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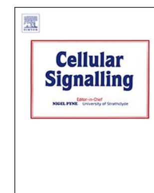

SMAD1/5 mediates bone morphogenetic protein 2-induced up-regulation of BAMBI expression in human granulosa-lutein cells☆

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ABSTRACT

Bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI) is a transforming growth factor β (TGF-β) type I receptor antagonist that negatively regulates TGF-β and bone morphogenetic protein (BMP) signaling. BAMBI has been shown to be regulated by TGF-β signaling; however, whether BAMBI can be regulated by BMP signaling remains to be determined. The aim of this study was to investigate the effect of BMP2 on the regulation of BAMBI expression in human granulosa-lutein cells and the underlying mechanisms. Both primary and immortalized human granulosa-lutein cells were used as research models. Using dual inhibition approaches, our results showed that BMP2 activated SMAD1/5/8 phosphorylation and up-regulated BAMBI mRNA levels, which was reversed by the BMP type I receptor inhibitors, DMH-1 and dorsomorphin, but not by SB431542 (activin/TGF-β type I receptor inhibitor). Moreover, the combined knockdown of SMAD1 and SMAD5 completely abolished the BMP2-induced up-regulation of BAMBI. Similarly, knockdown of SMAD4 reversed the BMP2-induced up-regulation of BAMBI. Pre-treatment with BMP2 inhibited the TGF-β1-induced phosphorylation of SMAD2/3 and up-regulation of MMP2, and these inhibitory effects were reversed by knockdown of endogenous BAMBI. Our findings indicate that BAMBI is a BMP-responsive gene and that BAMBI participates in the negative feedback regulation of TGF-β signaling in the human ovary.

1. Introduction

The transforming growth factor-β (TGF-β) superfamily consists of a group of growth factors that encompass a variety of physiological functions during embryonic development and tissue homeostasis [1]. The bone morphogenetic protein (BMP) family, which is the largest subfamily of the TGF-β superfamily, consists of multifunctional regulators of folliculogenesis and ovarian functions [2,3]. Similar to other members of the TGF-β superfamily, BMPs initiate cellular signaling through heteromeric combinations of two type I receptors (also known as activin receptor-like kinase, ALK) and two type II receptors (both are serine/threonine kinase receptors) [4]. Ligand-receptor binding results in downstream activation of signaling via the canonical phosphorylation of receptor-regulated Sma- and Mad-related (R-SMAD) proteins: the SMAD1/5/8 proteins. Phosphorylated SMAD1/5/8 proteins are further combined with the co-SMAD factor SMAD4, and they are translocated into the nucleus where they interact with tissue-specific transcription factors and target the genome via consensus SMAD-binding motifs to recruit chromatin remodeling machinery [5,6]. While the functional roles of BMPs in the regulation of ovarian functions have been studied extensively [2,3], the contributions of the specific R-SMADs, SMAD1/5/8 transcription factors to the human follicle function are less well characterized.

BMP signaling is regulated by the action of an intricate network of extracellular, intracellular and membrane modulators [7]. Bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI) is a transmembrane protein that is structurally similar to the TGF-β type I receptor while lacking the intracellular serine/threonine kinase domain. Thus, BAMBI acts as a pseudo-receptor to antagonize TGF-β/BMP signaling by inhibiting the formation of active ligand-receptor complexes [8]. In addition to being an antagonist of TGF-β/BMP signaling, BAMBI has been shown to function as a positive regulator of the Wnt/β-catenin pathway [9]. Currently, the physiological functions regulated by BAMBI or the aberrant expression of BAMBI-induced pathological

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conditions in humans are largely unknown. During Xenopus embryogenesis, BAMBI is co-expressed with BMP4, and it acts as a negative feedback regulator of BMP, activin and TGF-β signaling [8]. Surprisingly, targeted depletion of BAMBI in mice had no apparent abnormal phenotype or developmental defects that affected the embryos [10]. However, deregulation of BAMBI has been reported in various human diseases, including ovarian cancers, pathological fibrosis and metaplastic bone formation [11–13]. Recently, we showed that BAMBI was expressed in porcine ovarian follicles, and this BMP regulator could be negatively regulated by administration of FSH, indicating a possible functional role of BAMBI in the regulation of ovarian follicle development [14].

BMP2 is expressed in human ovarian follicles and corpus luteum, which play critical roles in modulating follicular and luteal functions [2,15,16]. In the hamster ovary, BMP2 stimulates primordial follicle formation by promoting the differentiation of somatic cells into pregranulosa cells and germ cell to oocyte transition [17]. Prior to follicle selection, BMP2 signaling maintains the granulosa cells in an undifferentiated state and inhibits cell responsiveness to FSH in hen granulosa cells [18]. In human granulosa cells, BMP2 up-regulates the expression of FSHR and P450 aromatase, whereas BMP2 down-regulates the expression of LHR and StAR [19]. Moreover, data from a clinical study suggests that BMP2 could potentially be used as an indicator of oocyte maturation [20]. During the luteal phase, BMP2 modulates cell-cell communication by down-regulating connexin 43 in human granulosa-lutein (hGL) cells [21]. Despite the important role of BMP2 in ovarian functions, whether BMP2 signaling has a negative feedback control system remains largely unknown. In the present study, we sought to investigate the effect of BMP2 on the regulation of BAMBI expression and the underlying molecular mechanism. Furthermore, we also examined the suppressive effects of BMP2-induced up-regulation of BAMBI on TGF-β1-activated cell signaling in hGL cells.

