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BMP6 increases TGF-β1 production by up-regulating furin expression in human granulosa-lutein cells

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ABSTRACT

Bone morphogenetic protein 6 (BMP6) and transforming growth factor-β1 (TGF-β1) are key intraovarian regulators that play essential roles in regulating mammalian follicular function and promoting oocyte maturation. Furin, a member of the subtilisin-like proprotein convertase family, promotes the activation of diverse functional proteins by cleaving protein precursors in the secretory pathway. The aim of this study was to investigate the effect and underlying molecular mechanisms by which BMP6 regulates the expression of furin to increase TGF-β1 production. Primary and immortalized (SVOG) human granulosa-lutein (hGL) cells were used as study models. Our results show that BMP6 significantly up-regulated the expression of furin and increased the production of TGF-β1 in hGL cells. Using dual inhibition approaches (kinase receptor inhibitors and small interfering RNA-targeted knockdown), we demonstrate that both activin receptor-like (ALK)2 and ALK3 are involved in the BMP6-induced up-regulation of furin. Additionally, knockdown of furin abolished BMP6-induced increases in TGF-β1 production. Moreover, knockdown of endogenous SMAD4 reversed the BMP6-induced increase in furin expression. These results indicate that the ALK2/3-mediated canonical SMAD signaling pathway is required for the stimulatory effect of BMP6 on furin expression, which in turn increases the production of TGF-β1 in hGL cells. Our findings provide insights into the molecular interactions and mechanisms of two intrafollicular growth factors in hGL cells.

1. Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily that play essential roles in embryonic patterning, tissue remodeling, and physiological homeostasis maintenance [1]. Recent studies have indicated that BMP signaling plays regulatory roles in the female reproductive system, including follicular development, ovarian steroidogenesis, ovulatory processes, and luteal function [2–4]. BMP6 belongs to the BMP subfamily and has been detected in the oocyte of primordial follicles and granulosa cells of antral follicles in mammals, such as humans [5,6]. Functional studies have shown that BMP6 is involved in primary/secondary follicle transition, dominant follicle selection, ovarian steroidogenesis, follicle atresia, the prevention of luteinization, and luteolysis [4,7,8]. Targeted depletion of *Bmp6* in female mice reduced the ovulation rate and impaired oocyte quality and embryo implantation, resulting in smaller litter sizes [9]. Furthermore, data from clinical studies suggest that dysregulated BMP6 signaling could be associated with women with ovulatory dysfunction, such as polycystic ovary syndrome (PCOS) [6]. BMP ligands initiate cellular action by binding to two constitutively active type I (also known as activin receptor-like receptors, ALKs) and type II receptors to phosphorylate the BMP receptor-responsive SMAD proteins SMAD1/5/8. These phosphorylated SMAD1/5/8 proteins then bind to a common SMAD, SMAD4, to form a complex that can translocate to the nucleus to regulate gene expression [10]. In mammals, five type II receptors (ActRII, ActRIIB, BMPRII, TβRII, and AMHRII) and seven type I receptors (ALK1-7) have been identified [11]. Studies have shown that type I receptors mainly determine the specificity of the downstream signaling pathway [12]. At present, which type I receptor and which downstream SMAD protein mediate BMP6-induced cellular functions in human granulosa cells remains to be determined.

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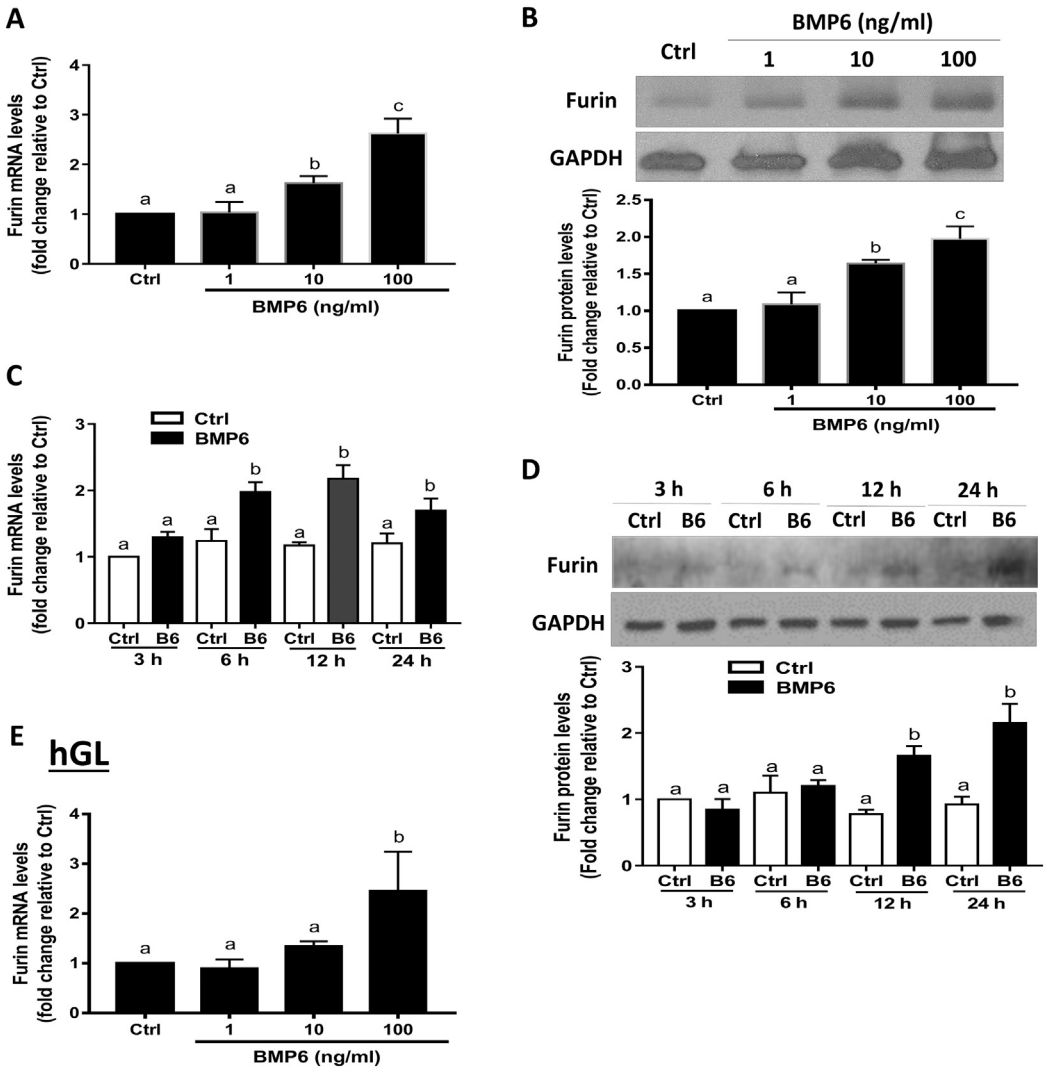

