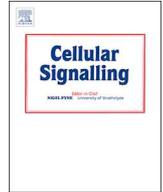




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# Bone morphogenetic protein 2 increases lysyl oxidase activity via up-regulation of snail in human granulosa-lutein cells

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## ABSTRACT

Lysyl oxidase (LOX) is a copper-dependent enzyme that maintains and stabilizes the extracellular matrix (ECM) by catalyzing the cross-linking of elastin and collagen. ECM within the ovarian follicle plays a crucial role in regulating follicular development and oocyte maturation. Bone morphogenetic protein 2 (BMP2) belongs to the BMP subfamily that has been shown to be involved in the process of ovarian folliculogenesis and luteal formation. To date, whether BMP2 regulates the activity of LOX during human follicular development remains to be elucidated. The aim of this study was to investigate the effect of BMP2 on the regulation of LOX expression and activity in human granulosa-lutein cells (hGL) and the underlying mechanisms. Using both primary and immortalized (SVOG cells) hGL cells, we demonstrated that BMP2 up-regulated the expression and activity of LOX and hence decreased the soluble collagens in cultured medium in hGL cells. Additionally, the mRNA and protein levels of two transcriptional factors, SNAIL and SLUG, were increased following cell exposure to BMP2. Knockdown of SNAIL, but not SLUG partially reversed BMP2-induced increases in LOX expression and activity. The BMP2-induced up-regulation of SNAIL expression was abolished by the pre-treatment with two BMP type I receptor inhibitors, dorsomorphin and DMH-1, but not SB431542. Moreover, knockdown of SMAD4 completely abolished BMP2-induced up-regulation of SNAIL expression and the subsequent increases in LOX expression and activity. Our results suggest that BMP2 increases LOX expression and activity via the up-regulation of SNAIL in hGL cells. These findings may provide insights into the functional role of BMP2 in the regulation of ECM formation during folliculogenesis.

## 1. Introduction

The mammalian folliculogenesis process heavily relied on the bi-directional communication between the oocyte and its surrounding somatic cells, granulosa and theca cell layers [1]. Cell-cell communication among these cells is required for oocyte maturation and follicular development, which is primarily dependent on the structure of the follicle. Extracellular matrix (ECM) maintains the follicular architecture and plays a crucial role in regulating mammalian folliculogenesis and oogenesis [2,3]. During the growing process, the primordial follicles grow into preovulatory follicles along with complex morphological and physical changes of cells within the developing follicles, which highlights the importance of ECM remodeling in various cellular processes, including morphology, communication, proliferation, differentiation,

and steroidogenesis [3]. Lysyl oxidase (LOX) is a copper-dependent enzyme that catalyzes the cross-linking of elastin and collagen, which further maintains and stabilizes the structure of ECM [4]. Previous studies have shown that LOX is expressed in both bovine and rat granulosa cells of the developing follicles [5,6]. In rat ovaries, the expression of LOX in the granulosa cells is positively correlated with the competence of the corresponding oocytes, suggesting a potential biomarker of LOX in evaluating the oocyte quality [7]. Several publications have appeared in recent years documenting the regulation of LOX expression and activity in the ovarian follicles. Animal studies have shown that FSH and 8-Bromo-cAMP suppress LOX mRNA expression and LOX enzyme activity in a dose-dependent manner [8]. In contrast, the LOX mRNA and activity are elevated during the ovulation process after hCG stimulation in rabbit and perch ovarian follicles [5,9]. In rat

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granulosa cells, members of the transforming growth factor- $\beta$  (TGF- $\beta$ ), including TGF- $\beta$ , activin A, and growth differentiation factor (GDF) 9, have been shown to increase the expression and activity of LOX [8]. Our recent studies also demonstrated that several TGF- $\beta$  superfamily members (TGF- $\beta$ 1, activin A, and GDF8) can increase LOX activity by up-regulating the expression of LOX in human granulosa-lutein (hGL) cells [10–12]. However, the functional role of intra-ovarian bone morphogenetic proteins (BMPs) in regulating LOX expression and activity in human granulosa cells has never been elucidated.

BMP2 is a member of BMP subfamily that is expressed in the human granulosa cell and corpus luteum to modulate follicular and luteal functions [1,13]. In hamster ovaries, BMP2 promotes primordial follicle formation by enhancing somatic cell differentiation and oocyte development and prevents the apoptotic cell death in the developing ovary [14]. In hen growing follicles, BMP2 signaling decreases FSH responsiveness by suppressing TGF- $\beta$ - and FSH-induced FSH receptor expression, which maintains the granulosa cells in an undifferentiated state [15]. In contrast, BMP2 up-regulates the expression of FSH receptor and aromatase, whereas BMP2 down-regulates the expression of LH receptor and steroidogenic acute regulatory protein in human granulosa cells [16]. Additionally, BMP2 can modulate cell-cell communication activity by down-regulating connexin 43 expression in human granulosa cells [17]. Indeed, a clinical study has suggested that BMP2 can be used as a potential marker for the competent oocytes [18].

SNAIL and SLUG are zinc-finger transcription factors that are first identified as the transcriptional suppressors to regulate epithelial-mesenchymal transition [19]. Both SNAIL and SLUG are expressed in mouse ovaries and pre-implantation embryos, and these transcription factors play essential roles in regulating mouse folliculogenesis, luteinization, and early embryonic development [20]. Despite the spatial-temporal expression and function of SNAIL and SLUG have been extensively studied in embryogenesis and organogenesis [21,22], limited information has been reported about the roles of these two transcription factors in regulating human ovarian function.

At present, the functional role of BMP2 in the modulation of ECM remodeling process during follicular development is completely unknown. The aim of this study was to investigate the effect of BMP2 on the regulation of LOX expression and activity as well as the involvement of SNAIL transcription factor in the underlying molecular mechanisms.

## 2. Materials and methods

### 2.1. Preparation of primary human granulosa-lutein cells

Primary hGL cells were obtained from follicular fluid samples of in vitro fertilization (IVF) patients. This study was under the approval from the University of British Columbia Research Ethics Board and the Institutional Review Board. Primary hGL cells were purified according to the protocol as previously described [23,24]. Briefly, cells were seeded in 12-well plates ( $2 \times 10^5$  cells per well) and cultured in DMEM/F-12 (Sigma-Aldrich Corp, St. Louis, Missouri) medium supplemented with 10% charcoal/dextran-treated fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin sulfate (Life Technologies, Inc., Carlsbad, California), and  $1 \times$  GlutaMAX (Life Technologies, Inc., Carlsbad, California). Cells were cultured in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C, and the culture medium was changed every other day in all experiments.

### 2.2. Immortalized human granulosa-lutein cells culture

A human granulosa cell line (SVOG) was used in our study. This human granulosa cell line was immortalized by transfection of primary hGL cells obtained from women undergoing IVF procedure using the SV40 large T antigen [25]. The SVOG cells were cultured in DMEM/F-12 medium with 10% FBS, 100 U/ml of penicillin, 100  $\mu$ g/ml of

streptomycin and  $1 \times$  GlutaMAX. The cells were maintained in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C, and the culture medium was changed every other day in all experiments. The cells were cultured in serum-free medium for 24 h before specific treatments.

