

# TGF $\beta$ Gene Members and Their Regulatory Factors in Granulosa Compared to Cumulus Cells in PCOS: A Case-Control Study

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## Abstract

**Objective:** Transforming growth factor-beta (TGF- $\beta$ ) superfamily and its members that include bone morphogenetic protein 15 (BMP15), anti-Mullerian hormone (AMH), growth /differentiation factor-9 (GDF9), and their respective receptors: BMPR1A, BMPR1B, and BMPR2 have been implicated as key regulators in various aspects of ovarian function. The abnormal function of the ovaries is one of the main contributing factors to polycystic ovarian syndrome (PCOS), so this study aimed to investigate the mRNA expression profile of these factors in granulosa (GCs) and cumulus cells (CCs) of those patients.

**Materials and Methods:** The case-control research was conducted on 30 women (15 infertile PCOS and 15 normo-ovulatory patients, 22 $\leq$ age  $\leq$ 38 years old) who underwent ovarian stimulation for *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycle. GCs/CCs were obtained during ovarian puncture. The expression analysis of the aforementioned genes was quantified using real-time polymerase chain reaction (PCR).

**Results:** AMH and BMPR1A expression levels were significantly increased in GCs of PCOS compared to the control group. In contrast, GDF9, BMP15, BMPR1B, and BMPR2 expressions were decreased. PCOS' CC showed the same expression patterns. GDF9 and AMH were effectively expressed in normal CCs, and BMP15 and BMPR1B in normal GCs (P<0.05).

**Conclusion:** Differential gene expression levels of AMH and its regulatory factors and their primary receptors were detected in granulosa and cumulus cells in PCOS women. Since the same antagonist protocol for ovarian stimulation was used in both PCOS and control groups, the results were independent of the protocols. This diversity in gene expression pattern may contribute to downstream pathways alteration of these genes, which are involved in oocyte competence and maturation.

**Keywords:** Cumulus Cell, Granulosa Cell, Polycystic Ovarian Syndrome, TGF-Beta Superfamily

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## Introduction

Polycystic ovary syndrome (PCOS), one of the most common causes of an-ovulatory, is a heterogeneous endocrine disorder in infertile women, affecting up to 6-20% of women of reproductive age (1).

PCOS is confirmed by existing at least two of three special features based on the Rotterdam criteria: clinical/ biochemical evidence of hyperandrogenism, oligomenorrhea/ anovulation, and polycystic ovaries (2). A wide variety of genetic alterations in differential tissues and cell types impact normal follicular development in this syndrome. Imbalanced follicle-stimulating hormone (FSH), luteinizing hormone (LH), and some key factors that are involved in the inappropriate progression of

folliculogenesis are the main molecular features associated with impaired and arrested development of the germinal vesicle stage of oocytes toward the dominant and mature follicles in polycystic ovaries (3, 4).

The delicate communication process between different types of cells in the mammalian follicles, namely oocyte and its surrounding somatic cells, granulosa (GCs), and cumulus cells (CCs), has a pivotal impact on the oogenesis process, oocyte maturation, cumulus expansion, and ovulation (5, 6). Paracrine signals of the oocyte are the potent stimulators in GCs/CCs, which regulate the above physiological processes and consequently affect female fertility (7).

Among the secreted factors by the oocyte or follicle somatic cells, transforming growth factor-beta (TGF- $\beta$ ) superfamily and its members, including anti-mullerian hormone (AMH), growth and differentiation factor 9 (GDF9), and bone morphogenetic protein 15 (BMP15) which are expressed in follicular stage-related manner, have been implicated as key regulators in various aspects of intra-ovarian development (8). On the other hand, alteration in expression of these factors, especially AMH, is related to pathological ovarian conditions, such as PCOS and Endometriosis (9).

AMH is now a quantitative marker of ovarian reserve as well as a diagnostic factor for PCOS (10). TGF- $\beta$  family members bind to their receptors in GCs/CCs which induces a cascade of several downstream protein-coding genes. Even though GCs are differentiated into CCs and both of them have a common progenitor during folliculogenesis, the mentioned genes show different expression and even regulation patterns in those specialized cell types (11, 12).