Fig. 1. BMP6 up-regulates the expression of furin in human granulosa-lutein cells. A and B, SVOG cells were treated with different concentrations (1, 10, or 100 ng/ml) of recombinant human BMP6 (BMP6) for 12 h (A) or 24 h (B), and furin mRNA (A) and protein (B) levels were examined using RT-qPCR (A) and western blot analysis (B), respectively. C and D, SVOG cells were treated with 50 ng/ml BMP6 for 3, 6, 12, or 24 h, and furin mRNA (C) and protein (D) levels were examined using RT-qPCR (C) and western blot analysis (D), respectively. E, Primary human granulosa-lutein (hGL) cells were treated for with different concentrations (1, 10, or 100 ng/ml) of BMP6 for 12 h, and furin mRNA levels were examined using RT-qPCR. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P < .05$). Ctrl, control; B6, BMP6.

TGF-β1 is a canonical member of the TGF-β superfamily that is expressed in the oocytes, granulosa cells and theca cells of growing follicles [13]. Studies using conditional knockout in mice and in vitro experiments have demonstrated that TGF-β1 is critical to regulating multiple reproductive functions, including folliculogenesis, steroidogenesis, cumulus expansion, oocyte maturation, and ovulation [14–19]. A recent association study performed in a cohort of Korean women showed that single nucleotide polymorphisms in the TGF-β1 gene were associated with the development and characteristics of PCOS, suggesting a potential role for TGF-β1 in the pathogenesis of this chronic disease [20]. Given the important regulatory role of TGF-β1 in the female reproductive system, the regulation of this growth factor has been a subject of considerable research. At present, how TGF-β1 is regulated in ovarian follicles is completely unknown. Studies performed using sequence analyses have shown that TGF-β1 is initially synthesized as a 390 amino acid precursor molecule called pre-pro-TGF-β1 [21]. Pre-pro-TGF-β1 is then proteolytically processed to yield mature TGF-β1 via cleavage at a consensus sequence motif, R–X–R–R [22]. In both in vivo and in vitro systems, the TGF-β1 precursor is effectively processed by the subtilisin–kexin family of proprotein convertases [23]. Furin (also known as PCSK3) is a member of the subtilisin-like proprotein convertase family that is the key enzyme responsible for the maturation process of various TGF-β superfamily members [24,25]. Previous studies on furin and substrate precursors in mammalian cultured cell lines have demonstrated that furin is capable of cleaving the precursor of TGF-β1 and subsequently processing the mature bioactive TGF-β1 protein [26]. In this study, we aimed to investigate the effect of BMP6 on the expression of furin, which can itself increase the production of TGF-β1 in human granulosa cells.

2. Materials and methods

2.1. Culture of primary and immortalized human granulosa-lutein cells

Primary human granulosa-lutein (hGL) cells were isolated from follicular fluid samples obtained from patients undergoing in vitro fertilization (IVF). All participants signed a written informed consent form. The study was approved by the Research Ethics Board of the University of British Columbia. All primary hGL cells were collected and purified according to a protocol as previously described [27,28]. Briefly, hGL cells were seeded in 12-well plates (2×10^5 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 μg/ml streptomycin sulfate (Life Technologies, Inc., Carlsbad, California), and 1× GlutaMAX (Life Technologies, Inc., Carlsbad, California). Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C, and the culture medium was changed every other day in all experiments. A human granulosa cell line, SVOG, was used in this study and was produced by transfecting primary hGLcells with the SV40 large T antigen [29]. Cells were counted using a hemocytometer, and cell viability was assessed by a 0.04% Trypan blue

