Bone Morphogenetic Protein 6 Induces Downregulation of Pentraxin 3 Expression in Human Granulosa Cells through the SMAD-Dependent Signaling Pathway in Women with Polycystic Ovary Syndrome

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Research Article

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Abstract

Background: Pentraxin 3 contributes to the formation of cumulus-oophorus complex, its level is considered to indicate the quality and potential of oocytes. Bone morphogenetic protein 6 is a key regulator of ovary follicular development and regulates female reproduction. It is unclear whether Pentraxin 3 is differentially expressed in granulosa cells derived from Polycystic ovary syndrome and whether Bone morphogenetic protein 6 affects Pentraxin 3 in human granulosa-lutein cells. To evaluate whether Pentraxin 3 is differentially expressed in the granulosa cells derived from women with Polycystic Ovary Syndrome and whether granulosa cell-derived Bone Morphogenetic Protein 6 can regulate the expression of Pentraxin 3 in hGL cells.

Materials and methods: The expression levels of BMP6 and PTX3 in granulosa cells were evaluated by RT-qPCR. The correlation between the expression levels of BMP6 and PTX3 and oocyte quality indexes were analyzed using clinical samples. The cells were incubated with BMP6 at different concentrations and times to check the expression of PTX3. TGF-β type 1 inhibitors and small interfering RNA targeting ALK2/3/6, SMAD1/5/8 and SMAD4 were used to study the involvement of SMAD dependent pathways.

Results: The levels of Bone Morphogenetic Protein 6 in hGL cells were negatively correlated with the corresponding oocyte maturation rate and high-quality embryo rate, whereas the levels of Pentraxin 3 were positively correlated with the corresponding oocyte maturation rate in women with Polycystic Ovary Syndrome. Additionally, the in vitro cell cultured results showed Bone Morphogenetic Protein 6 significantly inhibited the expression of Pentraxin 3 in KGN cells. Furthermore, using a dual inhibition approach (kinase inhibitors and small interfering RNAs), we identified the ALK2/ALK3 type I receptors and BMPR2/ACVR2A type II receptors and the downstream SMAD1/SMAD5-SMAD4 signaling pathway were responsible for the BMP6-induced cellular activities in hGL cells.

Conclusions: The suppressive effect of Bone Morphogenetic Protein 6 on Pentraxin 3 expression was mediated by ALK2/ALK3 type 1 receptors and BMPR2/ACVR2A type 2 receptors in granulosa cells through the SMAD1/5-SMAD4 dependent signaling pathway in women with Polycystic Ovary Syndrome. Our findings provides new insights into the understanding of the pathogenesis of Polycystic Ovary Syndrome-related ovulatory disorders.

Background

Ovulation is an essential event for mammals to maintain normal reproductive activities. The dysregulation of ovulation is one of the leading causes of female infertility. In humans, ovulation is regulated by gonadotropins and involves a series of steps, such as oocyte meiosis restart, cumulus-oocyte complex (COC) formation, extracellular matrix reconstruction, and COC expansion(1). This series of processes are essential for successful fertilization and early embryo development after the entrance of the oocyte into the oviduct. More importantly, successful ovulation largely depends on the proper expansion of COC under the control of gonadotropins and several growth factors(2). COC expansion is
initiated by the accumulation of hyaluronic acid derived from granulasa cells, and this process plays an important role in regulating oocyte maturation and ovulation. Moreover, the extent of COC expansion is a crucial morphological indicator for oocyte maturation and embryo quality in clinical assessment(3).

Pentraxin 3 (PTX3) belongs to the pentraxin superfamily and is expressed in various human tissues. As a multifunctional secreted protein, PTX3 regulates cell proliferation, angiogenesis, migration, and invasion and plays a vital role in the regulation of immune and inflammatory responses, matrix deposition, and female reproduction(4). PTX3 is a key factor that form a stable hyaluronic acid structure, which is essential for the mammalian ovulatory function(5). Indeed, the expression of PTX3 in granulosa cells is closely associated with the oocyte quality and subsequent fertilization rate(6). Gene inactivation of PTX3 led to a decrease in fertilization ability of the oocyte by destroying the structural integrity of COC. Similarly, the downregulation of PTX3 expression in granulosa cells resulted in a defect in female fertility(7). Considering the valuable role of PTX3 in follicular development and ovulation, relevant research regarding PTX3 modulation may help to find new targets for managing ovulation dysfunction.

The bone morphogenetic proteins (BMPs) are the largest members of the transforming growth factor β (TGF-β) superfamily growth factors, which are extremely important for maintaining female reproduction. Previous studies have shown that several BMPs, including BMP2, BMP4, BMP6, and BMP7 can regulate hyaluronic acid synthesis in human granulosa cells, leading to the occurrence of ovulation(8,9,10). BMPs phosphorylate and activate the intracellular signaling mediators Smad and Mad-related protein (SMAD)1/5/8 after binding to the functional receptors, TGF-β type I and type II receptors on the cell membrane. In particular, BMP6 has been shown to regulate mammalian follicle function, promote oocyte maturation, and maintain luteal function(12,13) by modulating intercellular communication in the ovary(14). Additionally, the results obtained from clinical studies have revealed that the dysregulation of BMP6 signaling in the ovary is related to the pathogenesis of polycystic ovary syndrome (PCOS) and ovulatory dysfunction(15). Taken together, BMP6 is an essential intraovarian regulator in maintaining normal follicular development.

PCOS is one of the most common causes of female infertility and is one of the most challenging research topics in female reproduction. However, the etiology of PCOS is still unclear. This study was proposed to evaluate the association between the expression of BMP6 and PTX3 in the human granulosa-lutein (hGL cells) and PCOS through both in clinical observation and in vitro cell culture experiments. By studying the molecular mechanism by which BMP6 regulates the expression of PTX3 in hGL cells, we aimed to understand the regulatory process of the ovulation event and identify new targets for treating ovulation disorders at the molecular level using clinical samples obtained from women with PCOS.