### 2.3. Antibodies and reagents

A polyclonal rabbit anti-LOX antibody (ab31238) was obtained from Abcam (Cambridge, MA). A monoclonal mouse anti- $\alpha$  tubulin antibody (sc-23,948) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal rabbit anti-SMAD4 (#9515), a monoclonal rabbit anti-SNAIL (#3879) and a monoclonal rabbit anti-SLUG (#9585) antibodies were obtained from Cell Signaling Technology (Danvers, MA). The horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit IgGs were purchased from Bio-Rad Laboratories (Hercules, CA). Recombinant human BMP2, dorsomorphin dihydrochloride (dorsomorphin) and DMH-1 (4-[6-[4-(1-Methylethoxy)phenyl]pyrazole [1, 5-a]pyrimidin-3-yl]-quinoline) were obtained from R&D Systems (Minneapolis, MN). SB431542 was obtained from Sigma-Aldrich Corp.

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

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Inc.) according to manufacturer's instructions. A total amount of 1.8  $\mu$ g of RNA was reverse transcribed into first-strand cDNA using the random primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI). Applied Biosystems 7300 Real-Time PCR System was used to perform the RT-qPCR. The total reaction system is 20  $\mu$ l which includes  $1 \times$  SYBR Green qPCR MasterMix (Applied Biosystems, Foster City, California), primer mixture (250 nM) and 20 ng of cDNA. The primers used in the experiments were: Lysyl oxidase (LOX), 5'-GCCTCAGGCTGCACAATTC-3' (sense) and 5'-TCAGAACACCAGGCCTGATTT-3' (antisense); SNAIL (SNAIL1), 5'-CCCAATCGGAAGCCTAACT-3' (sense), SLUG (SNAIL2), 5'-TTCGGACCCACACATTACCT-3' (sense) and 5'-GCAGTGAGGGCAA GAAAAG-3' (antisense); and 5'-GCTGGAAGGTAACTCTGGAT TAG-3' (antisense) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GAGTCAACGGATTTGGTCGT-3' (sense) and 5'-GACAAG CTCCCCGTTCTCAG-3' (antisense). All the experiments were repeated at least three times, and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Ct method ( $2^{-\Delta\Delta C_t}$ ) with GAPDH as the reference gene.

### 2.5. Western blot analysis

Cells were washed with warm phosphate-buffered saline (PBS) once and lysed by the cell lysis buffer (#9803, Cell Signaling) containing a protease inhibitor cocktail (P-2714, Sigma-Aldrich) on ice. A total amount of 30  $\mu$ g proteins were used for the western blot analysis. The samples were running in 10% SDS polyacrylamide gel electrophoresis and then transferred onto the polyvinylidene difluoride (PVDF) membranes (#1620177, Bio-Rad Laboratories Inc.). Tris-buffered saline containing 0.1% (vol/vol) Tween-20 (TBST) with 5% non-fat milk was used to block the PVDF membranes for 1 h, and then the membranes were incubated with relevant primary antibodies overnight at 4 °C. After the overnight incubation, the membranes were washed for 1 h using TBST and then probed with the appropriate HRP-conjugated secondary antibodies (Bio-Rad Laboratories Inc.). The immunoreactive bands were detected by an enhanced chemiluminescent substrate and X-ray film. The intensities of the bands were quantified using the Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD).

### 2.6. Small interfering RNA (siRNA) transfection

To knockdown the endogenous SMAD4, SNAIL and SLUG, 25 nM

ON-TARGET plus SMARTpool relevant siRNA, and ON-TARGET plus non-targeting control pool siRNA (transfection control) (Dharmacon, Lafayette, CO) were transfected into cells separately using the Lipofectamine RNAiMAX (Invitrogen, Life Technologies) according to the manufacturer's protocols.

### 2.7. Measurement of LOX activity

Following the specific treatments, the culture medium was collected to measure the activity of LOX immediately or stored at  $-80^{\circ}\text{C}$  freezer until it was assayed. The LOX activity was measured by an enzyme-linked fluorescent assay according to the manufacturer's (ab112139, Abcam). The inter- and intra-assay coefficients of variation for these assays were  $< 6\%$ , and the detection limit of LOX in solution is 40 ng. Each sample was measured three times, and LOX activity was normalized to the total cellular protein content in each sample, and the results were present as the fold change relative to control.

### 2.8. Measurement of soluble collagen

Following specific treatments, cultured medium in primary hGL cells was collected immediately for measurement of soluble collagen. Measurement of soluble collagen was performed using a Sircol Soluble Collagen Assay Kit (Biocolor, Carrickferaus, UK) according to the manufacturer's instructions. Each sample was measured in triplicate, and the concentration of soluble collagen were normalized to total cellular protein content and presented as values relative to those of the control group.

### 2.9. Statistical analysis

The results are presented as the mean  $\pm$  SEM of at least 3 independent experiments. PRISM software (GraphPad Software Inc., San Diego, CA) was used to perform a one-way ANOVA followed by a post-hoc Duncan's test for the multiple comparisons of means. Statistical significance was defined as  $P < .05$ .

## 3. Results

### 3.1. BMP2 increases LOX expression and activity in the SVOG and primary hGL cells

The follicular fluid levels of BMP2 range from 1 to 115 ng/ml in humans [1,18]. Based on the physiological relevance of BMP2, we chose the concentration levels of 1–100 ng/ml to conduct our in vitro study. To examine the effect of BMP2 on the expression of LOX in hGL cells, we treated the SVOG cells with vehicle control or different concentrations of recombinant human BMP2 (1, 10 or 100 ng/ml) for 12 h. The results showed that BMP2 significantly increased the mRNA levels of LOX in a dose-dependent manner (significantly increased effects with 10 and 100 ng/ml) (Fig. 1A). Similarly, the western blot analysis result showed BMP2 increased the protein levels of LOX in a concentration-dependent manner (Fig. 1B). Next, we performed the time-course study as we treated the cells with 50 ng/ml at different time points (3, 6 or 12 h). As shown in Fig. 1C, 50 ng/ml BMP2 significantly increased the mRNA levels of LOX at all the time points examined. Likewise, BMP2 significantly increased the protein levels of LOX at 12 and 24 h after treatment (Fig. 1D). To further confirm our findings in SVOG cells, we next used primary hGL cells obtained from women undergoing IVF to investigate the up-regulated effects of BMP2 on LOX expression. Similarly, treatment with BMP2 increased the protein levels of LOX in a concentration-dependent manner in primary hGL cells (Fig. 1E). To investigate whether BMP2-induced up-regulation of LOX expression correlates with an increase in LOX activity, we used an enzyme-linked immunoassay to measure the activity of LOX in the conditioned medium obtained from cells treated with BMP2. The results showed

that 100 ng/ml BMP2 significantly increased the LOX activity in SVOG cells (Fig. 1F). To further confirm the catalytic effects of BMP2 on collagen deposition in human granulosa cells, we used a soluble collagen assay to quantify the amounts of soluble collagen in the conditioned medium obtained from cultured primary hGL cells. The result showed that treatment with 50 ng/ml BMP2 significantly decreased the concentrations of soluble collagen in conditioned medium in primary hGL cells (Fig. 1G).