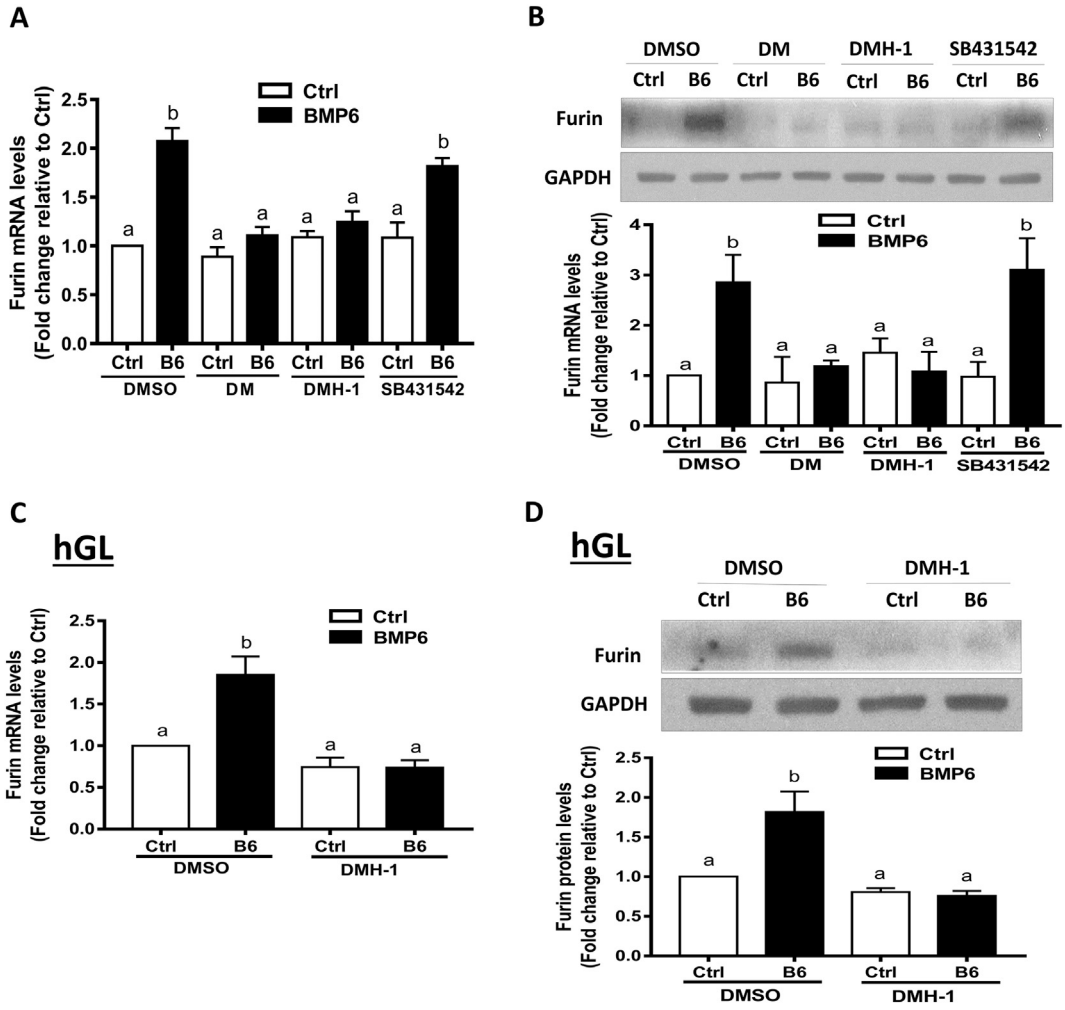

Fig. 2. BMP type I receptors (DM or DMH-1) reversed the BMP6-induced up-regulation of furin in SVOG cells. A and B, SVOG cells were treated with 50 ng/ml BMP6 in the presence of DMSO (a vehicle control), 5 μM dorsomorphin, 5 μM DMH-1 or 5 μM SB431542 for 12 h (A) or 24 h (B), and furin mRNA (A) and protein (B) levels were examined using RT-qPCR (A) and western blot analysis (B), respectively. C and D, Primary human granulosa-lutein cells were treated with 50 ng/ml BMP6 for 12 h (C) or 24 h (D) in the presence of DMSO or 5 μM DMH-1, and furin mRNA (C) and protein (D) levels were examined using RT-qPCR (C) and western blot analysis (D), respectively. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P < .05$). Ctrl, control; B6, BMP6; DM, dorsomorphin.

exclusion test. SVOG cells were seeded in 6-well plates (2–4 × 10^5 cells per well) and cultured in DMEM/F-12 (Sigma-Aldrich Corp.) medium supplemented with 10% 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 of 95% air and 5% CO_2 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 treatment.

2.2. Antibodies and reagents

A polyclonal rabbit anti-furin convertase antibody (PA1-062; diluted at 1:1000) was obtained from Thermo Fisher Scientific (Beverly, MA). A polyclonal rabbit anti-SMAD1/5/8 (N-18; sc-6013-R; diluted at 1:1000) antibody and a monoclonal anti-GAPDH antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal rabbit anti-SMAD4 (9515; diluted 1:1000) antibody, a polyclonal anti-phospho-SMAD1 antibody (Ser463/465)/SMAD5 (Ser463/465)/SMAD8 (Ser426/428) (9511; diluted at 1:1000), a polyclonal rabbit anti-phospho-SMAD2 (Ser465/467) (3101; diluted at 1:1000), and a monoclonal mouse anti-SMAD2 antibody (L16D3) (3103; diluted at 1:1000) were obtained from Cell Signaling Technology (Danvers, MA). Recombinant human BMP6 (P22004), recombinant human TGF-β1 (7754-BH), dorsomorphin dihydrocholoride (dorsomorphin) (3093), and 4-[6-[4-(1-Methylethoxy)phenyl]pyrazolo[1,5-*a*]pyrimidin-3-yl]-quinoline (DMH-1) (4126) were obtained from R&D System (Minneapolis, MN). SB431542 (301836-41-9) was obtained from Sigma-Aldrich.

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

Cells were washed with cold PBS, and total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies Inc.) according to the manufacturer's instructions. A total of 2 μg of RNA was reverse-transcribed into the first-strand cDNA with a random primer and Moloney Murine Leukemia Virus (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 was 20 μl, which included 1 × SYBR Green qPCR MasterMix (Applied Biosystems, Foster City, California), a primer mixture (250 nM) and 20 ng of cDNA. The specificity of each assay was validated using dissociation curve analysis and agarose gel electrophoresis of the PCR products. The following primers were used in the experiments: *FURIN*, 5′-CCTTCTTCCGTGGGGTTAG-3′ (sense) and

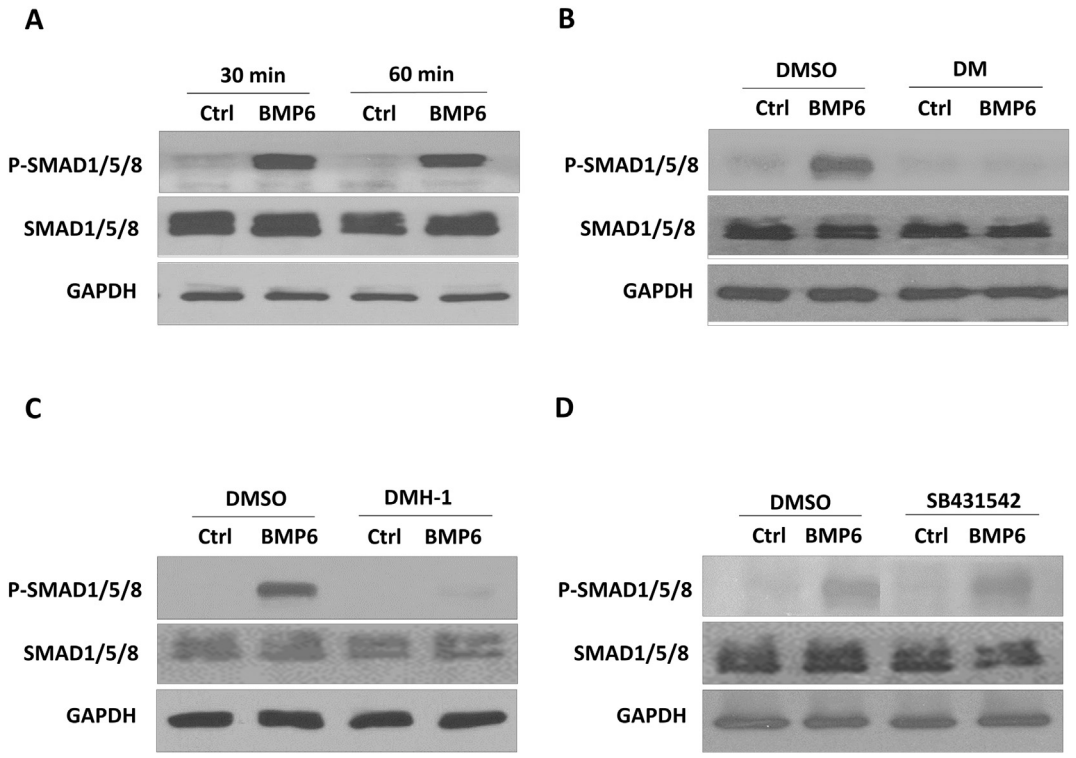

Fig. 3. BMP6 induces the phosphorylation of SMAD1/5/8 in SVOG cells. A, SVOG cells were treated with 50 ng/ml BMP6 for 30 or 60 min, and phosphorylated SMAD1/5/8 protein levels were examined using western blot analysis. B-D, SVOG cells were treated with 50 ng/ml BMP6 in the presence of DMSO, 5 μM dorsomorphin(B), 5 μM DMH-1 (C), or 5 μM SB431542 (D) for 30 min, and phosphorylated SMAD1/5/8 protein levels were examined using western blot analysis. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P < .05$). Ctrl, control; B6, BMP6; DM, dorsomorphin.