Materials And Methods

2.1. Patients included in this study
All participants underwent the initial cycle of assisted reproductive technology in vitro fertilization/embryo transfer (IVF/ET) at the reproductive and genetic center of Shandong University of Traditional Chinese Medicine from January 2018 to January 2022. Among the participants were 31 women with polycystic ovary syndrome (as a study group) and 31 patients with tubal factor infertility (as a control group). The diagnosis of PCOS was mainly based on the criteria established by the Rotterdam Society of Human Reproduction and Embryology/American Society of Reproductive Medicine, revised in 2003. All participants had no other endocrine system diseases, genital malformations, or chromosomal disorders and had not taken hormones or addictive drugs recently. All participants signed informed consent, and the study was approved by the ethics committee of the Reproductive and Genetic Center of Shandong University of Traditional Chinese Medicine, approval no. SDTCM/E2110-03. All procedures performed in studies involving human participants met the ethical standards of institutions and national research committees and the 1964 Declaration of Helsinki and its subsequent amendments or similar ethical standards.

2.2. Ovarian stimulation and oocyte retrieval

Patients undergoing IVF/ET treatment initially received controlled ovarian hyperstimulation, using medication that prevents the premature luteinization, including GnRH antagonists (ganirelix, Merck, Canada) in the follicular phase or triptorelin acetate (Synarel, Pfizer, Canada) in the luteal phase of the previous cycle to downregulate the pituitary gonadotropin-releasing hormone receptors and subsequent transduction pathways. On the second day of the menstrual cycle, appropriate human menopausal gonadotropin (hMG, Menopur, Ferring, Canada) or recombinant FSH (Puregon, Merck, Canada) were administered to stimulate the follicular growth. When the diameter of the leading follicle reached more than 18 mm or the diameters of at least three follicles reached more than 17 mm, hCG (Pregnyl, Merck) was administered to trigger final oocyte maturation. Oocytes were retrieved under vaginal ultrasound-guiding 34–36 h after the trigger, and corresponding follicular fluid was collected.

2.3. Oocyte scoring criteria

The embryologists collected all the retrieved oocytes and evaluated the maturation of oocytes based on the following four perspectives:

(1) Cumulus size: large cumulus cells and loose arrangement (1 point); small or clustered cumulus cells and closely arranged (0 points).

(2) Oocyte color and transparency: light and the transparency was good (1 point); dark and the transparency was dim (0 points).

(3) Radial crown arrangement: radial crown cells arranged radially (1 point); the radiation crown cells did not disperse, or the radiation crown was too divergent (0 points).

(4) Oocyte visible: the oocyte was visible under the microscope (1 point); the oocyte was fuzzy and light in color (0 points).
According to the abovementioned scoring standards, oocytes with a score of 3–4 were considered of high quality.

2.4. In vitro fertilization and embryo culture

Fertilization was determined by observing the formation of prokaryotes after 17 h of insemination. When two different prokaryotes containing nuclei (2 PN) were observed, successful fertilization was determined(16). The morphology and quality of embryos were evaluated based on the cleavage of cells after 72 h. Grade I embryos included 7–9 cells with a uniform germ layer and fragment rate of less than 10%. The fragment rate of grade II embryos was between 11% and 25%. The cell division of grade III embryos was irregular, and the fragmentation rate exceeded 25%(17) Grade I embryos were considered to be high-quality embryos. Grade I-II embryos were all considered available embryos. Those available embryos were used to perform fresh embryo transferred or frozen-thawed embryo transfer.

2.5. Clinical outcomes

The oocyte maturation rate was defined as the number of oocytes scored 3–4 divided by the total number of oocytes retrieved. The fertilization rate was defined as the number of fertilized oocytes divided by the total number of retrieved oocytes. The cleavage rate was defined as the number of cleaved embryos divided by the total number of fertilized eggs. The transferred embryo rate was defined as the number of transferred embryos divided by the number of all cleaved embryos. The high-quality embryo rate was defined as the number of high-quality embryos divided by the number of all available embryos. Clinical pregnancy was defined as the presence of a pregnancy sac and a fetal pole in the uterine cavity monitored by ultrasound at six weeks of gestation. The cumulative clinical pregnancy rate was defined as the number of clinical pregnancy divided by the total number of all transferred cycles. The main outcomes were measured by the high-quality oocyte rate and cumulative clinical pregnancy rate. The secondary outcomes were measured by the fertilization rate, cleavage rate, transferred embryo rate, and high-quality embryo rate.

2.6. Human ovarian granulosa cell line (KGN cells) culture

The human ovarian granulosa cell line (KGN cells) is a commonly used cell model in the in vitro experiments to study ovarian functions because these cells are easy to isolate, culture, and transfect and can be analyzed by immunocytochemical methods(18, 19). KGN cells were cultured at 37°C in 5% CO₂ and 95% air. Dulbecco's modified Eagle's medium/nutrient mixture F-12 ham (DMEM/F-12; Sigma Aldrich, USA) was used to culture the cells and supplemented with penicillin (100 U/mL, Invitrogen, life technologies, USA), streptomycin sulfate (100 µ G/mL, Invitrogen, Life Technologies, USA), glutamine (1x, Invitrogen, life technologies, USA), and 10% carbon/dextran treated fetal bovine serum (10%, HyClone, USA). The medium was changed every other day. All cells were resuspended in serum-free medium for 24 h before the experiment. For concentration-dependent studies, cells were treated with BMP6 at different concentrations (1, 10, or 100 ng/mL) for 24 h. For time course studies, cells were treated with 100 ng/mL BMP6 for 3, 6, 12, or 24 h. Cells were harvested for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis to examine messenger RNA (mRNA) and protein levels,
respectively. The PTX3 levels secreted in the culture medium were examined using an enzyme immunoassay.