Based on previous studies, alterations in the gene expression pattern of granulosa-cumulus cells play a definite role in the impaired final maturation of antral follicles to later-stage growth, which is one of the main contributing factors and underlying causes of polycystic ovaries (13).

AMH is secreted from ovarian cells in the growing follicles and can regulate the FSH-dependent follicle development. Previous studies showed that the expression and secretion of AMH from granulosa-cumulus cells are increased in preovulatory follicles, which contain atretic oocytes (14, 15).

GDF-9 and BMP-15, as other members of the TGF- $\beta$  superfamily, are known as growth promoters during normal folliculogenesis (16). A study of intraovarian interactions, regulatory mechanisms, and possible abnormalities which interfere with the balance between these molecular factors in patients suffering PCOS compared to women with normal oogenesis can provide a better overview of controlling and monitoring ovarian function that may be resulted in improving the fertility of such patients.

This study aimed to investigate the mRNA expression profile of *BMP15*, *GDF9*, *AMH* genes, and their respective receptors, which include: *BMPRIA*, *BMPR1B*, and *BMPR2*, in GCs and CCs of PCOS patients, compared to women with normal oogenesis undergoing controlled ovarian stimulation (COS) treatment cycles.

## Materials and Methods

The case-control research was endorsed by the Royan Institute Ethics Boards (IR.ACECR.ROYAN.REC.1394.86). Thirty patients (15 PCOS patients with infertility and 15 normo-ovulatory patients,  $22 \leq \text{age} \leq 38$  years old) were recruited to the study between November 2014 and April 2016 who underwent COS for IVF/ICSI at the Infertility Clinic (Royan Institute, Iran). Informed consent were obtained from all subjects.

Patients who met the following criteria were included in the control group: absence of clinical/ biochemical signs of hyperandrogenism or other endocrine diseases, polycystic ovaries, diabetes, with regular menstrual cycles ( $26 < \text{cycle length} < 32$ ), having normal hormone profile (TSH, FSH, and LH). All controls were undergoing ovarian stimulation treatment due to their tubal factor infertility.

Rotterdam diagnostic criteria are routinely used for PCOS phenotyping which includes: chronic oligo and/ or anovulation ( $< 26$  or  $> 35$  days of cycle length), biochemical or clinical evidence of hyperandrogenism (total testosterone concentration  $> 0.5$  ng/ml, hirsutism, acne, and alopecia), and the presence of 12 or more ovarian cysts with 2-10 mm diameter per cyst and/or ovarian volume  $\geq 10$  cm<sup>3</sup> on ultrasound (polycystic ovaries).

Inclusion criteria for all women (PCOS and control) were including the first IVF/ICSI cycle, ovarian stimulation by antagonist protocol,  $22 \leq \text{age} \leq 38$  years, and body mass index (BMI) of 18-28 kg/m<sup>2</sup>. The severe male factor, including oligo-astheno-teratozoospermia, retrieved sperm from testis, and semen freeze was excluded. The semen was analyzed in accordance with the criteria of the 5<sup>th</sup> edition of the World Health Organization (WHO) in 2010.

All hormone tests (FSH and LH levels) were carried out on days 2 to 3 of the menstrual cycle using electro-chemiluminescence immunoassay kits (ECLIA kits, Roche Diagnostics GmbH, Germany). Further evaluation of total testosterone concentrations in PCOS patients was performed by using ELISA kits (Monobind Inc., USA), and serum AMH levels were assessed using an electro-chemiluminescence immunoassay by Elecsys<sup>®</sup> and Cobas Immunoassay Analyzers (Roche GmbH).

## Control ovarian stimulation protocol

Down-regulation of the pituitary gland with GnRH antagonist protocol [the administration of 75–150 IU recombinant FSH (Gonal F; Merck Serono, or Puregon; MSD, the Netherlands)] was done for all women up to the day of human chorionic gonadotropin (hCG, Ovitrelle<sup>®</sup>, Merck-Serono) injection. The COS was performed considering the age of the women and the antral follicle count (AFC).

