic ovary syndrome. Fertil. Steril. 69, 236–241.
Bai, L., Chang, H.M., Cheng, J.C., Chu, G., Leung, P.C.K., Yang, G., 2017a. ALK2/ALK3-BMPR2/ACVR2A mediate BMP2-induced downregulation of pentraxin 3 expression in human granulosa-lutein cells. Endocrinology 158, 3501–3511.
Bai, L., Chang, H.M., Cheng, J.C., Chu, G., Leung, P.C.K., Yang, G., 2017b. Lithium chloride increases COX-2 expression and PGE2 production in a human granulosa-lutein SVOG cell line via a GSK-3β/β-catenin signaling pathway. Endocrinology 158, 2813–2825.
Bai, L., Chang, H.M., Cheng, J.C., Chu, G., Leung, P.C.K., Yang, G., 2018. Lithium chloride inhibits StAR and progesterone production through GSK-3β and ERK1/2 signaling pathways in human granulosa-lutein cells. Mol. Cell. Endocrinol. 461, 89–99.
Bayne, R.A., Kinnell, H.L., Coutts, S.M., He, J., Childs, A.J., Anderson, R.A., 2015. GDF9 is transiently expressed in oocytes before follicle formation in the human fetal ovary and is regulated by a novel NOBOX transcript. PloS One 10, e0119819.
Chang, H.M., Cheng, J.C., Huang, H.F., Shi, F.T., Leung, P.C., 2015a. Activin A, B and AB decrease progesterone production by down-regulating StAR in human granulosa cells. Mol. Cell. Endocrinol. 412, 290–301.
Chang, H.M., Cheng, J.C., Klausen, C., Leung, P.C., 2013a. BMP15 suppresses progesterone production by down-regulating StAR via ALK3 in human granulosa cells. Mol. Endocrinol. 27, 2093–2104.
Chang, H.M., Cheng, J.C., Klausen, C., Leung, P.C., 2015b. Recombinant BMP4 and BMP7 increase activin A production by up-regulating inhibin βA subunit and furin expression in human granulosa-lutein cells. J. Clin. Endocrinol. Metab. 100, E375–E386.
Chang, H.M., Cheng, J.C., Klausen, C., Taylor, E.L., Leung, P.C., 2014a. Effects of recombinant activins on steroidogenesis in human granulosa-lutein cells. J. Clin. Endocrinol. Metab. 99, E1922–E1932.
Chang, H.M., Cheng, J.C., Liu, Y., Klausen, C., Xu, C., Leung, P.C., 2016a. Activin A-induced increase in LOX activity in human granulosa-lutein cells is mediated by CTGF. Reproduction 152, 293–301.

Chang, H.M., Cheng, J.C., Taylor, E., Leung, P.C., 2014b. Oocyte-derived BMP15 but not GDF9 down-regulates connexin43 expression and decreases gap junction intercellular communication activity in immortalized human granulosa cells. Mol. Hum. Reprod. 20, 373–383.
Chang, H.M., Fang, L., Cheng, J.C., Taylor, E.L., Sun, Y.P., Leung, P.C., 2016b. Effects of growth differentiation factor 8 on steroidogenesis in human granulosa-lutein cells. Fertil. Steril. 105, 520–528.
Chang, H.M., Klausen, C., Leung, P.C., 2013b. Antimüllerian hormone inhibits follicle-stimulating hormone-induced adenylyl cyclase activation, aromatase expression, and estradiol production in human granulosa-lutein cells. Fertil. Steril. 100, 585–592 e1.
Chang, H.M., Qiao, J., Leung, P.C., 2016c. Oocyte-somatic cell interactions in the human ovary-novel role of bone morphogenetic proteins and growth differentiation factors. Hum. Reprod. Update 23, 1–18.
Chang, H.M., Wu, H.C., Sun, Z.G., Lian, F., Leung, P.C.K., 2019. Neurotrophins and glial cell line-derived neurotrophic factor in the ovary: physiological and pathophysiological implications. Hum. Reprod. Update 25, 224–242.
Chen, X.L., Gao, H.J., Wei, P., Song, X.X., Hu, Z.Y., Liu, Y.X., 2003. Regulatory effect of IFN-gamma on expression of TGF-beta 1, T beta R-II, and StAR in corpus luteum of pregnant rhesus monkey. Acta Pharmacol. Sin. 24, 435–441.
Chen, Y.J., Feng, Q., Liu, Y.X., 1999. Expression of the steroidogenic acute regulatory protein and luteinizing hormone receptor and their regulation by tumor necrosis factor alpha in rat corpora lutea. Biol. Reprod. 60, 419–427.
Cui, X., Shang, S., Lv, X., Zhao, J., Qi, Y., Liu, Z., 2019. Perspectives of small molecule inhibitors of activin receptor-like kinase in anti-tumor treatment and stem cell differentiation (Review). Mol. Med. Rep. 19, 5053–5062.