2.7. Preparation and culture of primary hGL cells

The primary hGL cells used in the experiment were all isolated from the remaining follicular fluid obtained from patients undergoing IVF treatment at the reproductive and genetic center of the affiliated hospital of Shandong University of Traditional Chinese Medicine. hGL cells were centrifuged and purified by density gradient centrifugation precipitation after collecting follicular fluid as previously described(20). These cells were then cultured in the same culture environment and medium as the KGN cell line.

2.8. Antibodies and reagents

Recombinant human BMP6 protein (507-BP), dorsomorphin dihydrochloride (dorsomorphin) (3093), and 4-[6-[4-(1-methylethoxy) phenyl] pyrazolo[1,5-a] pyrimidin-3-yl]-quinoline DMH-1 (DMH-1) (4126) were obtained from R&D Systems (Minneapolis, MN, USA). The TGF-β type I receptor inhibitor SB431542 was obtained from Sigma Aldrich (St Louis, MO, USA). The polyclonal rabbit anti-phospho-SMAD1 (Ser463/465), SMAD5 (Ser463/465), and SMAD8 (Ser426/428) (13820, diluted at 1:1000) antibodies and polyclonal rabbit anti-SMAD4 (9515, diluted at 1:1000) antibody were obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit (diluted at 1:5000) and goat anti-mouse IgGs (diluted at 1:5000) were obtained from Bio-Rad Laboratories (Hercules, CA). Horseradish peroxidase-conjugated donkey anti-goat IgG was obtained from Santa Cruz Biotechnology.

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

Cells were washed with cold phosphate-buffered saline (PBS), and total RNA was extracted from hGL cells using TRIzol Reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. Typically, 2 µg of RNA was used to produce first-strand cDNA with random primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, USA). Every 20 µL mixture contained 10 µL of 1X SYBR Green PCR Master Mix (Applied Biosystems, USA), 20 ng of cDNA template, and 250 nm primer. The primer sequences used in this study were as follows: PTX3, 5’-TCTCTGGTCTGCAGTGTGG-3’ (forward primer) and 5’-TGAAGAGCTTCCCATTCC-3’ (reverse primer); SMAD4, 5’-TGGCCCAGGATCAGTAGGT-3’ (forward primer) and 5’-CATCAACAATTCCAGCA-3’ (reverse primer); and GAPDH, 5’-GAGTCACGGATCAAGATTGGTCGT-3’ (forward primer) and 5’-GACAGACTTCTCTCTGATCGTCTCAG-3’ (reverse primer). The primers used for the TaqMan gene expression assays were as follows: PTX3 (Hs00173615_m1), ACVR1 (ALK2, Hs00153836_m1), BMPR1A (ALK3, Hs01034913_g1), BMPR1B (ALK6, Hs01010965_m1), SMAD1 (Hs01077084_m1); SMAD5 (Hs00195437_m1), and SMAD8 (Hs001195441_m1). SMAD4 (Hs00929647_m1) and GAPDH (Hs02758991_G2) (Applied Biosystems, Foster, CA). PCR was performed using the Applied Biosystems 7300 real-time fluorescent quantitative PCR system. Three independent experiments were performed using different cultures, and each sample was repeated three times. Relative quantitative analysis of mRNA levels was performed using the comparative cycle threshold (CT) method, with GAPDH used as a
reference gene and the calculation formula $2^{-\Delta \Delta Ct}$. All primers used in this study have passed the validation test.

2.10. Western blot analysis

After washing with cold PBS, the cells were lysed in lysis buffer (Cell Signaling) containing a protease inhibitor cocktail (Sigma–Aldrich). The cell lysates were centrifuged at 14000 rpm for 15 min at 4 °C, and then the supernatants were collected. The protein concentration in the supernatant was quantitatively determined using the DC protein assay (Bio Rad Laboratories). Equal amount of protein was separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene fluoride membrane. After 1 h of blocking with 5% nonfat dry milk in TBS buffer, the membranes were incubated overnight with relevant primary antibodies and then washed three times with TBST; the membranes were incubated for 1 h with appropriate peroxidase-conjugated secondary antibodies. The immunoreactive bands were detected by enhanced chemiluminescent substrate or super signal westfemto chemiluminescent substrate (Pierce, Rockford, IL). The membranes were stripped with stripping buffer at 50 °C for 30 min and reprobed with rabbit anti-SMAD1/5/8 or mouse anti-α-tubulin antibody as a loading control.

2.11. Small interfering RNA (siRNA) transfection

We used 25 nM ON-TARGETplus SMARTpool or 25 nM ON-ARGETplus nontargeting Pool (Thermo Fisher Scientific; Lafayette, CO, USA) to decrease the levels of ALK2, ALK3, ALK6, BMPR2, ACVR2A, ACVRR2B, SMAD1, SMAD5, SMAD8, or SMAD4 expression. Cells were cultured to 50% confluence in antibiotic-free DMEM/F12 medium containing 10% FBS and then transfected with 25 nM siRNA for 48 h using Lipofectamine RNAiMAX (13778–150; Invitrogen, Life Technologies). SiCONTROL Non-Targeting pool siRNA was used as the transfection control. The knockdown efficiency was confirmed by real-time quantitative RT-PCR or western blot analysis.

2.12. Measurement of BMP6 and PTX3 by enzyme-linked immunosorbent assay

The cell culture medium was collected for the enzyme-linked immunosorbent assay. BMP6 and PTX3 protein production levels in culture medium or follicular fluid were measured by an enzyme immunoassay Kit (R&D Systems) as per the manufacturer's instructions. The levels of BMP6 and PTX3 were normalized to the protein concentration of each cell lysate. Each sample was measured three times.

2.13. Statistical analysis

The results were analyzed using SPSS Version 22. Comparisons between the two groups were performed by Student’s t test or the Mann–Whitney U test for continuous variables and the $\chi^2$ test for categorical variables. Spearman correlation analysis was applied to identify correlations between BMP6/PTX3 expression and clinical indicators. Experimental results were presented as the mean ± SEM of at least three independent experiments. The results were analyzed by one-way analysis of variance followed by
Tukey’s multiple-comparison tests using PRISM software (GraphPad Software, San Diego, CA). Data were considered significantly different if the P-value < 0.05.