## Granulosa and cumulus cells isolation

The following steps were taken to isolate the follicular fluid and extract cumulus and granulosa cells from collected cumulus-oocyte complex (COC) as described previously (17, 18). Briefly, follicular fluid (FF) was obtained after hCG injection (34-36 hours) during ovarian puncture. COCs were collected from FF and repeatedly washed in culture medium G1V5<sup>™</sup> (Vitrolife AB, Sweden), then incubated at 37°C and 5% CO<sub>2</sub>. Just before ICSI, the COC was denuded with hyaluronidase enzyme, then CCs washed in free enzyme medium, and cold phosphate buffered saline (PBS), then the cells were pelleted by centrifugation twice for 8 minutes at 3000

rpm; the pellet was snap-frozen in liquid nitrogen then stored at -80°C (17).

After retrieval of COCs, aspirated FF was centrifuged for 10 minutes at 2000 rpm. The clear supernatant (without blood) was isolated, the pellet was solved in Tyrode's solution, and the suspension was added on Sill select gradient and centrifuged at 3000 rpm for 13 minutes. Granulosa cells (The layer formed between Sill select gradient and supernatant) were removed and washed with 3 ml of DMEM / HamF12 medium 10%. In the next step, the suspensions were centrifuged at 3000 rpm for 13 minutes. Afterward, the diluted hyaluronidase enzyme was added, and the suspension was incubated for 3 minutes; a culture medium was added to neutralize the effect of the enzyme, then the suspension was centrifuged for 5 minutes at 1500 rpm; Red blood cell lysing buffer was added to the cell suspension and incubated for 5 minutes, the solution was removed; granulosa cells were taken for extraction of RNA.

#### RNA extraction, cDNA synthesis, and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted, then purified by removing DNA genomic from the samples using the RNeasy Micro Kit (Qiagen, cat. no: 74004) as per the 'manufacturer's protocol which is also previously described (18).

After assessment of RNA quantity and quality by using the NanoDrop spectrophotometer (NanoDrop 2000 spectrophotometer), cDNA was synthesized using 20 nanograms of total RNA according to QuantiTect Whole Transcriptome Kit (Qiagen, Cat.No:207045) instruction and stored at -20°C until molecular analysis.

For granulosa cells' RNA extraction, TRIzol Reagent (Sigma-Aldrich) was used following the 'manufacturer's protocol. Genomic DNA contaminations were removed from all extracted RNA samples by treating them by DNaseI (Fermentas, Thermo Scientific, Germany). DNase I enzyme was inactivated by incubation for 7 minutes at 65°C with EDTA (50 mM, Fermentas, Germany). The purity of RNA was detected by NanoDrop spectrophotometer to determine A260/A280 ratio. RNA (1 µg) was reverse transcribed to cDNA using first-strand cDNA synthesis kit (K1632- Fermentas, Thermo Scientific, Germany) instruction, then stored at -20°C until molecular analysis.

#### Quantitative real time-polymerase chain reaction

A quantitative mRNA analysis was performed using the Step-One RT-PCR system for doing qRT-PCR (Applied Biosystems, USA). All reactions were run in duplicate. Evaluation of mRNA gene expression of *GDF9*, *BMP15*, *AMH*, *BMPRIA*, *BMPR1B*, *BMPR2* between the patient and control groups was done by calculation of  $\Delta C_t$  and  $2^{-\Delta C_t}$ . To perform this analysis, cDNAs were synthesized from granulosa and cumulus cells of patients and healthy individuals. The qPCR was performed with human-specific primers. The product size and primer sets are listed in Table 1. *GAPDH* primer was used as a control gene expression. PCR products were visualized using Gel Red ZellBio, Germany) staining and electrophoreses (1.7%-2% agarose, Paya pazhoh pars, Iran) under UV light by Molecular Imager<sup>®</sup> Gel Doc<sup>™</sup> XR+ (BioRad, USA).