### 3.2. BMP2 up-regulates the expression of SNAIL in the SVOG and primary hGL cells

To investigate whether BMP2 regulates the expression of SNAIL, we treated the SVOG cells with vehicle control or different concentrations (1, 10 or 100 ng/ml) of BMP2. The results showed that 10 or 100 ng/ml BMP2 significantly up-regulated the mRNA (at 12 h) and protein (at 24 h) levels of SNAIL (Fig. 2A and B). Additionally, the time-course studies showed that 50 ng/ml BMP2 increased the SNAIL mRNA levels starting at 1 h and the stimulatory effects persisted until 6 h after treatment (Fig. 2C). Similarly, 50 ng/ml BMP2 increased the SNAIL protein levels starting at 3 h and persisted until 12 h after treatment (Fig. 2D). Next, we used the primary hGL cells to further confirm the effect of BMP2 on SNAIL expression. The results showed that only 100 ng/ml BMP2 significantly increased the protein levels of SNAIL in primary hGL cells (Fig. 2E).

### 3.3. BMP2 up-regulates the expression of SLUG in the SVOG and primary hGL cells

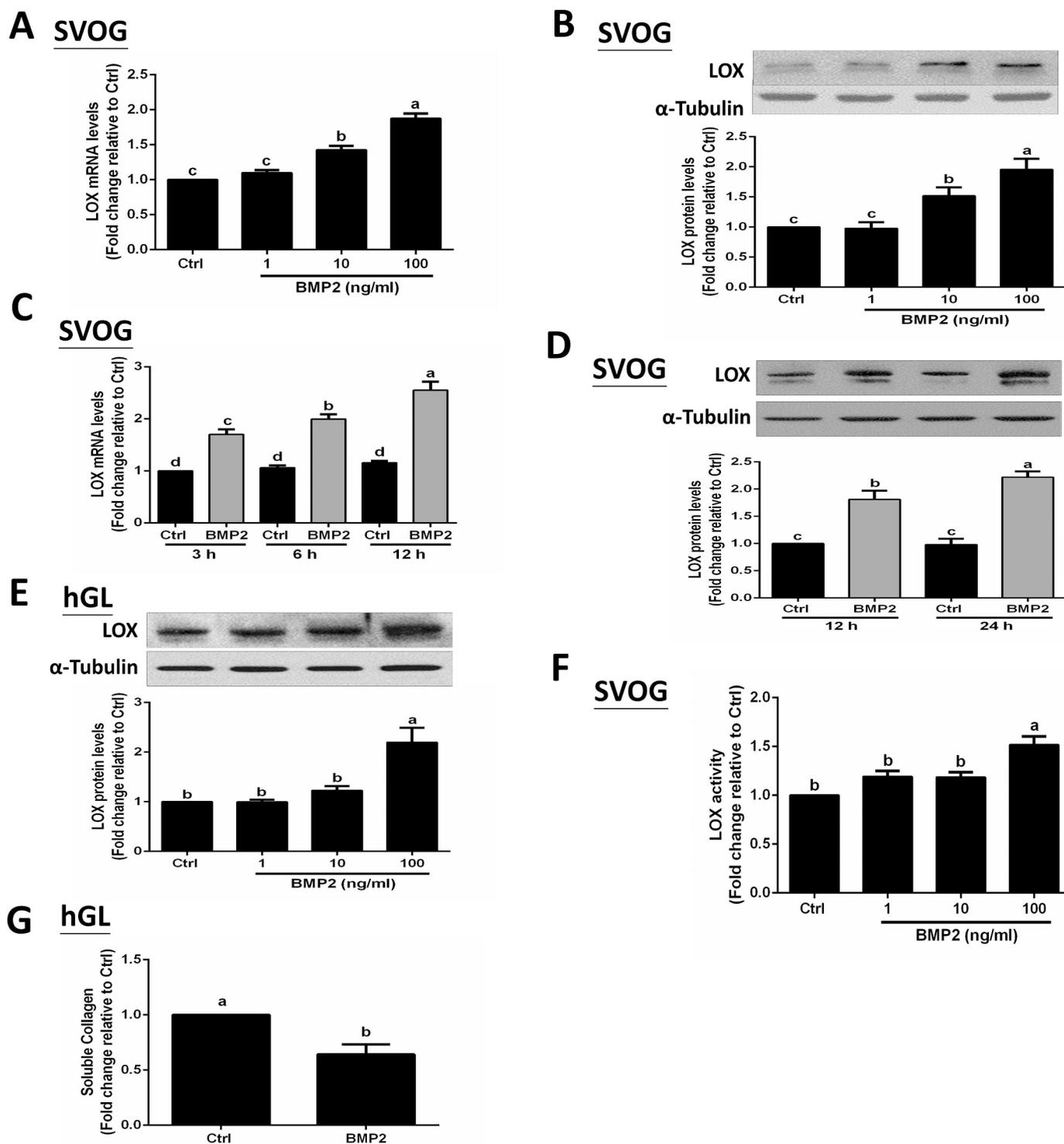
Since SNAIL and SLUG belong to the same SNAIL family that shares a similar DNA binding structure of zinc finger motif [26], we next investigated the effect of BMP2 on the expression of SLUG in hGL cells. As shown in Fig. 3A and B, treatment with 100 ng/ml BMP2 for 12 h or 24 h significantly increased the mRNA (12h) and protein (24 h) levels of SLUG in SVOG cells. Additionally, the time-course studies showed that 50 ng/ml BMP2 increased the SLUG mRNA levels starting at 1 h and the stimulatory effects persisted until 6 h after treatment (Fig. 3C). Similarly, 50 ng/ml BMP2 increased the SLUG protein levels started at 3 h and persisted until 12 h after treatment (Fig. 3D). We also used the primary hGL cells to further confirm the effect of BMP2 on SLUG expression. The results showed that both 10 ng/ml and 100 ng/ml BMP2 significantly increased the protein levels of SLUG in primary hGL cells (Fig. 3E).