Results

3.1. Clinical data analysis

3.1.1. Baseline characteristics of recruited patients

This study included 31 patients with polycystic ovary syndrome and 31 patients with tubal factor infertility (with a normal endocrine function). There were a total of 71 embryo transfer cycles. The baseline characteristics of patients are displayed in Table 1. There was no significant difference in patient age, infertility duration and causes, and body mass index (BMI) between the two groups (P > 0.05). Additionally, there was no significant difference in the duration of Gonadotropin (Gn) administration (P > 0.05). However, a significantly lower total dose of Gn administration during stimulation cycle was found in the PCOS group than that in the control group (P < 0.05). Moreover, significantly higher levels of basal LH, basal testosterone, and Anti-Mullerian Hormone (AMH) were found in the PCOS group (P < 0.001). The number of retrieved oocytes was also higher in the PCOS group (P < 0.001).
Table 1
Baseline Characteristics of Recruited Patients.

<table>
<thead>
<tr>
<th></th>
<th>PCOS group</th>
<th>Control group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
<td>31</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Female age at oocyte retrieval(years)</td>
<td>30.10 ± 3.38</td>
<td>30.52 ± 3.01</td>
<td>0.61</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>2.97 ± 1.78</td>
<td>2.74 ± 1.41</td>
<td>0.58</td>
</tr>
<tr>
<td>Infertility type</td>
<td></td>
<td></td>
<td>0.31</td>
</tr>
<tr>
<td>Primary infertility</td>
<td>16(51.6%)</td>
<td>12(38.7%)</td>
<td></td>
</tr>
<tr>
<td>Secondary infertility</td>
<td>15(48.4%)</td>
<td>19(61.3%)</td>
<td></td>
</tr>
<tr>
<td>Duration of COS</td>
<td>11.45 ± 1.65</td>
<td>11.74 ± 1.12</td>
<td>0.42</td>
</tr>
<tr>
<td>Total Gn dose administered</td>
<td>2804.19 ± 515.75</td>
<td>3174.20 ± 595.67</td>
<td>0.01*</td>
</tr>
<tr>
<td>AMH level (ng/ml)</td>
<td>6.75 ± 1.69</td>
<td>2.48 ± 0.91</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>24.19 ± 4.52</td>
<td>23.15 ± 3.51</td>
<td>0.31</td>
</tr>
<tr>
<td>Basic LH level (IU/L)</td>
<td>8.83 ± 4.87</td>
<td>4.52 ± 1.39</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Basic T level (ng/ml)</td>
<td>1.15 ± 0.45</td>
<td>0.34 ± 0.18</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>No. of oocytes retrieved</td>
<td>24.78 ± 13.87</td>
<td>10.68 ± 3.11</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD for continuous variables and n (%) for dichotomous variables. All P values were assessed with the use of Student’s t test or χ². **p < 0.01, *p < 0.05

3.1.2. Clinical outcomes

We conducted statistical analysis on the relevant indicators of oocyte retrieval and embryo transfer cycle of the two groups. The results are presented in Table 2. In the oocytes removed from the two groups, the high-quality oocytes rate of the PCOS group was significantly lower than that of the control group (50.9% vs. 70.1%, P < 0.001). Although there was no significant difference between the two groups in the fertilization rate, the cleavage rate of the PCOS group was still significantly lower than that of the control group (59.3% vs. 74.1%, P < 0.001). There was a significantly lower available embryo rate in the PCOS group (36.8% vs. 52.7%, P < 0.001); however, there was no significant difference in high-quality embryo rate between the two groups (Table 2). Additionally, the cumulative pregnancy rate of the PCOS group was lower than that of the control group (28.6% vs. 52.8%, P = 0.038).
<table>
<thead>
<tr>
<th></th>
<th>PCOS group</th>
<th>Control group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
<td>31</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Oocyte maturation rate (%)</td>
<td>50.9% (391/768)</td>
<td>70.1% (232/331)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>67.2% (516/768)</td>
<td>67.7% (224/331)</td>
<td>0.88</td>
</tr>
<tr>
<td>Cleavage rate (%)</td>
<td>59.3% (306/516)</td>
<td>74.1% (166/224)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Available embryo rate (%)</td>
<td>36.8% (190/516)</td>
<td>52.7% (118/224)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>High-quality embryo rate (%)</td>
<td>35.3% (67/190)</td>
<td>28.0% (33/118)</td>
<td>0.18</td>
</tr>
<tr>
<td>Cumulative clinical pregnancy rate (%)</td>
<td>28.6% (10/35)</td>
<td>52.8% (19/36)</td>
<td>0.04*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD for continuous variables and n (%) for dichotomous variables. All P values were assessed with the use of χ². **p < 0.01, *p < 0.05

3.2. Increased expression of BMP6 and decreased expression of PTX3 in the granulosa cells and follicular fluid obtained from patients with PCOS

To determine whether the expression levels of BMP6 and PTX3 in the granulosa cells of PCOS patients differed from those of control patients, we measured the mRNA levels of BMP6 and PTX3 of granulosa cells using RT-qPCR. The results are presented in Figs. 1A and 1B showing that BMP6 mRNA levels were upregulated, whereas PTX3 mRNA levels were downregulated in the hGL cells of PCOS patients. Additionally, using an enzyme-linked immunosorbent assay, we found that the BMP6 protein levels were increased, whereas the PTX3 protein levels were decreased in the hGL cells obtained from PCOS patients compared with those from the control group (P < 0.05) (Figs. 1C and 1D). Similarly, the BMP6 protein levels were increased, whereas the PTX3 protein levels were decreased in follicular fluid samples obtained from PCOS patients compared with those from control group (P < 0.05, Figs. 1E and 1F).