**Table 1:** Sequence of the primers used for quantitative polymerase chain reaction (qPCR) experiments

Gene	Primer sequence (5'→3')	Annealing temperature (°C)	Size (bp)
<i>GDF9</i>	F: AGAAGTCACCTCTACAACACTG R: AACGGTAGTAATGCGATCCA	60	132
<i>BMP15</i>	TGTGAACTCGTGCTTTTCATG R: CTCAATCAGGGGCAAAGTAGG	60	102
<i>AMH</i>	AAGCTGCTCATCAGCCT R: TGCTTGCTGGTCTTTATTGG	60	194
<i>BMPRIA</i>	GAACTATTGCCAAACAGATTCAGA R: TGCCGCTATGAAACCAAGTAT	60	200
<i>BMPR1B</i>	ATTGTCAGCACAGACGGATATTGTT R: GAGGCAGTGTAGGGTGTAGGTCTT	60	199
<i>BMPR2</i>	TGCCGCTATGAAACCAAGTAT R: GACTCACCTATCTGTATACTGCT	60	184
<i>GAPDH</i>	AGAAGGCTGGGGCTCATTG R: TGATGGCATGGACTGTGGT	60	228

## Statistical analysis

Data obtained were analyzed using the Independent Samples t test, using IBM SPSS software version 22. The one-sample Kolmogorov-Smirnov (K-S) test was used to determine whether the variables come from the normal distribution in each group. Data are presented as mean  $\pm$  SD at the level of  $P < 0.05$ .

## Results

### Demographic information

Table 2 presents all the demographic data. Age, BMI, and duration of infertility were similar between the PCOS and control groups ( $P > 0.05$ ). However, there were significant differences among them considering the main PCOS features and characteristics, such as the ratio of LH/FSH, LH and AMH levels (Table 2). The PCOS subjects showed higher LH level than normo-ovulatory females (control group) ( $P < 0.01$ ).

### The expression level of *GDF9*, *BMP15*, *AMH* and their receptors in GCs and CCs from PCOS women compared to non-PCOS women

As shown in Figure 1, all amplified RT-PCR products were at the expected size for *GDF9*, *BMP15*, *AMH*, *BMPR1B*, *BMPR1A*, and *BMPR2* genes.

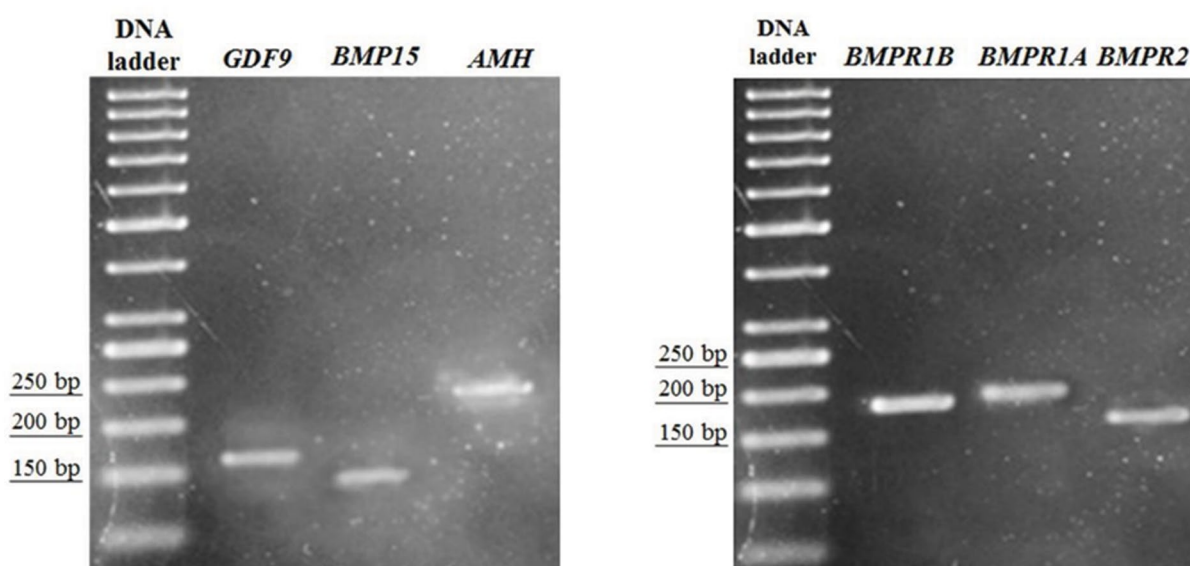
In GCs isolated from PCOS subjects, an overexpression of *AMH* ( $P < 0.001$ ) and *BMPR1A* ( $P < 0.001$ ) was observed. In contrast, the level of *GDF9* ( $P > 0.001$ ), *BMP15*, *BMPR1B*, and *BMPR2* gene expression were significantly decreased ( $P < 0.001$ , Fig.2A).