Ding, Z.Y., Jin, G.N., Wang, W., Sun, Y.M., Chen, W.X., Chen, L., Liang, H.F., Datta, P.K., Zhang, M.Z., Zhang, B., Chen, X.P., 2016. Activin A-smad signaling mediates connective tissue growth factor synthesis in liver progenitor cells. Int. J. Mol. Sci. 17, 408.
Dow, M.P., Bakke, L.J., Cassar, C.A., Peters, M.W., Pursley, J.R., Smith, G.W., 2002. Gonadotrophin surge-induced upregulation of mRNA for plasminogen activator inhibitors 1 and 2 within bovine periovulatory follicular and luteal tissue. Reproduction 123, 711–719.
Evans, L.W., Muttukrishna, S., Knight, P.G., Groome, N.P., 1997. Development, validation and application of a two-site enzyme-linked immunosorbent assay for activin-AB. J. Endocrinol. 153, 221–230.
Findlay, J.K., 1986. Angiogenesis in reproductive tissues. J. Endocrinol. 111, 357–366.
Harada, K., Shintani, Y., Sakamoto, Y., Wakatsuki, M., Shitsukawa, K., Saito, S., 1996. Serum immunoreactive activin A levels in normal subjects and patients with various diseases. J. Clin. Endocrinol. Metab. 81, 2125–2130.
Inman, G.J., Nicolas, F.J., Callahan, J.F., Harling, J.D., Gaster, L.M., Reith, A.D., Laping, N.J., Hill, C.S., 2002. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol. Pharmacol. 62, 65–74.
Knight, P.G., Satchell, L., Glister, C., 2012. Intra-ovarian roles of activins and inhibins. Mol. Cell. Endocrinol. 359, 53–65.
Lau, C.P., Ledger, W.L., Groome, N.P., Barlow, D.H., Muttukrishna, S., 1999. Dimeric inhibins and activin A in human follicular fluid and oocyte-cumulus culture medium. Hum. Reprod. 14, 2525–2530.
Li, H., Chang, H.M., Shi, Z., Leung, P.C.K., 2018. SNAIL mediates TGF-β1-induced downregulation of pentraxin 3 expression in human granulosa cells. Endocrinology 159, 1644–1657.
Lie, B.L., Leung, E., Leung, P.C., Auersperg, N., 1996. Long-term growth and steroidogenic potential of human granulosa-lutein cells immortalized with SV40 large T antigen. Mol. Cell. Endocrinol. 120, 169–176.
Lindholm, A., Bixo, M., Eliasson, M., Hudecova, M., Arnadottir, R., Holte, J., Poromaa, I.S., 2010. Tissue plasminogen activator and plasminogen activator inhibitor 1 in obese and lean patients with polycystic ovary syndrome. Gynecol. Endocrinol. 26, 743–748.
Liu, C., Chang, H.M., Yi, Y., Fang, Y., Zhao, F., Leung, P.C.K., Yang, X., 2019. ALK4-SMAD2/3-SMAD4 signaling mediates the activin A-induced suppression of PTX3 in human granulosa-lutein cells. Mol. Cell. Endocrinol. 493 110485.
Liu, K., Brändström, A., Liu, Y.X., Ny, T., Selstam, G., 1996. Coordinated expression of tissue-type plasminogen activator and plasminogen activator inhibitor type 1 during corpus luteum formation and luteolysis in the adult pseudopregnant rat. Endocrinology 137, 2126–2132.
Liu, K., Feng, Q., Gao, H.J., Hu, Z.Y., Zou, R.J., Li, Y.C., Liu, Y.X., 2003. Expression and regulation of plasminogen activators, plasminogen activator inhibitor type-1, and steroidogenic acute regulatory protein in the rhesus monkey corpus luteum. Endocrinology 144, 3611–3617.
Liu, P.P., Chang, H.M., Cheng, J.C., Leung, P.C., 2016. Activin A upregulates PTGS2 expression and increases PGE2 production in human granulosa-lutein cells. Reproduction 152, 655–664.
Liu, Y.X., 2004. Plasminogen activator/plasminogen activator inhibitors in ovarian physiology. Front. Biosci. 9, 3356–3373.
Liu, Y.X., Chen, Y.X., Shi, F.W., Feng, Q., 1995. Studies on the role of plasminogen activators and plasminogen activator inhibitor type-1 in rat corpus luteum of pregnancy. Biol. Reprod. 53, 1131–1138.
Liu, Y.X., Liu, X.M., Nin, L.F., Shi, L., Chen, S.R., 2013. Serine protease and ovarian paracrine factors in regulation of ovulation. Front. Biosci. 18, 650–664.
Mather, J.P., Moore, A., Li, R.H., 1997. Activins, inhibins, and follistatins: further thoughts on a growing family of regulators. Proc. Soc. Exp. Biol. Med. 215, 209–222.