3.3. Correlation between the mRNA levels of BMP6 and PTX3 in hGL cells and clinical outcomes in patients with PCOS.

To investigate the effects of the expression changes in BMP6 and PTX3 in the granulosa cells of patients with PCOS on the clinical outcomes of IVF treatment, we conducted a correlation study using the Spearman correlation analysis. The results showed that the mRNA levels of BMP6 in hGL cells were negatively correlated with the oocyte maturation rate (r=-0.580, P < 0.01, Fig. 2A) and high-quality embryo rate (r=-0.440, P < 0.05, Fig. 2D). Whereas the mRNA levels of PTX3 were positively correlated with the oocyte maturation rate (r = 0.448, P < 0.05, Fig. 2G).
3.4. BMP6 suppresses the expression of PTX3 in KGN and hGL cells

To investigate the effect of BMP6 on the expression of PTX3 in the human ovary, we used recombinant human BMP6 (BMP6) to treat KGN cells. The results showed that treatment with different concentrations (1, 10, or 100 ng/mL) of BMP6 for 6 h, significantly decreased the mRNA levels of PTX3 in a concentration-dependent manner using in vitro KGN cell culture (Fig. 3A). We further treated KGN cells with 100 ng/mL BMP6 for different time points (3, 6, 12, or 24 h), and the time course experiments demonstrated that the suppressive effects of BMP6 on the mRNA levels of PTX3 started at 3 h and persisted until 24 h after treatment (Fig. 3B). Similarly, the western blot analysis results revealed that BMP6 treatment for 12 h decreased the protein levels of PTX3 in a concentration-dependent manner in KGN cells (Fig. 3C). To confirm the physiological relevance of the results obtained from immortalized cell line, we used primary hGL cells isolated from follicular fluid samples obtained from IVF patients. Similar to the results obtained from KGN cells, the level of PTX3 mRNA was significantly decreased in Primary hGL cells after treatment with different concentrations of BMP6 (1, 10, or 100 ng/mL) for 6 h (Fig. 3D).

3.5. The BMP type I receptors ALK2/ALK3 but not ALK6 mediate the BMP6-induced downregulation of PTX3 in KGN cells

Several TGF-β Type I receptors (activin receptor-like kinases or ALKs) have been shown to transduce the BMP signaling(21, 22). Whereas, several kinase inhibitors have been shown to inhibit the activity of ALKs. For instance, DMH-1 selectively inhibits ALK2/3, dorsomorphine (DM) inhibits ALK2/3/6, and SB431542 inhibits ALK4/5/7(23, 24). Our results showed that pretreatment with DM (10 µM) or DMH-1 (0.25 µM) for 1 h reversed the suppressive effect of BMP6 on PTX3 mRNA levels in KGN cells (Figs. 4A and 4B). However, pretreatment with 5 µM SB431542 did not have this effect (Fig. 4C). To further determine which ALKs were involved in this process, a siRNA-based inhibition approach was conducted to knock down the type I receptors, ALK2, ALK3, or ALK6. Previous studies have verified that transfection of cells with siRNA targeting ALK2, ALK3, or ALK6 for 48 h significantly reduced the mRNA levels of specific ALKs. Specifically, the results showed that knockdown of ALK2 or ALK3 partially reversed the suppressive effect of BMP6 on the mRNA levels of PTX3 (Figs. 4D and 4E), whereas knockdown of ALK6 had no such effect (Fig. 4F). Notably, the combined knockdown of ALK2 and ALK3 completely reversed the suppressive effect of BMP6 on the expression of PTX3 (Fig. 4G). These results indicate that the type I receptors ALK2 and ALK3 are required to mediate the BMP6-induced downregulation of PTX3 expression in KGN cells.

3.6. BMPR2 and ACVR2A are required to mediate the suppressive effect of BMP6 on the expression of PTX3 in KGN cells

A similar siRNA approach was used to determine which type II receptor (BMPR2, ACVR2A, or ACVR2B) mediates the BMP6-induced signaling in KGN cells(24). The results showed that knockdown of BMPR2 and ACVR2A partially reversed the BMP6-induced downregulation of PTX3 mRNA (Figs. 5A and 5B). However, knockdown of ACVR2B did not have such effect (Fig. 5C). In addition, we found that the
simultaneous knockdown of BMPR2 and ACVR2A completely reversed the BMP6-induced downregulation of PTX3 mRNA (Fig. 5D). However, either concomitant knockdown of BMPR2 and ACVR2B (Fig. 5E) or concomitant knockdown of ACVR2A and ACVR2B (Fig. 5F) did not have such effect. These results indicate that the type II receptors BMPR2 and ACVR2A, but not ACVR2B, are required to mediate the suppressive effect of BMP6 on the expression of PTX3 in KGN cells.

3.7. ALK2/ALK3 type I receptors and BMPR2/ACVR2A type II receptor mediate BMP6-induced phosphorylation of SMAD1/5/8 in KGN cells

Previous studies have shown BMP6 may induce the activation of downstream SMAD1/5/8 proteins(25). To further determine which receptors were involved in BMP6-induced SMAD1/5/8 phosphorylation, we treated KGN cells with type I receptor inhibitors or knocked down the aforementioned type I and type II receptors using siRNA to analyze phosphorylated SMAD1/5/8 protein levels using Western blot analysis. Our results showed that pretreatment with DM or DMH-1 significantly decreased BMP6-induced increase in phosphorylated SMAD1/5/8 protein levels in KGN cells. However, pretreatment with SB431542 did not have this effect (Fig. 6A). In addition, knockdown of ALK2, ALK3, or combined knockdown of ALK2 and ALK3 significantly reversed the BMP6-induced increase in phosphorylated SMAD1/5/8 protein levels, whereas knockdown of ALK6 did not have such effect (Figs. 6B and 6C). Interestingly, knockdown of BMPR2 (but not ACVR2A or ACVR2B) reversed the BMP6-induced increase in phosphorylated SMAD1/5/8 protein levels (Fig. 6D to 6F). Our results further showed that combined knockdown of BMPR2 and ACVR2A or combined knockdown of BMPR2 and ACVR2B reversed the BMP6-induced increase in phosphorylated SMAD1/5/8 protein levels, while the combined knockdown of BMPR2 and ACVR2A reversed the increase in phosphorylated SMAD1/5/8 protein levels to a greater extent, indicating the important role of BMPR2 and ACVR2A in regulating SMAD1/5/8 activity. (Fig. 6G and 6H).