On the other hand, CCs from PCOS patients display the same patterns as observed in GCs genes expression increased expression of *AMH* ( $P < 0.001$ ) and *BMPR1A* ( $P < 0.038$ ); decreased expression of *GDF9* ( $P < 0.001$ ), *BMP15* ( $P = 0.005$ ), *BMPR1B* ( $P < 0.004$ ), and *BMPR2* ( $P < 0.001$ ) mRNA significantly (Fig.2B).

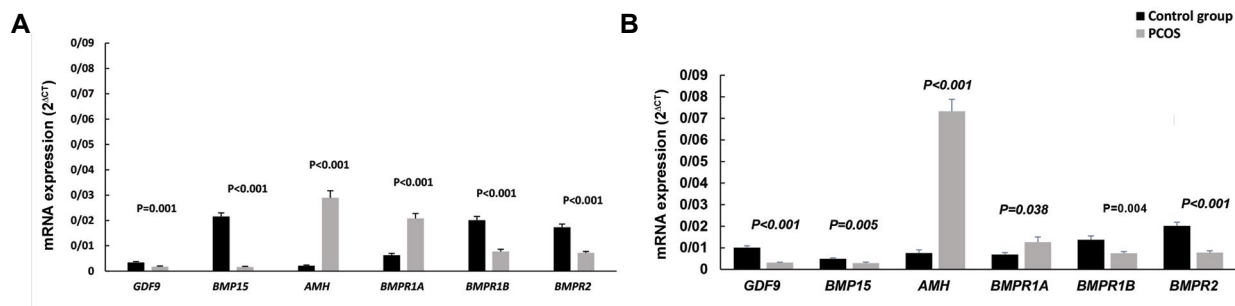
**Table 2:** Demographic information

Parameters	PCOS group	Control group	P value
Age (Y)	28.87 $\pm$ 6.5	28.87 $\pm$ 6.5	0.9
BMI (kg/m <sup>2</sup> )	24.5 $\pm$ 4.5	25.82 $\pm$ 4.5	0.2
Infertility duration (Y)	2.5-10	3-15	0.6
Number of oocyte	11.5 $\pm$ 4.5	8 $\pm$ 3	0.7
AMH (ng/ $\mu$ l)	12.43 $\pm$ 5.9*	4.7 $\pm$ 2.9	0.0001
FSH (IU/I)	6.71 $\pm$ 3.3	6.42 $\pm$ 3.15	0.6
LH (IU/I)	14.35 $\pm$ 11.55*	3.85 $\pm$ 3.35	0.01
TSH (IU/I)	2.23 $\pm$ 1.65	2.45 $\pm$ 2.15	0.5

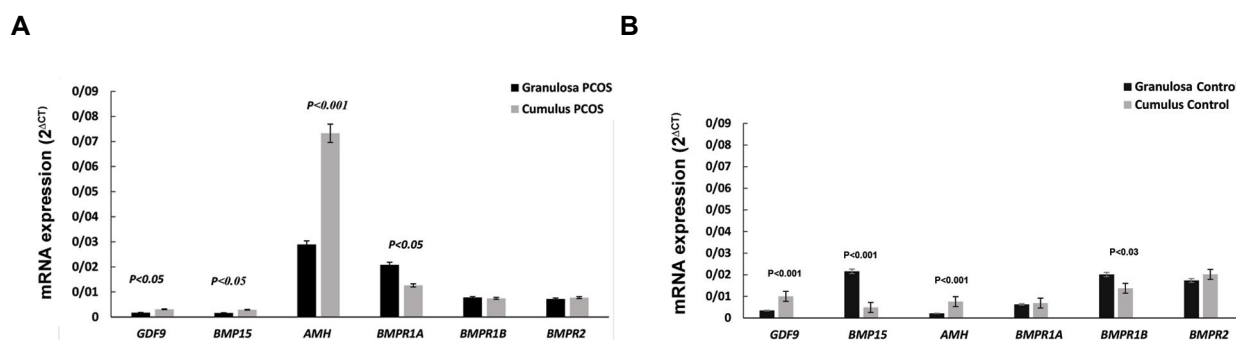
The results are reported as mean  $\pm$  SD. AMH; Anti-mullerian hormone, BMI; Body mass index, FSH; Follicle-stimulating hormone, TSH; Thyroid stimulating hormone, and \*;  $P < 0.05$ .