5′-GCAGTTGCAGCTGTCATGTT-3′ (antisense); and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5′-ATGGAAATCCCATCACCAT CTT-3′ (sense) and 5′-CGCCCCACTTGATTTTGG-3′ (antisense). Alternatively, a TaqMan gene expression assay was used to detect human TGF-β1, ACVR1 (ALK2), BMPR1A (ALK3), BMPR1B (ALK6), SMAD4 and GAPDH (Hs01931883_s1, Hs00153836_m1, Hs01034913_g1, Hs01010965_m1, Hs01077084_m1, Hs00195437_m1, Hs00195441_m1, Hs00929647_m1 and Hs02758991_g1, respectively; Applied Biosystems). Each 20 μl TaqMan reaction contained 4 μl of cDNA, 5 μl of RNase-free water, 10 μl of 2× TaqMan gene expression master mix (Applied Biosystems) and 1 μl of 20× TaqMan gene expression assay. The specificity of each assay was validated by dissociation curve analysis and agarose gel electrophoresis of PCR products. Assay performance was validated by evaluating amplification efficiencies using means of calibration curves and ensuring that the plot of log input amount vs. ΔCq (also known as ΔCt) had a slope < 0.1. Three separate experiments were performed on different cultures, and each sample was assayed in triplicate. A mean value was used to determine the mRNA levels using the comparative Cq method with the formula $2^{-\Delta\Delta Cq}$ ($2^{-\Delta\Delta Ct}$). GAPDH was used as the reference gene.

2.4. Western blot analysis

Following treatment, 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 Laboratories Inc.). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked for 1 h in Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dried milk and then incubated overnight at 4 °C with the relevant primary antibodies. After they were washed, the membranes were incubated with the peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories Inc.). Immunoreactive bands were detected using enhanced chemiluminescence reagents or SuperSignal West Femto Chemiluminescence Substrate (Pierce) followed by exposure to CL-XPosure film (Thermo Fisher). The membranes were stripped with stripping buffer at 50 °C for 30 min and re-probed with total SMAD1/5/8 or GAPDH antibodies as loading controls. Films were scanned and quantified by densitometry using Scion imaging software (Scion Corp). Furin and SMAD4 levels were normalized to GAPDH levels, whereas phosphorylated SMAD1/5/8 levels were normalized to total SMAD1/5/8 levels.

2.5. Small interfering RNA transfection

We performed transient knockdown assays with an ON-TARGET *plus* SMART pool targeting control pool or a separate ON-TARGET *plus* SMART pool targeting ALK2, ALK3, ALK6, SMAD4, or Furin (Thermo Fisher Scientific). Cells were pre-cultured in antibiotic-free DMEM/F-12 medium containing 10% fetal bovine serum until they were 50–60% confluent and then transfected with 25 nM siRNA using Lipofectamine RNA iMAX (Life Technologies) for 24 or 48 h, as previously described [30,31]. The knockdown efficiency of each target was confirmed using RT-qPCR or western blot analysis.

2.6. Measurement of TGF-β1

Following the specified treatments, cell lysis was assayed immediately, or the lysates were stored at −80 °C until assayed. The accumulation levels of TGF-β1 in the conditioned media were measured according to the manufacturer's instructions using a solid phase sandwich enzyme immunoassay (EIA) kit (DB100B) obtained from R&D