3.8. SMAD1 and SMAD5 mediate the suppressive effect of BMP6 on PTX3 expression in KGN cells

To further determine which SMAD mediates the suppressive effect of BMP6 on the expression of PTX3, we used specific siRNAs to knock down SMAD1, SMAD5, and SMAD8, respectively. The knockdown efficiency of each SMAD was verified using RT-qPCR (Figs. 7A, 7B and 7C). In particular, single knockdown of SMAD1, SMAD5, or SMAD8 did not affect the suppressive effect of BMP6 on the expression of PTX3 in KGN cells (Figs. 7D, 7E and 7F). Interestingly, combined knockdown of SMAD1 and SMAD5 reversed the suppressive effect of BMP6 on the mRNA levels of PTX3 (Fig. 7G). However, combined knockdown of SMAD1 and SMAD8 or SMAD5 and SMAD8 did not have such effect (Figs. 7H and 7I). These results showed that smad1 and smad5 were key molecules to mediate the suppressive effect of BMP6 on the expression of PTX3 in KGN cells.
3.9. SMAD4 mediates the BMP6-induced suppressive effect on PTX3 expression in KGN cells

In most tissues, phosphorylated SMAD1/5/8 proteins bind to SMAD4 and translocate to the nucleus to regulate the expression of the related target genes(26). To further investigate the role of SMAD4 in the BMP6-induced downregulation of PTX3 expression, we knocked down SMAD4 using a specific siRNA. The knockdown efficiency and specificity of siSMAD4 were examined using RT-qPCR (Fig. 8A). Notably, knockdown of SMAD4 completely reversed the suppressive effect of BMP6 on PTX3 mRNA levels in KGN cells (Fig. 8B). Similarly, knockdown of SMAD4 completely reversed the suppressive effect of BMP6 on accumulative PTX3 protein levels in KGN cells (Fig. 8C).

Discussion

PCOS is one of the leading causes of anovulatory infertility. The development of assisted reproductive technology has solved the fertility problems of such women to a certain extent. However, patients with PCOS still faces several challenges. Although more ovarian follicles can be picked up than in patients with a normal ovarian reserve, the number of oocytes with good capacity is lower. The oocytes of PCOS patients do not fully acquire the ability to become mature, more likely leading to follicular development arrest and ovulation disorder(27). A high-quality oocyte indicates that it has a high potential ability to undergo meiosis, become mature, and form a normal embryo after fertilization, which leads to a higher possibility of pregnancy(28). During the late stage of folliculogenesis, mural granulosa cells, cumulus cells, and the oocyte work together to induce cumulus expansion, which results in a mature and competent oocyte.

PTX3 is a multifunctional glycoprotein related to the innate immune response, inflammation regulation, angiogenesis, and the formation and remodeling of the extracellular matrix. In the ovary, PTX3 is involved in the cumulus expansion process and is considered a marker gene of oocyte developmental potential(29). Zhang et al. found that the expression level of PTX3 in cumulus cells is closely related to the fertilization ability of the corresponding oocyte and the subsequent development of an embryo with implantation potential(30). An animal study using the transcriptional analysis indicated that the oocytes with higher expression of PTX3 had higher potential for division and fertilization in buffalo(31). Most studies suggest that plasma level of PTX3 is associated with the pathogenesis of obesity, low-grade inflammation, and insulin resistance in patients with PCOS. However, the molecular mechanisms underlying the correlation between PTX3 level and PCOS(32, 33, 34) have not been determined. Among the patients included in this study, there was no significant difference in the body mass index between the PCOS group and the control group. Future studies will be required to investigate the correlation of the PTX3 levels and obese PCOS patients. In addition, whether the PTX3 levels are correlated with insulin resistance status and hyperandrogenemia in PCOS patients remains to be elucidated. Pan et al. found that women with PCOS had higher PTX3 levels, indicating that the relatively low inflammatory status in PCOS patients with a dysfunctional pituitary gland and ovarian hyperandrogenism(35). However, to our
knowledge, only few studies on PTX3 levels in follicular fluid samples and granulosa cells obtained from PCOS patients have been carried out.

In this study, we found that the expression levels of BMP6 derived from follicular fluid and granulosa cells were higher in PCOS patients. In contrast, the expression levels of PTX3 were lower in PCOS patients. Based on observation of clinical data, we found that the rates of high-quality follicles, cleavage, available embryos, and cumulative pregnancy were lower in the PCOS group than those in the control group. Although the rate of high-quality embryos did not differ between the two groups. Using the Spearman correlation analysis, our results showed that the expression levels of BMP6 were negatively correlated with oocyte maturation and high-quality embryo, while the expression levels of PTX3 were positively correlated with oocyte maturation. These results indicate that the relatively high expression of BMP6 and low expression of PTX3 in patients with PCOS might contribute to poor oocyte quality and embryonic development potential. In addition, the results obtained from our in vitro experiments revealed that BMP6 downregulated the expression of PTX3 in hGL cells. In this regard, we may propose that the increased activity of BMP6 in PCOS patients suppresses the expression of PTX3, leading to oocyte development arrest and ovulation disorder. Previous studies have shown that gene mutations in BMP and its receptors or disruption of BMP-mediated signaling may result in reproductive disorders(36).