### 3.4. SNAIL, but not SLUG, mediates BMP2-induced up-regulation of LOX expression in hGL cells

A previous study has shown that SNAIL is involved in the formation of ECM [27]. To demonstrate whether SNAIL or SLUG mediates BMP2-induced up-regulation of LOX expression, small interfering RNAs targeting SNAIL and SLUG were used to knockdown the endogenous SNAIL and SLUG. As shown in Fig. 4A and C, knockdown of SNAIL partially reversed the BMP2-induced increases in mRNA (Fig. 4A) and protein (Fig. 4C) levels of LOX. Whereas, knockdown of SLUG did not have such effects (Fig. 4B and D). Notably, knockdown of SNAIL partially reversed BMP2-induced increase in LOX activity in SVOG cells (Fig. 4E), indicating that SNAIL but not SLUG is involved in BMP2-induced increases in LOX expression and activity.

### 3.5. DMH-1 and dorsomorphin, but not SB-431542, inhibit BMP2-induced up-regulation of SNAIL expression

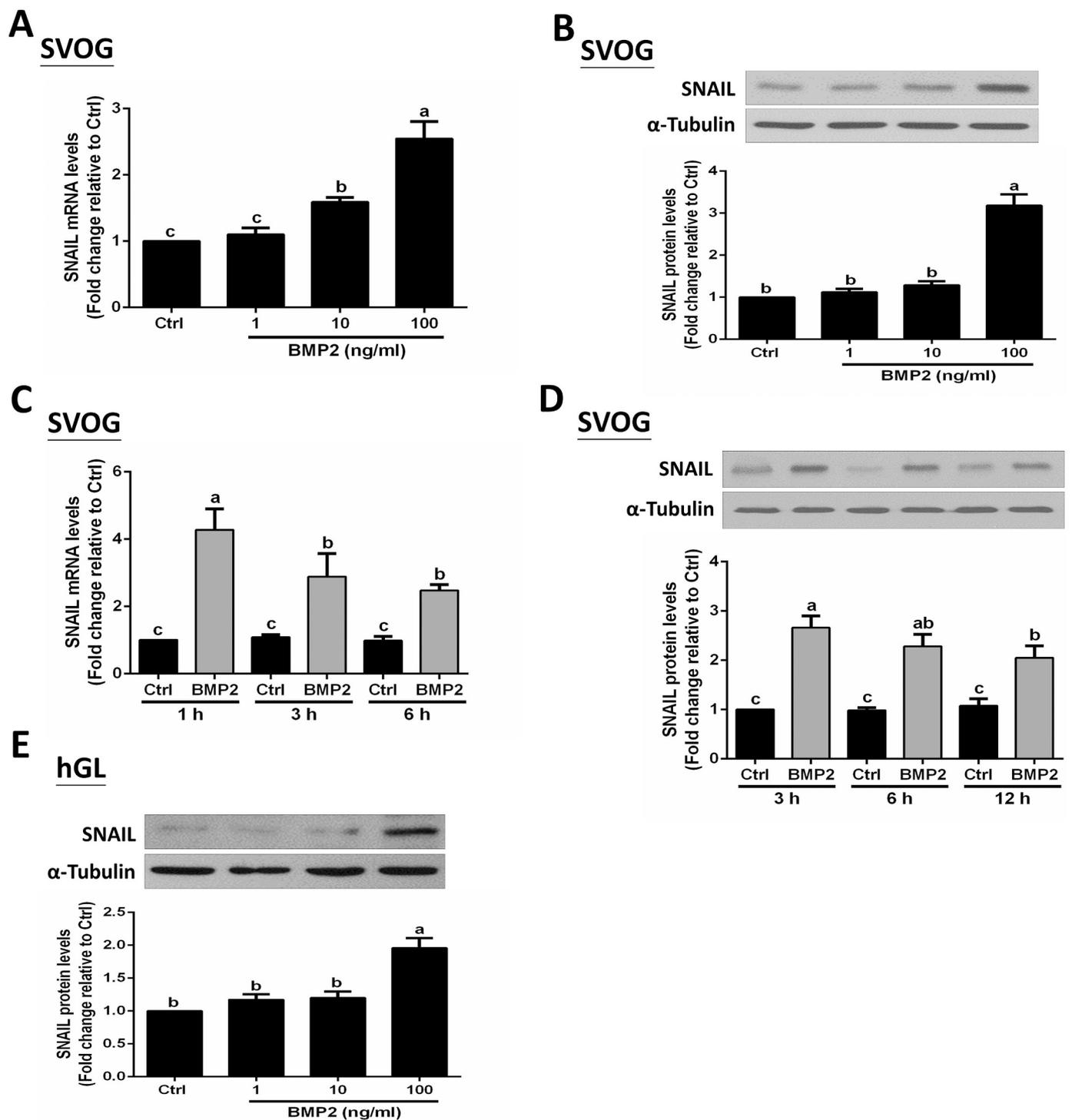
In the canonical BMP signaling pathway, BMP2 activates the SMAD1/5/8 signaling via binding to the BMP type I receptors in diverse cells. The pharmacological inhibitors of type I receptor have been shown to prohibit BMP2-induced activation of SMAD1/5/8 signaling in



**Fig. 1.** BMP2 increases LOX expression and activity in hGL cells. A and B, SVOG cells were treated with vehicle control or different concentrations (1, 10 or 100 ng/ml) of BMP2 for 12 h or 24 h, the mRNA (12 h) and protein (24 h) levels of LOX were examined using RT-qPCR (A) and western blot analysis (B), respectively. C and D, SVOG cells were treated with 50 ng/ml BMP2 for 3, 6 or 12 h, and the mRNA (3, 6 or 12 h) and protein (12 or 24 h) levels of LOX were examined using RT-qPCR (C) and western blot analysis (D), respectively. E, Primary hGL cells were treated with vehicle control or different concentration (1, 10 or 100 ng/ml) of BMP2 for 24 h, the protein levels of LOX were examined using western blot analysis. F, The SVOG cells were treated with vehicle control or different concentration of BMP2 (1, 10 or 100 ng/ml), and the activity of LOX was examined using an enzyme fluorescent assay. G, Primary hGL cells were treated with vehicle control or 50 ng/ml BMP2 for 24 h, and the concentrations of soluble collagen were measured using a soluble collagen assay. The results are expressed as the mean ± SEM of at least three independent experiments. Values without common letters are significantly different ( $P < .05$ ). Ctrl, control; hGL, human granulosa-lutein; SVOG, human granulosa cell line.

human granulosa-lutein cells [28]. To determine whether BMP type I-mediated downstream signaling pathway is involved in BMP2-induced up-regulation of SNAIL expression, three specific inhibitors (DMH-1,

dorsomorphin and SB431542) were utilized in this study. The SVOG cells were pre-treated with three different inhibitors for 30 mi and then treated with 50 ng/ml BMP2 for an additional 12 h. The results showed