Murakami, M., Ikeda, T., Saito, T., Ogawa, K., Nishino, Y., Nakaya, K., Funaba, M., 2006. Transcriptional regulation of plasminogen activator inhibitor-1 by transforming growth factor-beta, activin A and microphthalmia-associated transcription factor. Cell. Signal. 18, 256–265.
Muttukrishna, S., Fowler, P.A., Groome, N.P., Mitchell, G.G., Robertson, W.R., Knight, P.G., 1994. Serum concentrations of dimeric inhibin during the spontaneous human menstrual cycle and after treatment with exogenous gonadotrophin. Hum. Reprod. 9, 1634–1642.
Ny, T., Bjersing, L., Hsueh, A.J., Loskutoff, D.J., 1985. Cultured granulosa cells produce two plasminogen activators and an antiactivator, each regulated differently by gonadotropins. Endocrinology 116, 1666–1668.
Orio, F., Palomba, S., Cascella, T., Tauchmanovà, L., Nardo, L.G., Di Biase, S., Labella, D., Russo, T., Savastano, S., Tolino, A., Zullo, F., Colao, A., Lombardi, G., 2004. Is plasminogen activator inhibitor-1 a cardiovascular risk factor in young women with polycystic ovary syndrome? Reprod. Biomed. Online 9, 505–510.
Pangas, S.A., Li, X., Robertson, E.J., Matzuk, M.M., 2006. Premature luteinization and cumulus cell defects in ovarian-specific Smad4 knockout mice. Mol. Endocrinol. 20, 1406–1422.
Pate, J.L., 1994. Cellular components involved in luteolysis. J. Anim. Sci. 72, 1884–1890.
Piek, E., Ju, W.J., Heyer, J., Escalante-Alcalde, D., Stewart, C.L., Weinstein, M., Deng, C., Kucherlapati, R., Bottinger, E.P., Roberts, A.B., 2001. Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. J. Biol. Chem. 276, 19945–19953.
Rabinovici, J., Spencer, S.J., Jaffe, R.B., 1990. Recombinant human activin-A promotes proliferation of human luteinized preovulatory granulosa cells in vitro. J. Clin. Endocrinol. Metab. 71, 1396–1398.
Roberts, V.J., Barth, S., el-Roeiy, A., Yen, S.S., 1994. Expression of inhibin/activin system messenger ribonucleic acids and proteins in ovarian follicles from women with polycystic ovarian syndrome. J. Clin. Endocrinol. Metab. 79, 1434–1439.
Saksela, O., Rifkin, D.B., 1988. Cell-associated plasminogen activation: regulation and physiological functions. Annu. Rev. Cell Biol. 4, 93–126.
Sidis, Y., Fujiwara, T., Leykin, L., Isaacson, K., Toth, T., Schneyer, A.L., 1998. Characterization of inhibin/activin subunit, activin receptor, and follistatin messenger ribonucleic acid in human and mouse oocytes: evidence for activin's paracrine signaling from granulosa cells to oocytes. Biol. Reprod. 59, 807–812.
Stocco, C., Telleria, C., Gibori, G., 2007. The molecular control of corpus luteum formation, function, and regression. Endocr. Rev. 28, 117–149.
Tuuri, T., Erämaa, M., Hildén, K., Ritvos, O., 1994. Activin-binding protein follistatin messenger ribonucleic acid and secreted protein levels are induced by chorionic gonadotropin in cultured human granulosa-luteal cells. Endocrinology 135, 2196–2203.
van Mourik, J.A., Lawrence, D.A., Loskutoff, D.J., 1984. Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. J. Biol. Chem. 259, 14914–14921.
Wu, Y.T., Chang, H.M., Huang, H.F., Sheng, J.Z., Leung, P.C., 2017. Bone morphogenetic protein 2 regulates cell-cell communication by down-regulating connexin43 expression in luteinized human granulosa cells. Mol. Hum. Reprod. 23, 155–165.
Yu, C., Zhang, Y.L., Fan, H.Y., 2013. Selective Smad4 knockout in ovarian preovulatory follicles results in multiple defects in ovulation. Mol. Endocrinol. 27, 966–978.
Yu, P.B., Hong, C.C., Sachidanandan, C., Babitt, J.L., Deng, D.Y., Hoyng, S.A., Lin, H.Y., Bloch, K.D., Peterson, R.T., 2008. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat. Chem. Biol. 4, 33–41.
Zhang, H., Klausen, C., Zhu, H., Chang, H.M., Leung, P.C., 2015. BMP4 and BMP7 suppress StAR and progesterone production via ALK3 and SMAD1/5/8-SMAD4 in human granulosa-lutein cells. Endocrinology 156, 4269–4280.
Zhang, X.Y., Chang, H.M., Taylor, E.L., Leung, P.C.K., Liu, R.Z., 2018. BMP6 down-regulates GDNF expression through SMAD1/5 and ERK1/2 signaling pathways in human granulosa-lutein cells. Endocrinology.
Zhang, Y.E., 2009. Non-Smad pathways in TGF-beta signaling. Cell Res. 19, 128–139.