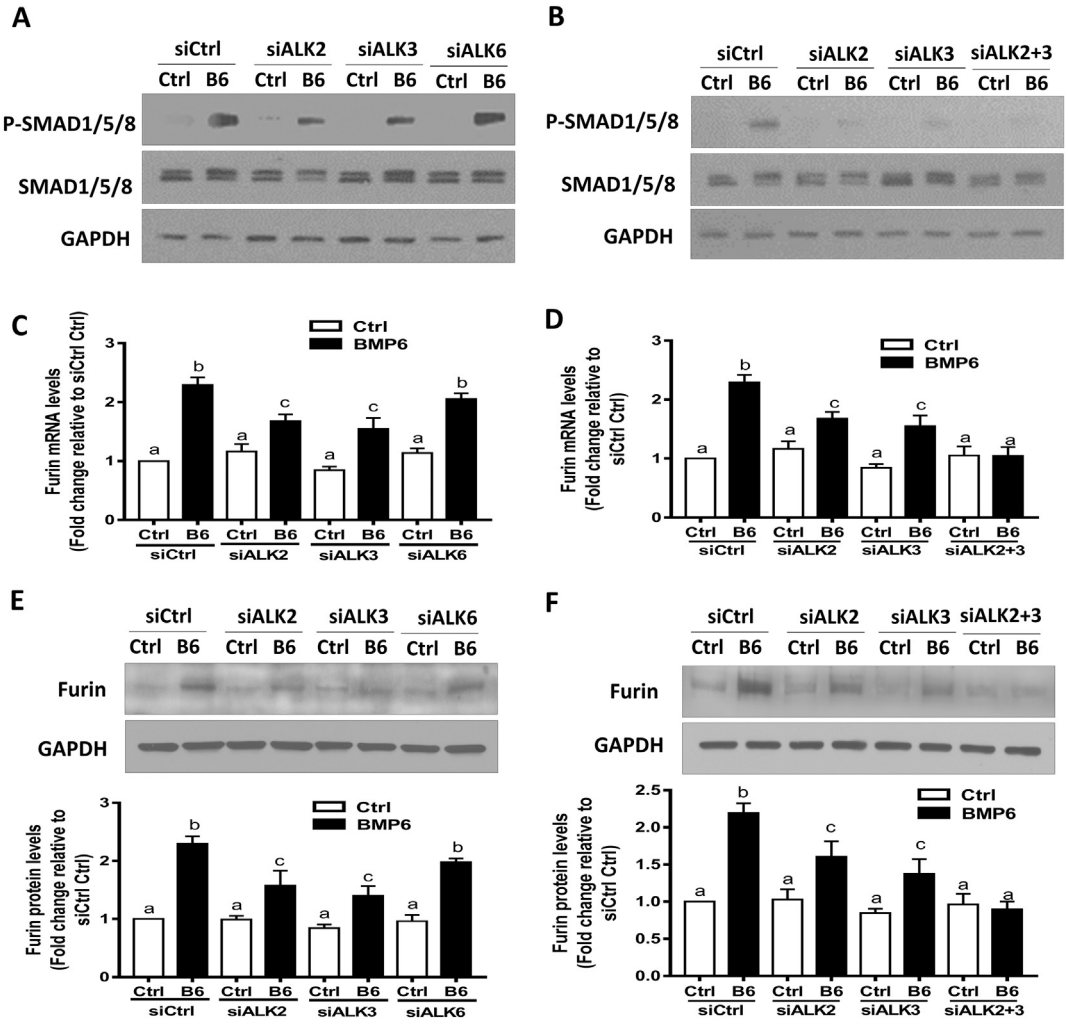

Fig. 4. ALK2 and ALK3 mediate BMP6-induced increases in SMAD1/5/8 phosphorylation and furin expression in SVOG cells. A, C, and E, SVOG cells were transfected with a 25 nM control siRNAs (siCtrl), or an equivalent amount of an siRNA targeting ALK2 (siALK2), ALK3 (siALK3), or ALK6 (siALK6) for 48 h and then treated with 50 ng/ml BMP6 for 60 min (A), 12 h (C) or 24 h (E). The levels of phosphorylated SMAD1/5/8 protein (A), furin mRNA (C), and furin protein (E) were examined using RT-qPCR (C) and western blot analysis (A and E). B, D, and F, SVOG cells were transfected with specific siRNAs (siCtrl, siALK2, siALK3, or siALK2 + 3) for 48 h, and then treated with 50 ng/ml BMP6 for 60 min (B), 12 h (D) or 24 h (F). The levels of phosphorylated SMAD1/5/8 protein (B), furin mRNA (D), and furin protein (F) were examined using RT-qPCR (D) and western blot analysis (B and F). The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P < .05$). ALK, activin receptor-like receptor; Ctrl, control; B6, BMP6.

Systems. The inter- and intra-assay coefficients of variation for this assay were < 10%, and the minimum detectable dose of TGF-β1 ranged from 1.7–15.4 pg/ml. Each sample was measured in triplicate, and secreted TGF-β1 levels were normalized to the total cellular protein content.

2.7. Statistical analysis

Results were analyzed by one-way ANOVA followed by a Tukey's multiple comparison test using PRISM software (GraphPad Software, Inc. San Diego, CA). The results are presented as the mean ± SEM of at least 3 independent experiments performed on different cultures. A significant difference was defined as $P < .05$.

3. Results

3.1. BMP6 up-regulates the expression of furin in human granulosa-lutein cells

Animal studies and studies using clinical samples obtained from humans have shown that the ranges of BMP6 concentrations in the serum of mice, cattle, and humans are 55.46–128.7 pg/ml, 2.75–8.14 ng/ml, and 0.5–2.75 ng/ml, respectively [32,33]. Based on previous granulosa cell culture-related studies [6,34,35], we used a concentration range of 1–100 ng/ml for BMP6 in our in vitro study performed in hGL cells. We first investigated the effects of BMP6 on furin expression in human granulosa cells by treating SVOG cells with increasing concentrations (1, 10, or 100 ng/ml) of recombinant human BMP6 (BMP6) for 12 h or 24 h. As shown in Fig. 1A, treatment with BMP6 for 12 h increased furin mRNA levels in a concentration-dependent manner. Similarly, furin protein levels increased in response to exposure to different concentrations of BMP6 (1, 10, or 100 ng/ml) for 24 h (Fig. 1B). Based on results showing that 10 or 100 ng/ml BMP6 significantly increased the expression of furin, we next chose a concentration of 50 ng/ml BMP6 to perform the time course experiments. As shown in Fig. 1C and D, treating SVOG cells with 50 ng/ml BMP6 increased furin mRNA (effect started from 6 h) and protein (effect started from 12 h) levels, and these stimulatory effects persisted until 24 h. To further investigate the physiological role of BMP6 in the regulation of furin, we used primary hGL cells to confirm the results. Consistent with the results obtained using SVOG cells, treatment with BMP6 (100 ng/ml) for 12 h significantly increased furin mRNA levels in