Given the potential role of BMP6 in the pathogenesis of ovulation dysfunction, a comprehensive understanding of the molecular mechanism underlying the response of PTX3 to BMP6 in cells is essential for developing therapeutic strategies for treating patients with PCOS. Similar to the TGF-β family, BMP induces cellular response signal transduction through interactions with type I and II membrane-bound serine/threonine kinase receptors(37, 38). Although BMP6 plays a key role in human fertility, the cellular receptors that mediate BMP6 biological activity remain largely unknown. Previous studies have confirmed that ALK type I receptors and BMPR2, ACVR2A, or ACVR2B type II receptors may mediate BMP-induced phosphorylation of SMAD1/5/8. Upon phosphorylation and activation, SMADs bind to SMAD4 and translocate to the nucleus to regulate target gene expression(24, 39)). To understand the involvement of the two receptor types in the molecular regulatory mechanism of BMP6 on PTX3 expression, we conducted a series of experiments. Our results showed that the addition of dorsomorphin (an inhibitor of ALK2/ALK3/ALK6) or DMH-1 (an inhibitor of ALK2/ALK3) completely reversed the BMP6-induced increase in SMAD1/5/8 phosphorylation and inhibition of PTX3 expression, indicating that ALK2 or ALK3 may be involved in BMP6-induced cellular activities. Using a siRNA-based inhibition approach, we further confirmed that ALK2 and ALK3 were the functional type I receptors that mediate the BMP6-induced cell activities in human granulosa cells. Using the same methods, our results showed that BMPR2 or ACVR2A may participate in the BMP6-induced cell effects, indicating that BMPR2 and ACVR2A are the functional type II receptors in the molecular regulatory mechanism of BMP6 on PTX3 expression. However, BMPR2 may be the major type II receptor for BMP6-induced cell activity.

Receptor-activated SMADs or receptor-regulated SMADs (R-SMADs), including SMAD 1, SMAD 5, and SMAD 8 are required for BMP-mediated cellular activities(40). Using a similar inhibition approach, we demonstrated that SMAD1 and SMAD5 are the functional signal transduction molecules that mediate the
inhibition of BMP6 on PTX3 expression in KGN cells. In addition, our results also showed that SMAD4 is an indispensable mediator in the BMP6-induced downstream signaling pathway that resulted in the inhibition of PTX3 expression.

In summary, we demonstrated that the expression of BMP6 in hGL cells was higher, while the expression of PTX3 was lower in hGL cells obtained from patients with PCOS. Additionally, the expression of BMP6 was negatively correlated, while the expression of PTX3 was positively correlated with the oocyte maturation and pregnancy outcome in PCOS patients during their IVF/ET treatment. Using in vitro cell culture system, we also demonstrated that BMP6 suppressed the expression of PTX3 at the transcriptional and translational levels in hGL cells. Moreover, the suppressive effect of BMP6 on PTX3 expression was mediated by the ALK2/ALK3 type I receptors and BMPR2/ACVR2A type II receptors through the SMAD1/5/8-SMAD4 signaling pathway. Our findings shed light into the physiological role and potential molecular mechanisms by which BMP6 and PTX3 regulate ovarian function and ovulation, which may be involved in the pathogenesis of PCOS.

Declarations

**Ethics approval and consent to participate**

The reproductive ethics committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine (ref approval no. SDTCM/E2110-03) authorized the research and agreed to exempt the informed consents. All procedures were carried out in accordance with relevant guidelines and regulations and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Consent for publication**

Not applicable.

**Availability of Data and materials**

All data generated or analyzed during this study are included in this published article.

**Competing interests**

The authors declare that they have no conflict of interest.

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**Authors’ Contributions**
FL and HW conceived and designed this study. XX contributed to the acquisition and analyses of data. XX and LD contributed to drafting the manuscript. JL was responsible for sample collection. HMC and PCKL contributed to manuscript revision. All authors read and approved the final submitted version of this manuscript.

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References


**Figures**
Figure 1

Comparison of the expression of BMP6 and PTX3 in human granulosa-lutein (hGL) cells and follicular fluid between PCOS and control groups. (A–B) The mRNA levels of BMP6 and PTX3 in hGL cells of PCOS patients and control patients examined using RT-qPCR. (C–E) The protein levels of BMP6 and PTX3 in the granulosa cells of PCOS and control patients examined using western blot analysis. (F–G) The accumulated protein levels of BMP6 and PTX3 in the follicular fluid samples obtained from PCOS.
patients and control patients examined using an ELISA. The results were expressed as mean ± standard error of at least three independent experiments with different letters indicating statistically significant difference (P < 0.05).

Figure 2
Correlation between the mRNA levels of BMP6 and PTX3 in hGL cells and clinical outcomes in patients with PCOS. (A-F) Spearman correlation analyses were used to investigate the correlation between the mRNA levels of BMP6 and the oocyte maturation rate (A), cleavage rate (B), available embryo rate (C), high-quality embryo rate (D), cumulative clinical pregnancy rate (E), and fertilization rate (F) in PCOS patients. (G-L) Spearman correlation analyses were used to investigate the correlation between the mRNA levels of PTX3 and the oocyte maturation rate (G), cleavage rate (H), available embryo rate (I), high-quality embryo rate (J), cumulative clinical pregnancy rate (K), and fertilization rate (L) in PCOS patients.

Figure 3

The effects of BMP6 on the expression of PTX3 in KGN and hGL cells. (A) KGN cells were treated with vehicle control or different concentrations (1, 10, or 100 ng/mL) of BMP6 for 6 h, and the mRNA levels of PTX3 were examined using RT-qPCR. (B) KGN cells were treated with 100 ng/mL BMP6 for 3, 6, 12, or 24 h, and the mRNA level of PTX3 were examined using RT-qPCR. (C) KGN cells were treated for 6 h with vehicle control or different concentrations (1, 10, or 100 ng/mL) of BMP6, and the accumulated protein levels of PTX3 were measured using an enzyme immunoassay. (D) The primary hGL cells were treated with different concentrations (1, 10, or 100 ng/mL) of BMP6 for 6 h, and the mRNA level of PTX3 were
examined using RT-qPCR. The results were expressed as mean ± standard error of at least three independent experiments with different letters indicating statistically significant differences (P < 0.05).