**Fig. 2.** BMP2 up-regulates the expression levels of SNAIL in hGL cells. A and B, SVOG cells were treated with vehicle control or different concentrations of BMP2 (1, 10 or 100 ng/ml) for 12 or 24 h, the mRNA (12 h) and protein (24 h) levels of SNAIL were examined using RT-qPCR and western blot analysis, respectively. C and D, SVOG cells were treated with 50 ng/ml BMP2 for 1, 3, 6 or 12 h, the mRNA (C) and protein (D) levels of SNAIL were examined using RT-PCR and western blot analysis, separately. E, Primary hGL cells were treated with vehicle control or different concentrations (1, 10 or 100 ng/ml) of BMP2 for 6 h, the protein levels of SNAIL were examined using western blot analysis. The results are expressed as the mean  $\pm$  SEM of at least three independent experiments. Values without common letters are significantly different ( $P < .05$ ). Ctrl, control; hGL, human granulosa-lutein; SVOG, human granulosa cell line.

that DMH-1 (Fig. 5A) or dorsomorphin (Fig. 5B) completely abolished BMP2-induced increases in SNAIL mRNA levels, whereas SB431542 did not have such effect in SVOG cells (Fig. 5C). Consistent with the results of mRNA, DMH-1 or dorsomorphin but not SB431542 completely

abolished BMP2-induced increases in SNAIL protein levels (Figs. 5D-F). These results indicated that BMP type I receptor-mediated signaling is required for BMP2-induced up-regulation of SNAIL expression in hGL cells.

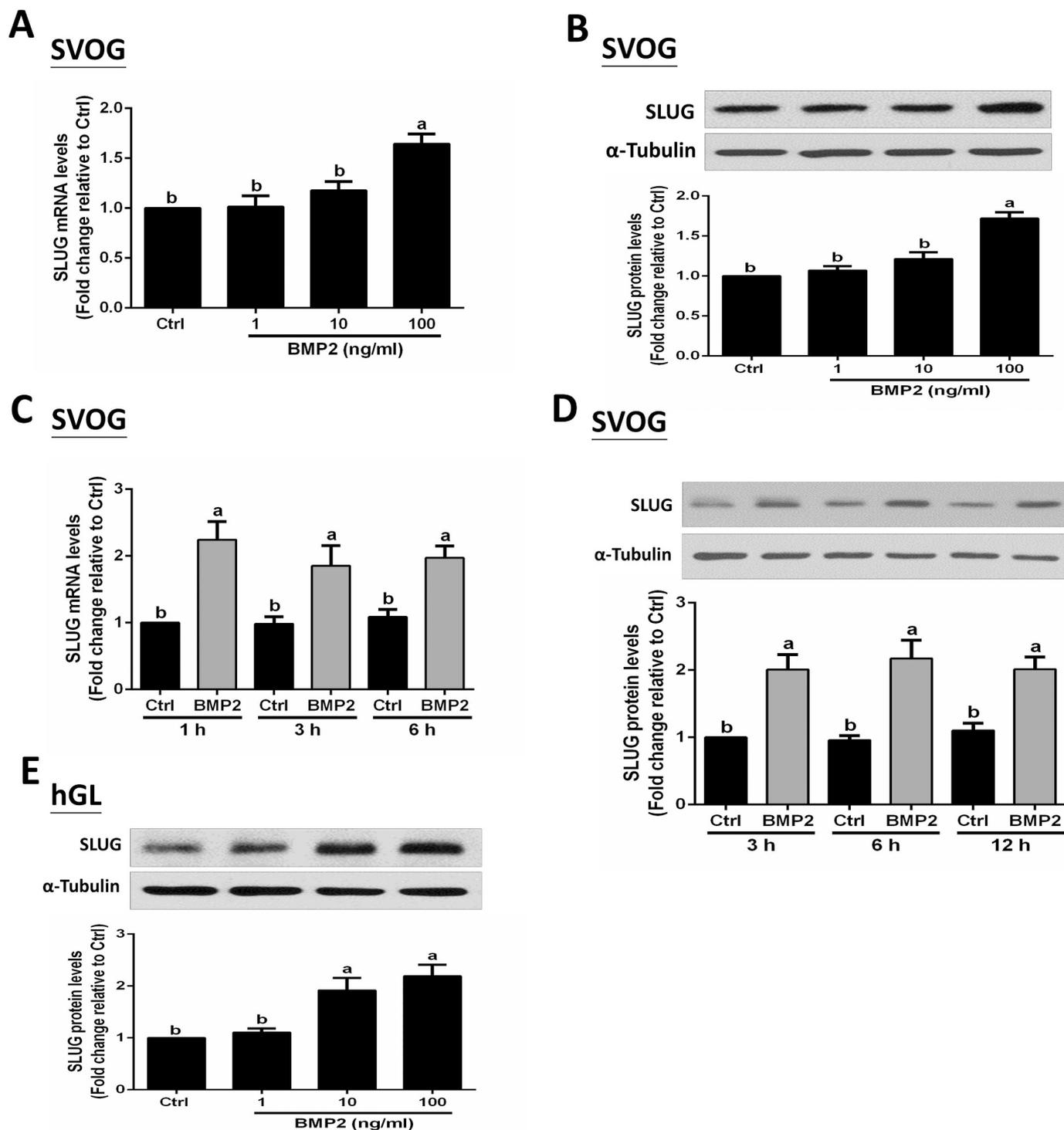
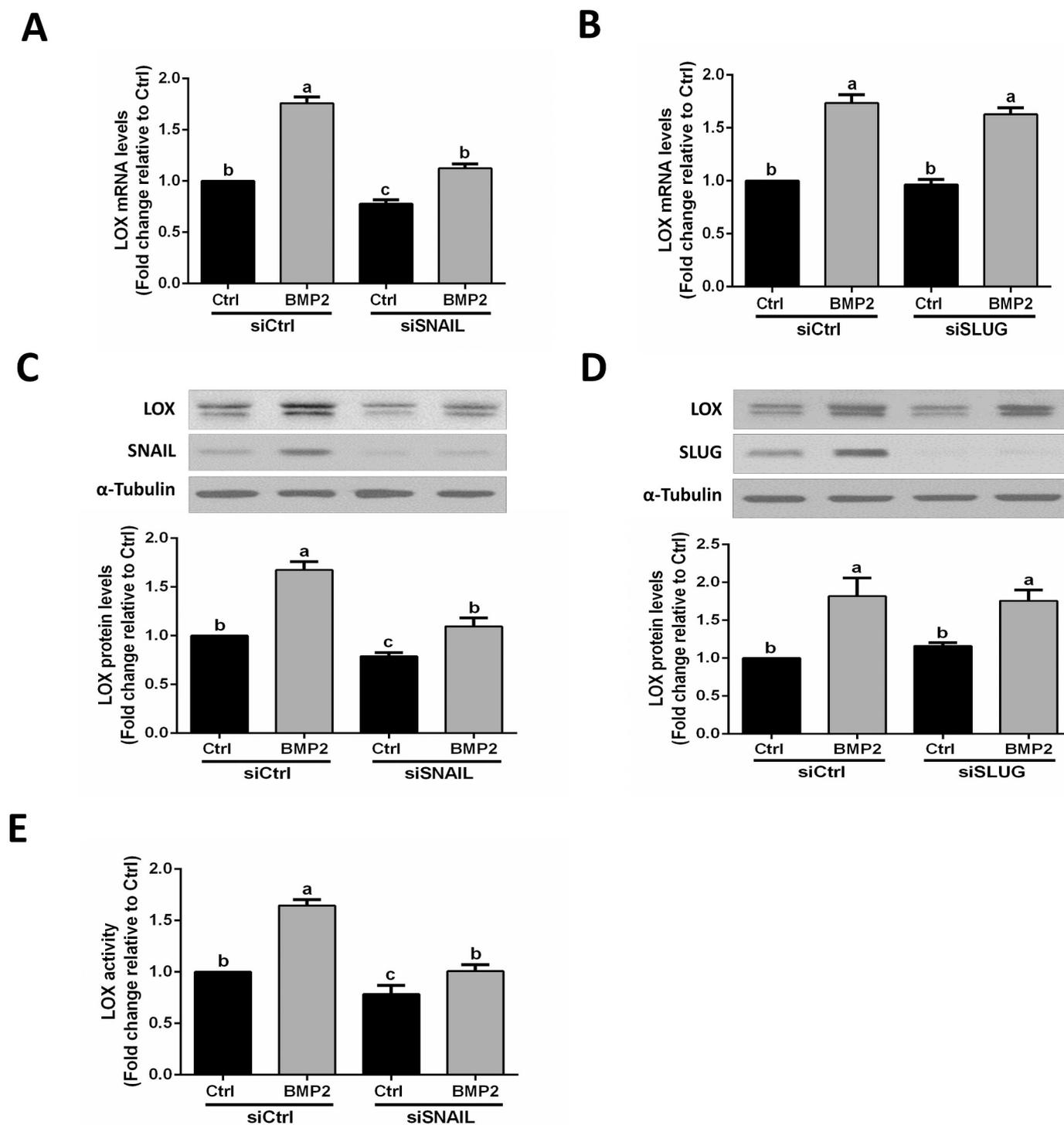