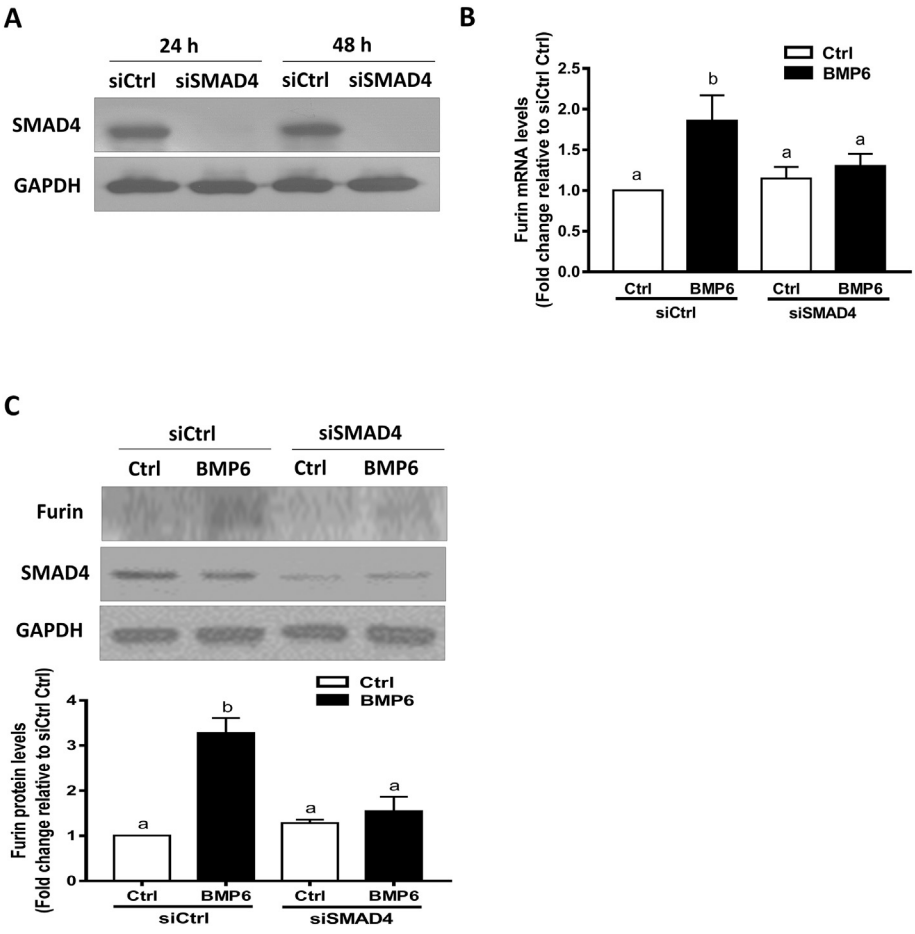

Fig. 5. Knockdown of SMAD4 abolishes the BMP6-induced up-regulation of furin in SVOG cells. A, SVOG cells were transfected with 25 nM siCtrl or 25 nM siSMAD4 for 24 or 48 h, and SMAD protein levels were examined using western blot analysis. B and C, SVOG cells were transfected with 25 nM siCtrl or 25 nM siSMAD4 for 48 h and then treated with 50 ng/ml BMP6 for an additional 12 or 24 h. The resulting furin mRNA (B) and protein (C) levels were examined using RT-qPCR (B) or western blot analysis (C). The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P < .05$). Ctrl, control.

primary hGL cells (Fig. 1E).

3.2. The BMP type I receptor inhibitors dorsomorphin and DMH-1 reverse the BMP6-induced up-regulation of furin in human granulosa-lutein cells

In many cells, BMPs initiate cellular functions by binding to both type I and type II receptors, with type I receptors mainly determining the specificity of the downstream signaling pathway [12]. At present, three type I receptors, including ALK2, ALK3, and ALK6, have been shown to mediate BMP-induced cellular functions [12]. To investigate the molecular mechanisms by which BMP6 up-regulates the expression of furin, we used several small molecule inhibitors of BMP type I receptor kinases to probe the cellular functions of BMP signaling in vitro. Dorsomorphin is a specific inhibitor of ALK2/ALK3/ALK6, and DMH-1 is a selective inhibitor of ALK2/ALK33. SB431542 is a potent inhibitor of ALK4/ALK5/ALK7 [36–38]. Our results showed that pretreatment with 5 μM dorsomorphin or 5 μM DMH-1 for 1 h completely abolished the BMP6 (50 ng/ml for 12 h)-induced increase in furin mRNA levels observed in SVOG cells. However, pretreatment with 5 μM SB431542 did not have a similar effect (Fig. 2A). Consistent with the results obtained for the furin mRNA, pretreatment with dorsomorphin or DMH-1 but not SB431542 completely abolished the BMP6 (50 ng/ml for 24 h)-induced increase in furin protein levels observed in SVOG cells (Fig. 2B). Similarly, experiments performed using primary hGL cells showed that pretreatment with 5 μM DMH-1 completely abolished BMP6-induced increases in furin mRNA (Fig. 2C) and protein (Fig. 2D) levels. These results indicate that BMP type I receptors (most likely ALK2 and ALK3) are required for the BMP6-induced up-regulation of furin in hGL cells.

3.3. BMP6 increases the protein levels of phosphorylated SMAD1/5/8 in SVOG cells

Next, we investigated BMP6-induced downstream signaling in hGL cells by examining the protein levels of phosphorylated SMAD1/5/8. We treated SVOG cells with 50 ng/ml BMP6 for 30 or 60 min, and the results showed that BMP6 significantly increased phosphorylated BMP6 protein levels at the two examined time points (Fig. 3A). Additionally, pretreatment with 5 μM dorsomorphin (Fig. 3B) or 5 μM DMH-1 (Fig. 3C) abolished BMP6-induced increases in phosphorylated SMAD1/5/8 protein levels in SVOG cells. However, pretreatment with 5 μM SB431542 had no such effect (Fig. 3D).

3.4. ALK2 or ALK3 mediates BMP6-induced increases in phosphorylated SMAD1/5/8 and furin protein levels

To further explore which specific ALK(s) mediate(s) BMP-6 induced increases in phosphorylated SMAD 1/5/8 protein levels, siRNA-mediated inhibition approaches were used to knock down specific ALKs. Western blot analyses showed that knocking down either ALK2 or ALK3 partially abolished the BMP6-induced increase in phosphorylated SMAD1/5/8 protein levels (Fig. 4A). However, knockdown of ALK6 did not have a similar effect (Fig. 4A). Furthermore, dual knockdown of ALK2 and ALK3 (siALK2 + 3) completely abolished the BMP6-induced increase in phosphorylated SMAD 1/5/8 protein levels (Fig. 4B). Next, we investigated the functional roles of these BMP type I receptors in the regulation of furin expression in hGL cells. The results showed that single knockdown of either ALK2 or ALK3 but not ALK6 partially abolished BMP6-induced increases in furin mRNA (Fig. 4C) and protein (Fig. 4E) levels. Similarly, dual knockdown of ALK2 and ALK3 completely abolished BMP6-induced increases in furin mRNA (Fig. 4D) and