**Fig.1:** Results of RT-PCR for mRNA expression of *GDF9*, *BMP15*, *AMH*, *BMPR1B*, *BMPR1A*, and *BMPR2* genes (DNA ladder 50 base pair). RT-PCR; Reverse transcription polymerase chain reaction.



**Fig.2:** Expression level of *GDF9*, *BMP15*, *AMH* and their receptors in polycystic ovarian syndrome (PCOS) women and control group. **A.** Gene expression of granulosa cell from PCOS women compared to control group. **B.** Gene expression of cumulus cells from PCOS women compared to control group.



**Fig.3:** Differential gene expression profile in granulosa compared to cumulus cells. **A.** Gene expression profile in granulosa cells compared to cumulus cells in polycystic ovarian syndrome (PCOS) group. **B.** Gene expression profile in granulosa cells compared to cumulus cells in control groups.

### Differential gene expression profile in GCs compared to CCs in control and PCOS women

Comparing CCs and GCs from PCOS patients revealed *GDF9*, *BMP15* ( $P<0.05$ ) and *AMH* ( $P<0.001$ ) have higher expression in cumulus cells; in contrast, *BMPR1A* ( $P<0.05$ ) expression level is higher in GCs of such patients (Fig.3A). On the other hand, *BMP15* ( $P<0.001$ ) and *BMPR1B* ( $P<0.03$ ) are effectively expressed in normal granulosa cells compared to normal CCs, and *AMH* ( $P<0.001$ ) and *GDF9* ( $P<0.001$ ) have higher mRNA expression in CCs of control than PCOS patients (Fig.3B).

### Discussion

The differential gene expression levels of *AMH* and its regulatory factors that include *BMP15* and *GDF9*, as well as their primary receptors: *BMPR2*, *BMPR1B*, and *BMPR1A*, were detected in granulosa and cumulus cells in PCOS women compared to the control group. In addition, these genes showed different expression patterns in two ovarian cells (GCs and CCs).

Granulosa cells show phenotypic heterogeneity in the developing ovarian follicle. During folliculogenesis, the heterogeneous GC populations differentiate and divide into mural GCs and CCs. Their function is associated

with their position in the follicles and proximity to the oocyte resulting in different properties and gene expression patterns (19, 20). The mural GCs have a great steroidogenic function, a high level of LH receptors, and mainly are involved in the acquisition of signals from outside follicle; however, the cumulus cells provide essential regulatory factors for oocytes to promote final cytoplasmic and nuclear maturation (21, 22).

The resumption of oocyte meiosis is regulated by activating some genes in the oocyte itself and a broad signaling network in other follicular cells (23). The increased expression of *BMP15* and *BMPR1B* receptors in the periovulatory phase of ovarian cells showed their implication in the ovulatory process. On the other hand, these BMP proteins, the largest subfamily belonging to the TGFβ superfamily, regulate numerous main biological processes in the ovarian follicle, including cell differentiation, proliferation, and apoptosis (24). Also, the expression of *BMPR1B* is correlated with serum oestradiol (E2) level in normal folliculogenesis (25). Alterations in BMPs signaling are involved in some folliculogenesis disorders such as PCOS (24). In *Bmp15* knockout mice, uni- and bi-lateral cysts developed, ovulation rate was decreased and female mice were subfertile due to reduced developmental potential of oocytes and ovulation (26). The reduced expression of this ligand and its receptor in

PCOS patients, as shown in the present study, has revealed the association mechanism of PCOS-related anovulation and impaired oocyte competency and maturation.