2. Materials and methods

2.1. Cell culture

An immortalized human granulosa cell line (SVOG, non-tumorigenic) that was previously established by our laboratory using SV40 large T antigen transfection was used as the cell model in the present study [22]. The SVOG cells were cultured in DMEM/F12 medium (Sigma-Aldrich, Oakville, ON, Canada) with 5% charcoal/dextran-treated fetal bovine serum (HyClone Laboratories Inc., Logan, UT, USA), 100 U/ml penicillin (Life Technologies Inc./BRL, Grand Island, NY, USA), 100 mg/ml streptomycin sulfate (Life Technologies) and 1 × GlutaMAX (Life Technologies). The cultured cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Primary hGL cells were isolated from the follicle fluid obtained from in vitro fertilization (IVF) patients with informed consent, following approval from the University of British Columbia Research Ethics Board. The hGL cells were purified using Ficoll Paque density centrifugation as previously described [23,24]. The purified hGL cells were seeded (2×10^5 cells per well in 12-well plates) and were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was changed every other day in all of the experiments.

2.2. Antibodies and reagents

Polyclonal rabbit anti-SMAD1/5/8 (N-18; sc-6031-R) and a monoclonal mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (G-9; sc-365062) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal rabbit *anti*-phospho-SMAD1 $^{(Ser463/465)}$/SMAD5 $^{(Ser463/465)}$/SMAD8 $^{(Ser465/467)}$ (D5B10) antibody, rabbit *anti*-phospho-SMAD2 (Ser$^{465/467}$, #3101), rabbit *anti*-phospho-SMAD3 (Ser $^{423/425}$, # 9520), rabbit *anti*-SMAD3 (C67H9), rabbit *anti*-SMAD4 (D3M6U) and monoclonal mouse *anti*-SMAD2 antibody (L16D3, #3103) were obtained from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated goat *anti*-rabbit and goat *anti*-mouse secondary antibodies were obtained from Bio-Rad (Richmond, CA, USA). Recombinant human BMP2, recombinant human TGF-β1, dorsomorphin dihydrochloride (dorsomorphin), and DMH-1 (4-[6-[4-(1-methylethoxy) phenyl] pyrazolo [1, 5-*a*]pyrimidin-3-yl]-quinoline) were obtained from R & D Systems (Minneapolis, MN, USA). SB431542 was obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA).

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

The total RNA of the cells was extracted using TRIzol reagent (Invitrogen Life Technologies, Inc., Grand Island, NY, USA) according to the manufacturer's instructions. A total of 2 μg of RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase, random primers and dNTP (Promega, Madison, WI, USA). Each 20-μl qPCR sample contained 10 μl of 2 × SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 20 ng of cDNA and 250 nM of each specific primer. The primers used in this study were as follows: *BAMBI*, 5′-GGCCTCAGGACAAGGAAACAG-3′ (sense) and 5′-CGGAACCACAACTCTTTGGAAG-3′ (antisense) and *GAPDH*, 5′-GAGTCAACGGATTTGGTCGT-3′ (sense) and 5′-GACAAGCTTCC CGTTCTCAG-3′ (antisense). Alternatively, TaqMan gene expression assays (Applied Biosystems) for *SMAD1* (Hs01077084_m1), *SMAD5* (Hs00195437_m1), *SMAD8* (Hs00195441_m1), and *GAPDH* (Hs02758991_g1) were performed on corresponding cDNA samples. All of the experiments were repeated at least three times, and each sample was assayed in triplicate. Relative quantification of the mRNA levels was performed using the comparative cycle threshold (Ct) method with *GAPDH* as the reference gene and the calculation formula $2^{-\triangle\triangle Ct}$.

2.4. Western blot

Cell lysis buffer (Cell Signaling) containing a protease inhibitor cocktail (Sigma-Aldrich) was used to lyse the cells. A total of 30 μg of protein was separated by 10% SDS-PAGE and then was transferred onto the polyvinyl difluoride membranes (PVDF). The membranes were blocked for 1 h with 5% non-fat dry milk in Tris-buffered saline (TBS) and then were incubated overnight at 4 °C with the indicated primary antibodies diluted in 5% non-fat milk-TBS. Next, the membranes were washed three times with TBST and then were incubated for 1 h with appropriate peroxidase-conjugated secondary antibodies (diluted at 1:5000 in TBST with 5% non-fat dried milk). Finally, the immunoreactive bands were detected using an enhanced chemiluminescent substrate and X-ray film. The intensities of the bands were quantified using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

2.5. Small interfering RNA (siRNA) transfection

To knockdown endogenous SMAD1, SMAD5, SMAD8, SMAD4, or BAMBI, we transfected cells using 25 nM ON-TARGET*plus* SMARTpool *SMAD1*, *SMAD5*, *SMAD8*, *SMAD4*, or *BAMBI* siRNAs (Dharmacon, Lafayette, CO, USA) as previously described [25]. Briefly, the SVOG cells were pre-cultured to 50% confluency in antibiotic-free DMEM/F12 medium containing 10% charcoal/dextran-treated fetal bovine serum and then were transfected with 25 nM siRNA using Lipofectamine RNA iMAX (Life Technologies) for 48 h. siCONTROL Non-Targeting Pool siRNA (Dharmacon) was used as the transfection control. The knockdown efficiency of each target was examined using RT-qPCR or Western blot analysis.

2.6. Statistical analysis

Data are presented as the mean ± SEM of at least three

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