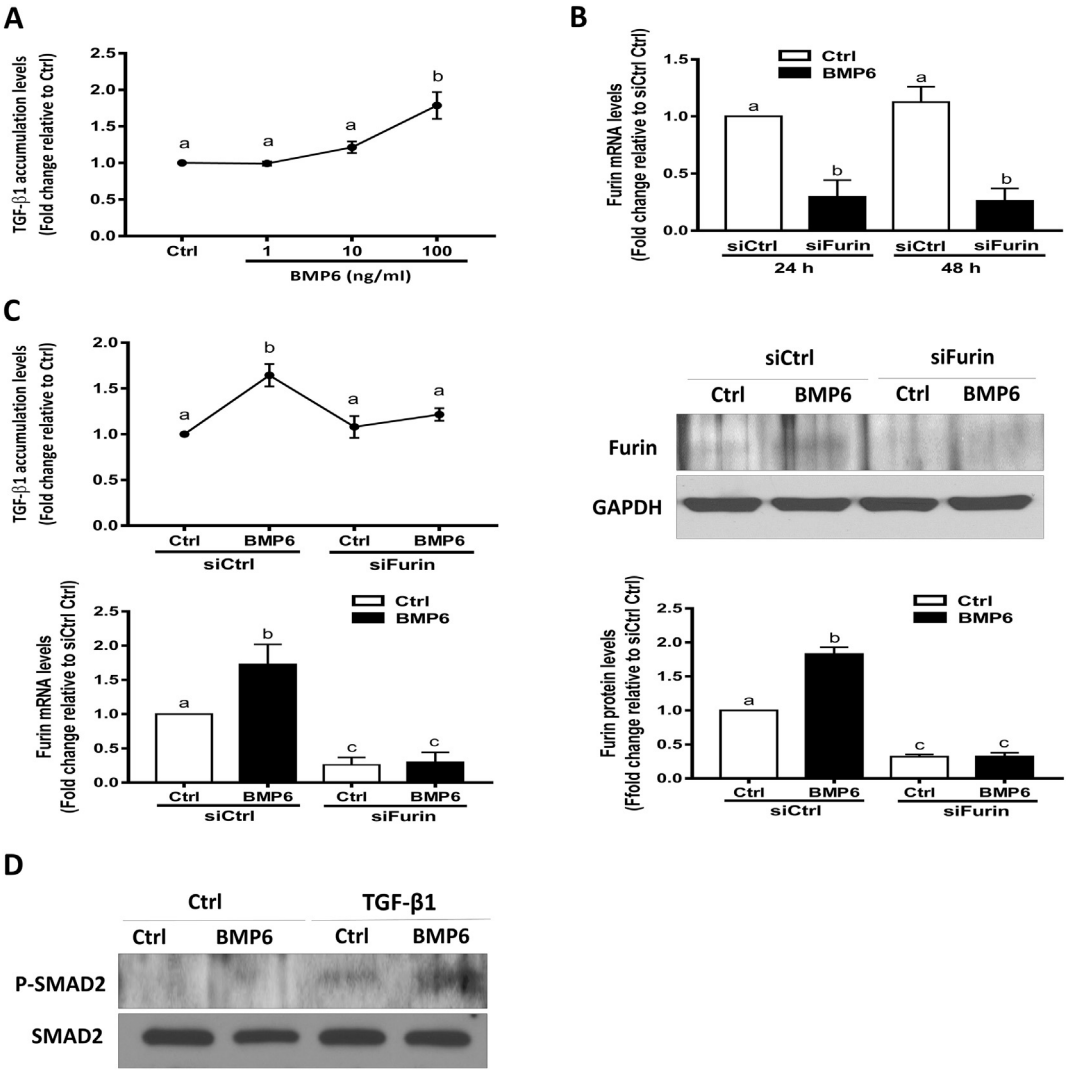

Fig. 6. Knockdown of furin abolished BMP6-induced increases in TGF-β1 production in SVOG cells. A, SVOG cells were treated with vehicle control or different concentrations of BMP6 (1, 10, or 100 ng/ml) for 24 h, and the levels of mature TGF-β1 protein in the conditioned medium were examined using an enzyme immunoassay. B, SVOG cells were transfected with 25 nM siCtrl or 25 nM siFurin for 24 or 48 h, and furin mRNA levels were examined using RT-qPCR. C, SVOG cells were transfected with 25 nM siCtrl or 25 nM siFurin for 48 h, and then treated with 50 ng/ml BMP6 for an additional 24 h. The levels of mature TGF-β1 protein in the conditioned medium were examined using an enzyme immunoassay. Furin mRNA and protein levels were examined using RT-qPCR and western blot analysis, respectively. D, SVOG cells were treated with vehicle control or 50 ng/ml BMP6 for 24 h, and then were treated with 5 ng/ml TGF-β1 for an additional 60 min. The phosphorylated SMAD2 protein levels were examined using western blot analysis. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P < .05$). Ctrl, control.

protein (Fig. 4F) levels in SVOG cells. These results indicate that either ALK2 or ALK3 mediates the observed BMP6-induced increases in phosphorylated SMAD1/5/8 and furin expression in hGL cells.

3.5. The SMAD signaling pathway is required for the BMP6-induced up-regulation of furin in SVOG cells

In the canonical SMAD-dependent signaling pathway, receptor-responsive SMAD proteins recruit a common SMAD (co-SMAD), SMAD4, to further regulate gene transcription [39]. To confirm the role of the SMAD signaling pathway in the BMP6-mediated regulation of furin expression, SVOG cells were treated with BMP6 after SMAD4 knockdown. The knockdown efficiency of SMAD4 in SVOG cells is demonstrated in Fig. 5A. Notably, the siRNA-mediated depletion of SMAD4 abolished BMP6-induced increases in furin mRNA (Fig. 5B) and protein (Fig. 5C) levels. These results indicate that the canonical SMAD signaling pathway is required for the BMP6-induced up-regulation of furin in hGL cells.

3.6. Furin is required for the BMP6-induced up-regulation of TGF-β1 production in SVOG cells

Finally, we examined the regulatory effect of BMP6 on the production of TGF-β1 in hGL cells. As shown in Fig. 6A, treatment with different concentrations of BMP6 (1, 10, or 100 ng/ml) for 24 h increased mature TGF-β1 protein levels in the conditioned medium of SVOG cells. To investigate whether furin is involved in the production of TGF-β1 in hGL cells, we examined the levels of accumulated mature TGF-β1 proteins after furin was depleted. Knockdown efficiency experiments showed that transfection with a 25 nM siRNA targeting furin (siFurin) for 24 or 48 h significantly decreased furin mRNA levels by up to 80% to 90% relative to the results obtained using the control siRNA (siControl) (Fig. 6B). Notably, knocking down furin for 48 h completely abolished the BMP6-induced increase in mature TGF-β1 protein levels in SVOG cells (Fig. 6C). These results indicate that furin is required for the BMP6-induced up-regulation of TGF-β1 production in hGL cells.

3.7. BMP6 enhances TGF-β1-induced increases in phosphorylated SMAD2 protein levels

To further investigate the functional impact of these effects on cell signaling, we searched a downstream signaling transducer activated by TGF-β1. SMAD2 is a well-known downstream molecule of TGF-β1 in hGL cells [18]. Next, TGF-β1 (5 ng/ml, 60 min) was used to induce the activation of SMAD2 following pretreatment with BMP2 (50 ng/ml) for 24 h. The results showed that pretreatment with BMP2 enhanced the increase in phosphorylated SMAD2 protein levels induced by TGF-β1 (Fig. 6D).

4. Discussion

Both TGF-β1 and BMP6 are critical intrafollicular growth factors that play essential regulatory roles during follicular development, oocyte maturation, steroidogenesis, cell-cell interactions, and ovulation [3,4]. Dysregulated TGF-β or BMP signaling has been demonstrated to be associated with several pathological ovarian diseases [4,40]. However, how these two growth factors are interactively regulated has never been fully explained. In the present study, we provide the first data showing that the oocyte- and granulosa cell-derived growth factor BMP6 up-regulates the biosynthesis and maturation of TGF-β1 in hGL cells. This stimulatory effect most likely arises as a result of the BMP6-induced up-regulation of the proprotein convertase furin, which further induces the proteolytic processing and production of mature TGF-β1 protein. This proposal was based on experimental results showing that knocking down furin abolished BMP6-induced increases in TGF-β1 production in hGL cells. Furthermore, our in vitro functional study confirmed that pretreatment with BMP2 enhanced TGF-β1-induced increases in phosphorylated SMAD2 protein, the downstream signal transducer that mediates the cellular activities in response to TGF-β1. Our findings suggest a novel paracrine/autocrine mechanism by which BMP6 can regulate the production of TGF-β1 by promoting its processing enzyme (furin) in hGL cells. As such, this mechanism may provide an additional layer of modulatory control over locally secreted growth factors and could be extended to other intraovarian growth factors processed by furin or other proprotein convertases. These findings also support the notion that furin is the proprotein convertase that is responsible for the mature processing of TGF-β1, as has been shown in other studies performed using different cell models.