Fig. 3. BMP2 up-regulates the expression of SLUG in hGL cells. A and B, SVOG cells were treated with vehicle control or different concentrations (1, 10 or 100 ng/ml) of BMP2 for 12 or 24 h, the mRNA (12 h) and protein (24 h) levels of SLUG were examined using RT-qPCR and western blot analysis, respectively. C and D, SVOG cells were treated with 50 ng/ml BMP2 for 1, 3, 6 or 12 h, the mRNA (C) and protein (D) levels of SLUG were examined using RT-PCR and western blot analysis, respectively. E, Primary hGL cells were treated with vehicle control or different concentrations (1, 10 or 100 ng/ml) of BMP2 for 24 h, the protein levels of SLUG were examined using western blot analysis. The results are expressed as the mean  $\pm$  SEM of at least three independent experiments. Values without common letters are significantly different ( $P < .05$ ). Ctrl, control; hGL, human granulosa-lutein; SVOG, human granulosa cell line.

3.6. SMAD4 is required for BMP2-induced up-regulation of SNAIL expression and increases in LOX expression and activity in SVOG cells

SMAD4 is a common regulator in the canonical SMAD-dependent signaling pathway. Upon BMP binding with its receptors, the induced phosphorylation of SMAD1/5/8 further bind to the SMAD4, which

forms a complex and translocates into the nucleus to regulate the target gene expression [29]. To investigate the involvement of the SMAD4 in BMP2-induced up-regulation of LOX and SNAIL expression in hGL cells, the specific siRNA targeting SMAD4 was utilized to knockdown the endogenous SMAD4. The results showed that knockdown of SMAD4 completely reversed BMP2-induced up-regulation of LOX (Fig. 6A and