**Figure 4**

**ALK2 and ALK3 type I receptor are required to mediate BMP6-induced downregulation of PTX3 expression in KGN cells.** (A-C) KGN cells were pretreated with dimethyl sulfoxide (DMSO), 10 μM DM (A),
0.25 μM DMH-1 (B), or 10 μM SB431542 for 1 h, and then cells were treated with 100 ng/mL BMP6 for another 6 h. The mRNA levels of PTX3 were examined using RT-qPCR. (D-G) KGN cells were transfected with siCtrl, siALK2 (D), siALK3 (E), siALK6 (F), or combined siALK2 and siALK3 (G) for 24 h, and then cells were treated with 100 ng/mL BMP6 for another 6 h. The mRNA levels of PTX3 were examined using RT-qPCR. The results are expressed as mean±standard error of at least three independent experiments. Different letters indicate statistically significant differences (P < 0.05).
**Figure 5**

**BMPR2 and ACVR2A type II receptors are required to mediate the BMP6-induced downregulation of PTX3 expression in KGN cells.** (A-C) KGN cells transfected with siCtrl, siBMPR2 (A), siACVR2A (B), or siACVR2B (C) for 48 h, and then cells were treated with 100 ng/mL BMP6 for another 6 h. The mRNA levels of PTX3 were examined using RT-qPCR. (D-F) KGN cells were transfected with siCtrl, combined siBMPR2 and siACVR2A (D), combined siBMPR2 and siACVR2B (E), or combined siACVR2A and siACVR2B (F) for 48h, and then cells were treated with 100 ng/mL BMP6 for another 6 h. and the mRNA levels of PTX3 were examined using RT-qPCR. The results are expressed as mean±standard error of at least three independent experiments. Different letters indicate statistically significant differences (P < 0.05).

**Figure 6**
ALK2/ALK3 type I receptors and BMPR2 type II receptor mediate BMP6-induced phosphorylation of SMAD1/5/8 in KGN cells. (A) KGN cells were pretreated for 1 h with DMSO, DM (10 µM), DMH-1 (0.25 µM), or SB431542 (10 µM), and then cells were treated with 100 ng/mL BMP6 for another 6 h. The levels of phosphorylated SMAD1/5/8 protein levels were examined using Western blot analysis. (B) KGN cells were transfected with siCtrl, siALK2, siALK3, or siALK6 for 24 h, and then cells were treated with 100 ng/mL BMP6 for another 1 h. The phosphorylated SMAD1/5/8 protein levels were examined using Western blot analysis. (C) KGN cells were transfected with siCtrl, siALK2, siALK3 or combined siALK2 and siALK3 for 24 h, and then cells were treated with 100 ng/mL BMP6 for another 1 h. The phosphorylated SMAD1/5/8 protein levels were examined using Western blot analysis. (D-F) KGN cells were transfected with siCtrl, siBMPR2 (D), siACVR2A (E), or siACVR2B (F) for 48 h, and then cells were treated with 100 ng/mL BMP6 for another 6 h. The phosphorylated SMAD1/5/8 protein levels were examined using Western blot analysis. (G-H) KGN cells were transfected with siCtrl, combined siBMPR2 and siACVR2A, combined siBMPR2 and siACVR2B, or combined siACVR2A and siACVR2B for 48 h, and then cells were treated with 100 ng/mL BMP6 for another 6 h. The phosphorylated SMAD1/5/8 protein levels were examined using Western blot analysis.
SMAD1 and SMAD5 mediate the suppressive effect of BMP6 on PTX3 expression in KGN cells. (A-C) KGN cells were transfected with 25nM siCtrl, 25nM SMAD1 siRNA (siSMAD1) (A), 25 nM SMAD5 siRNA (siSMAD5) (B), or 25 nM SMAD8 siRNA(siSMAD8) (C) for 24 h. The mRNA levels of SMAD1, SAMD5 and SMAD8 were examined using RT-qPCR. (D-F) KGN cells were transfected with 25 nM siCtrl, 25 nM siSMAD1 (D), 25 nM siSMAD5 (E), or 25 nM siSMAD8 (F) for 24 h, and then cells were treated with 100
ng/mL BMP6 for another 6 h. The mRNA levels of PTX3 were examined using RT-qPCR. (G-I) KGN cells were transfected with 25 nM siCtrl, concomitant 25 nM siSMAD1 and siSMAD5 (G), concomitant 25 nM siSMAD1 and siSMAD8 (H), or concomitant 25 nM siSMAD5 and siSMAD8 (I) for 24h and then treated with 100 ng/mL BMP6 for 6h. The mRNA levels of PTX3 were examined using RT-qPCR. The results are expressed as SEM±mean of at least three independent experiments. Different letters indicate significant differences (P<0.05).

Figure 8

**SMAD4 mediates the suppressive effect of BMP6 on PTX3 expression in KGN cells.** (A) KGN cells were transfected with 25 nM siCtrl or 25 nM SMAD4 siRNA (siSMAD4) for 24 h, and the mRNA levels of SMAD4 were examined using RT-qPCR. (B) KGN cells were transfected with 25 nM siCtrl or 25 nM siSMAD4 for 24 h, and then cells were treated with 100 ng/mL BMP6 for another 6 h. The mRNA levels of PTX3 were examined using RT-qPCR. (C) KGN cells were transfected with 25 nM siCtrl or 25 nM siSMAD4 for 24 h, and then cells were treated with 100 ng/mL BMP6 for another 6 h. The accumulative protein levels of PTX3 were examined using an enzyme-linked immunosorbent assay. The results are expressed as SEM±mean of at least three independent experiments. Different letters indicate significant differences (P < 0.05).