Intriguingly, a similar interactive regulatory effect has been shown in human ovarian follicles, as our previous studies revealed that theca cell-derived BMP4 and BMP7 increased activin A production by up-regulating the expression of inhibin βA and furin in human granulosa cells [41]. However, we did not demonstrate that BMP6 up-regulated the transcription of the TGF-β1 precursor in hGL cells. Studies have shown that TGF-β1 induces its own transcription in normal and transformed cells, in that the autoregulation of its transcript by its ligand may provide a positive feedback loop for further developmental regulation [42]. In addition to autoregulation, the transcription of TGF-β1 is selectively responsive to several products of oncogenes and immediate early genes, including AP-1 and BMAL1 [43,44]. In glioma-initiating cells, furin cleaved and promoted the activation of pro-TGF-β1 and pro-TGF-β2; TGF-β2 in turn increased furin levels via an ALK5-dependent pathway [45]. In cardiac fibroblasts, TGF-β1 amplified its activating convertase furin, which was required for TGF-β1-induced increases in MMP activity [46]. In a CHO-derived cell line, when pro-TGF-β1 was co-expressed with furin, the 55-kDa pro-TGF-β1 was converted into 44 and 12.5 kDa molecules corresponding to the pro-region and the mature monomer, respectively, suggesting that the TGF-β1 precursor was efficiently and correctly processed by furin [47]. Furthermore, an in vitro study showed that the addition of a potent furin inhibitor blocked 80% of TGF-β1 processing mediated by endogenous enzymes [25]. Collectively, previous studies and the results obtained in our studies suggest that the stimulatory effect of BMP6 on the production of TGF-β1 is mainly due to the up-regulation of the processing enzyme furin.

Members of the TGF-β superfamily induce cellular activities via ligand-receptor interactions with type I and type II receptors [48]. To date, only a limited number of receptors (five type II receptors and seven type I receptors) have been identified, whereas there are a relatively large number of TGF-β superfamily ligands (> 40 ligands). Therefore, promiscuous interactions must occur between these ligands and their corresponding receptors. Indeed, ligands in the BMP subfamily may initiate cellular activities by binding to differential subsets of receptors to give rise to cell-specific intracellular signaling in various tissues [49]. Studies have demonstrated that the dysregulation of BMP-mediated signaling and naturally occurring BMP gene mutations are associated with various female reproductive diseases [4]. Obtaining a more comprehensive understanding of the ligand-receptor interactions of and molecular mechanisms underlying BMP-induced cellular functions would promote the development of therapeutic strategies for BMP-related disorders. In the present study, we used two inhibition approaches (kinase receptor inhibitors and small interfering RNAs) to investigate the involvement of BMP type I receptors in BMP6-induced cellular actions in hGL cells. Our results show that either ALK2 or ALK3 mediates the phosphorylation of SMAD1/5/8 proteins. Additionally, the activation of SMAD1/5/8-SMAD4 signaling contributed to the BMP6-induced up-regulation of furin expression in hGL cells. Based on indirect evidence, previous studies have demonstrated that ALK2, ALK3, and ALK6 are the putative binding receptors for BMP6 [50]. An in vitro study performed using different cell lines showed that different cell types may express different cell type-specific type I and type II receptors and that different type II receptors may recruit different type I receptors into the BMP6 ligand-receptor complex [51]. For instance, BMP6 strongly bound to ALK2 type I receptors as well as BMPRII and ACVRII type I receptors in C2C12 and MC3T3-E1 cells [51]. However, BMP6 bound to ALK6 (it also bound to ALK2 and ALK3 but less efficiently) type I receptors and BMPRII type II receptors in ROB-C26 cells [51]. In contrast, our results show that both dorsomorphin (an ALK2, ALK3, and ALK6 inhibitor) and DMH-1 (an ALK2 and ALK3 inhibitor) completely abolished BMP6 activity. Additionally, the combined targeted depletion of ALK2 and ALK3 completely abolished BMP6-induced cellular signaling and actions. Our findings indicate that ALK 2 and ALK3 but not ALK6 are functional type I receptors for BMP6 in hGL cells. Taken together, which type I receptors interact with BMP6 is dependent on the expression levels of type I and type II receptors, and the cellular activity of BMP6 is cell type- and species-specific.

Our recent studies have shown that immortalized hGL cells (SVOG cells) may use differential subsets of type I receptors to transduce the intracellular signals in response to various BMP ligands. Specifically, BMP2 signals act through ALK2 and ALK3, BMP4 signals through ALK3 and ALK6, BMP7 signals through ALK2 and ALK3, and BMP15 signals through ALK3 [52–54]. Based on their structural similarities, BMP6 is most similar to BMP5, BMP7, and BMP8 and different from the BMP subgroup *Drosophila* decapentaplegic gene products (DPPs), BMP2 and BMP4 [55]. However, in vitro studies performed using cell models suggest that BMP6, BMP2, and BMP7 exert their cellular activities in a similar manner by binding to the same subsets of type I receptors to induce a SMAD-dependent signaling pathway in hGL cells.

The limitation of this study is that our results were obtained from the experiments using primary hGL cells and immortalized hGL cells. All these cells were isolated from patients undergoing IVF treatment that had been exposed to high concentrations (pharmacological effects) of human chorionic gonadotropin. These cells are luteinized that have different characteristics of cellular activities compared to the granulosa cells of the growing follicles. Future studies will be required to confirm these intrafollicular functions using animal models. For instance, it will be of great interest to overcome this limitation by generating granulosa cell-specific conditional mutants and examining the impact on follicular function.

In summary, the results of the present study demonstrate that BMP6 increases the production of TGF-β1 by up-regulating the expression of furin in human granulosa cells, most likely via an ALK2/ALK3-mediated SMAD1/5/8-SMAD4 signaling pathway. These results provide new insights into the mechanisms by which an intrafollicular TGF-β superfamily member affects the processing and production of another member through paracrine or autocrine mechanisms in human granulosa cells.

Disclosure statement

The authors have nothing to disclose.

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