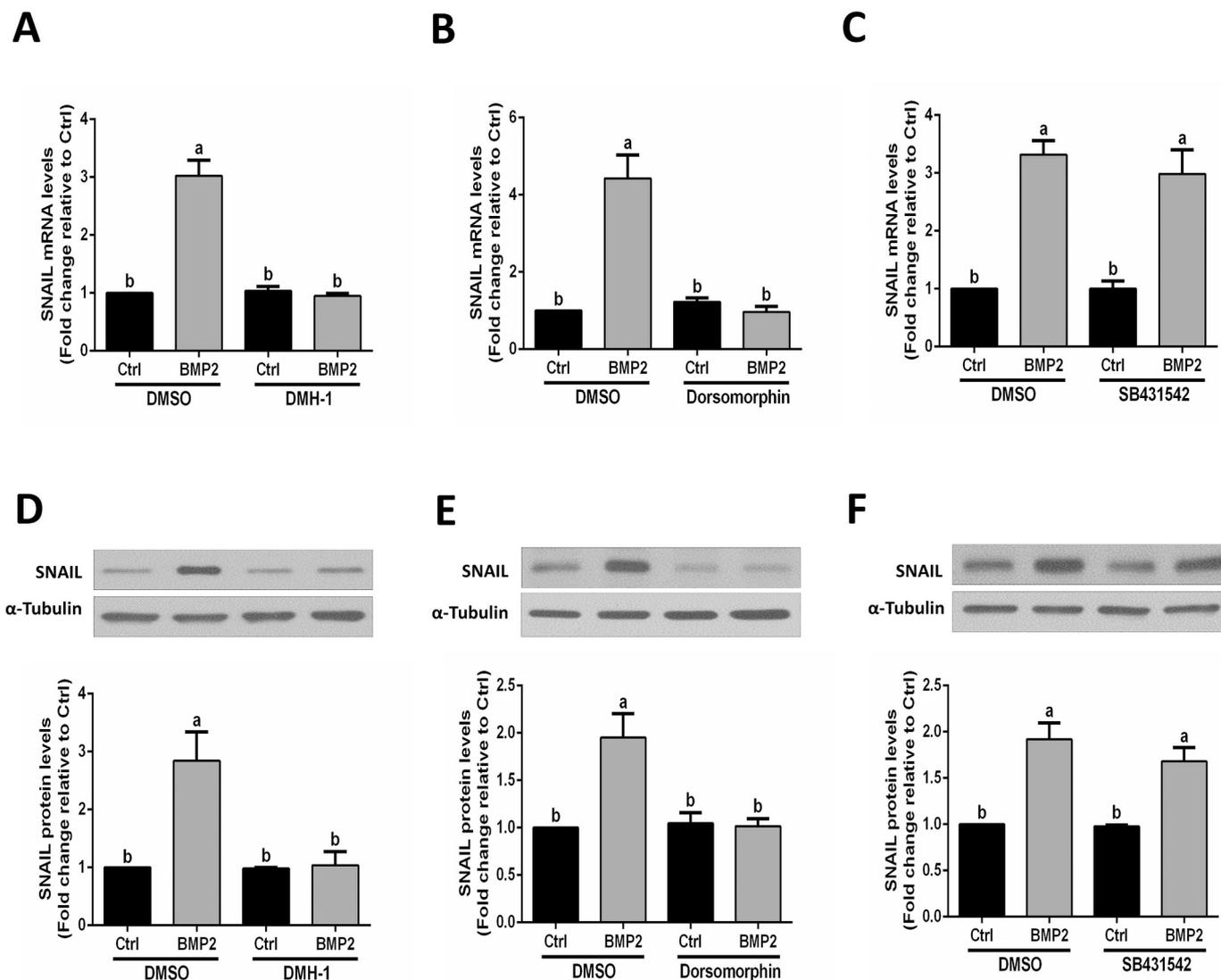


**Fig. 4.** SNAIL mediates BMP2-induced increases in LOX expression and activity in SVOG cells. A and B, SVOG cells were transfected with 25 nM of siCtrl, siSNAIL (A) or siSLUG (B) for 48 h, and then treated with 50 ng/ml BMP2 for an additional 12 h. The mRNA levels of LOX were examined using RT-qPCR. C and D, SVOG cells were transfected with 25 nM of siCtrl, siSNAIL (C) or siSLUG (D) for 48 h, and then treated with 50 ng/ml BMP2 for an additional 24 h. The protein levels of LOX were examined using western blot analysis. E, SVOG cells were transfected with 25 nM of siCtrl or siSNAIL for 48 h, and then treated with 50 ng/ml BMP2 for an additional 24 h. The activity of LOX was examined using an enzyme fluorescent assay. The results are expressed as the mean  $\pm$  SEM of at least three independent experiments. Values without common letters are significantly different ( $P < .05$ ). Ctrl, control; siCtrl, control small interfering RNA; siSNAIL, SNAIL small interfering RNA; siSLUG, SLUG small interfering RNA.

C) and SNAIL (Fig. 6B and D), at both mRNA and protein levels. Notably, knockdown of SMAD4 completely reversed the BMP2-induced increase in LOX activity (Fig. 6E). These findings indicate that SMAD4 is required for BMP2-induced up-regulation of SNAIL expression and increases in LOX expression and activity in SVOG cells.

#### 4. Discussion

In mammals, the LOX enzyme-mediated ECM formation plays an essential role in the regulation of various biological functions including reproduction [3,30]. The dysregulation of LOX has been shown to be

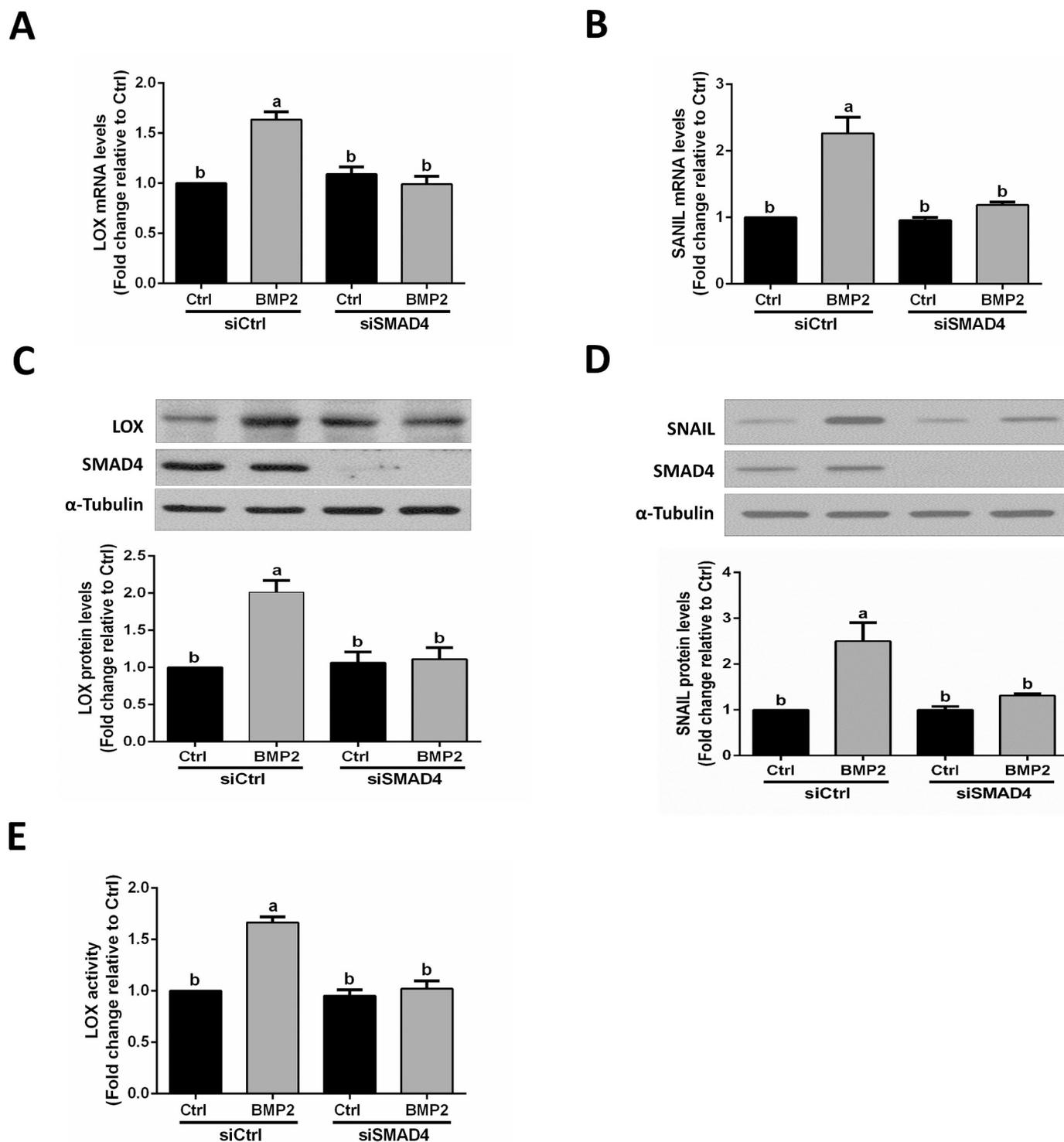


**Fig. 5.** The effects of inhibitors DMH-1, dorsomorphin and SB-431542 on the BMP2-induced up-regulation of SNAIL expression in SVOG cells. A-F, SVOG cells were pre-treated with DMSO (vehicle control), DMH-1, dorsomorphin or SB-431542 for 1 h, and then treated with 50 ng/ml BMP2 for an additional 12 or 24 h. The mRNA (A, B and C) and protein (D, E and F) levels of SNAIL were examined using RT-qPCR (12 h) and western blot analysis (24 h), respectively. The results are expressed as the mean  $\pm$  SEM of at least three independent experiments. Values without common letters are significantly different ( $P < .05$ ). DMSO, dimethylsulfoxide; SVOG, human granulosa cell line.

correlated with certain pathological events, including two common causes of female infertility, endometriosis and polycystic ovary syndrome (PCOS) [8,31,32]. In particular, exogenous DHEA up-regulates the expression of LOX which contributes to one of the causes of the pathogenesis of PCOS in a rat model [8]. Given the critical role of LOX in the development of physiological and pathological conditions, the study regarding the regulation of LOX expression and activity has been a subject of considerable research. In the present study, using primary and immortalized human granulosa-lutein cells as a study model, our experimental data showed that cells exposed to different concentrations of BMP2 displayed an up-regulation of LOX expression and increase in LOX activity. Furthermore, we demonstrated that treatment with BMP2 indeed decreased the concentrations of soluble collagen in conditioned medium in hGL cells. Consistent with our previous studies, we have demonstrated that other TGF- $\beta$  superfamily members, TGF- $\beta$ 1, GDF8 and activin A can increase the expression and activity in hGL cells. Taken together, these findings suggest that these intraovarian growth factors may be involved in the regulation of ECM formation in the periovarian follicles, most likely through the up-regulation of LOX expression and activity in human granulosa cells. However, we did not

provide the evidence that BMP2 directly modulates the intrafollicular ECM formation and stabilization because of lacking human tissues (cumulus-oocyte complex or human ovary). The presented methodology has certain limitations and further *in vivo* study performed using animal models to demonstrate the functional role of BMP2 in regulating follicular ECM formation will be of great interest.

Since the aberrant LOX expression and activity have been implicated in the pathogenesis of gynecological diseases (PCOS and endometriosis), a comprehensive understanding of the molecular mechanism of the cellular response to BMP2-induced up-regulation of LOX is crucial for developing new pharmacological strategies for clinical treatment. Previous studies have shown that several TGF- $\beta$  superfamily members can increase the expression of SNAIL and SLUG in a variety of cells. Intriguingly, only SNAIL is involved in the regulatory effects of TGF- $\beta$  superfamily members on diverse cellular physiological functions [33,34]. In the current study, we also demonstrated that both SNAIL and SLUG were up-regulated following BMP2 exposure. However, knockdown of endogenous SNAIL but not SLUG partially abolished BMP2-induced increases in LOX expression and activity in hGL cells. These results indicate that although SNAIL and SLUG are close relatives



**Fig. 6.** SMAD4 mediates BMP2-induced up-regulation of SNAIL expression and increase in LOX expression and activity in SVOG cells. A-D, SVOG cells were transfected with 25 nM of siCtrl or siSMAD4 for 48 h, and then treated with 50 ng/ml BMP2 for an additional 12 or 24 h. The mRNA and protein levels of LOX (A and C) and SNAIL (B and D) were examined using RT-qPCR and western blot analysis, respectively. E, SVOG cells were transfected with 25 nM of siCtrl or siSMAD4 for 48 h, and then treated with 50 ng/ml BMP2 for an additional 24 h. The activity of LOX was examined using an enzyme fluorescent assay. The results are expressed as the mean  $\pm$  SEM of at least three independent experiments. Values without common letters are significantly different ( $P < .05$ ). Ctrl, control; siCtrl, control small interfering RNA; siSMAD4, SMAD4 small interfering RNA.

and display a similar transcriptional regulatory pattern, their effects vary with respect to the different target genes. Moreover, knockdown of SNAIL decreased the basal expression levels of LOX, indicating a positive role for SNAIL in maintaining basal LOX expression and activity in hGL cells. At present, the detailed molecular mechanism of how SNAIL regulates LOX remains poorly understood. It has been well-established

that SNAIL can recognize the canonical E-box sequences and bind to the proximal E-box elements at the promoter of the target genes [35]. Notably, it has been demonstrated that the LOX promoter contains an E-box sequence, indicating that SNAIL may directly bind to LOX promoter and regulate gene transcription in hGL cells [36]. In addition, data generated from our experiments showed that knockdown of SNAIL only

partially abolished BMP2-induced up-regulation of LOX expression, indicating that there might be other transcription factors required for the regulatory process of LOX expression induced by BMP2. Further research is needed to investigate the involvement of other transcription factors in the BMP2-induced up-regulation of LOX expression in hGL cells.

Recent years have witnessed increased interest and extensive studies on members of TGF- $\beta$  superfamily functions firmly linking major cellular signaling mediated by SNAIL to implement diverse biological processes in multiple disease contexts [37]. Indeed, the up-regulation of SNAIL in different cells are dependent on diverse signaling pathways. In human trophoblast cells, TGF- $\beta$ 1 and activin A up-regulate the expression of SNAIL via the SMAD2/3 signaling pathway [34,38]. Interestingly, both integrin  $\beta$ 3 and SNAIL are involved in promoting endometrial cancer cells migration. However, activin B up-regulates the expression of integrin  $\beta$ 3 via SMAD2/3 signaling pathway, while activin B up-regulates the expression of SNAIL via MEK-ERK1/2 signaling pathway [39,40]. Our previous study has demonstrated that both DMH-1 and dorsomorphin (BMP type I receptor inhibitors) inhibit BMP2-induced phosphorylation of SMAD1/5/8 and the target gene expression in hGL cells [28]. In the current study, we showed that both pharmacological inhibitors DMH-1 and dorsomorphin also completely inhibited BMP2-induced up-regulation of SNAIL expression in hGL cells. Collectively, these findings suggest that the canonical SMAD1/5/8 signaling pathway is involved in BMP2-induced up-regulation of SNAIL expression. In addition, the SMAD1/5/8 transcription factors activated by BMP receptors form trimeric complexes with the common SMAD, SMAD4 to target specific genes for cell functions [41]. Notably, our SMAD4 knockdown experiments showed that SMAD4 is required for BMP2-induced up-regulation of SNAIL expression in hGL cells. Taken together, our results indicate that BMP2-induced up-regulation of SNAIL expression is most likely mediated via the SMAD1/5/8-SMAD4 signaling pathway in hGL cells. The advancements in our understanding of the molecular interactions and mechanisms that underlie BMP2 and LOX may provide insights into physiological and pathophysiological roles of these intrafollicular molecules (BMP2 and LOX), which increases opportunities to achieve more efficient and safe therapies for related ovarian disorders.

In conclusion, the present study has demonstrated that BMP2 up-regulates the expression of the LOX, SNAIL and SLUG in hGL cells. SNAIL, but not SLUG, mediates the BMP2-induced increases in LOX expression and activity. Additionally, DMH-1 and dorsomorphin abolish BMP2-induced up-regulation of SNAIL expression. Furthermore, knockdown of SMAD4 completely reverses BMP2-induced up-regulation of SNAIL expression and increases in LOX expression and activity. Our in vitro study indicates that the transcription factor SNAIL mediates BMP2-induced increases in LOX expression and activity, most likely via the SMAD-dependent pathway in hGL cells. These findings provide insight into the physiological roles and the underlying molecular mechanisms of the intra-ovarian BMP2 in regulating the formation of ECM during the periovulatory stage.

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## Disclosure statement

The authors have nothing to disclose.

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