The study performed by Kedem-Dickman et al. (27) showed a reverse association between GC-cumulus *AMH* expression and oocyte maturity in preovulatory follicles. It means that corresponding GC-cumulus cells of the Germinal Vesicle oocyte stage express higher *AMH* mRNA levels than the Metaphase stage ones. They suggested that *AMH* meiosis inhibition, which had been confirmed previously (28), was the mechanism by which the oocytes may fail to resume meiosis.

Some recent studies showed that high levels of *AMH* decrease the FSH receptors number and reduces the sensitivity of GC to FSH, which consequently prevents the provoke activation of critical genes in steroidogenesis processes such as *P450scc* and *CYP19A1* genes (29).

PCOS is a disease in which *AMH* has putative roles in its pathophysiology (30). Usually, it is the first cause of anovulation infertility in women (31). It seems that *AMH* contributes to the mechanisms leading to antral follicle arrest, anovulation, and oligomenorrhoea/ amenorrhoea (32, 33). Taken together, these studies suggest that increased *AMH* levels in PCOS women create an *AMH*-dominated microenvironment, which counteracts the action of FSH on follicles selection (34). The increased pre-antral follicle number, which is the primary source of *AMH*, is not merely contributing factor, but also elevated *AMH* concentration in PCOS is largely due to increased *AMH* production by individual follicles (35).

These studies have shown indirect confirmation of *AMH*'s role in PCOS. Further observations elucidated the mechanism by which *AMH* expression is up-regulated in GCs. A limited number of BMPs such as *BMP15* can bind to *BMPR1B* and *BMPR2* receptors, and the activated receptors recognize *SMAD1*, which is translocated to the *AMH* promoter sequence. This mechanism significantly causes the *AMH* expression induction in human GCs (36, 37). In our study, mRNA expression of *BMP15* in cumulus cells of PCOS patients is higher than in granulosa ones, and *AMH* expression was also higher in these cells. However, when CCs/GCs of PCOS are compared to normal oogenesis ones, this pattern was not observed. The high level of *AMH* expression in PCOS may be due to an additional source of *BMP15* secretion, especially from the corresponding oocyte, which could induce *AMH* expression in CCs/GCs of such patients.

In PCOS, however, *BMPRIA* in GCs and *GDF9*, *BMP15* and *AMH* in CCs have a substantial expression. These distinct alterations in the follicular microenvironment of PCOS patients may negatively interfere with intra-follicular signals such as the follicle sensitivity to FSH, compromise the dominant follicle selection and the other functions of the GC/CC cells from the viewpoint of the oocyte maturation aspect and reproductive processes (38, 39).

On the other hand, in ovarian cells, the most important

receptor for *AMH* is *BMPR-1A*. Therefore some members of the TGF- $\beta$  superfamily share their signaling pathway with *AMH*, and target genes involved in folliculogenesis and GCs differentiation could also be regulated by *AMH* (40).

To explore whether gene expression pattern is diverse between cumulus cells and granulosa cells, we analyzed the expression profile. Data showed a differential overexpression of *GDF9*, *BMP15*, and *AMH* in infertile PCOS cumulus cells in comparison to PCOS granulosa cells. However, *AMH* and *GDF9* have the same pattern in normal cyclic cumulus cells, but *BMP15* and *BMPR1B* significantly decreased in granulosa cells of normal patients. Therefore, the gene expression profile in both GC and CC cells showed interesting diversity. Since the same antagonist protocol for ovarian stimulation was used in both PCOS and control groups, the results were independent of the protocols.

## Conclusion

To conclude, based on the previous results, increased gene expression of *AMH* and *BMPRIA* and decreased gene expression of *GDF9*, *BMP15*, and *BMPR1B* may contribute to alterations in the downstream pathway, which are involved in ovulatory function, follicular growth, and oocyte maturation. The results of the current study are important for understanding the mechanisms of follicular growth arresting and anovulation cycles in patients suffering from PCOS. It will be considered to screen the epigenetic regulatory mechanism of the promoter regions of the TGF $\beta$  family members and corresponding receptors in future studies.

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## Authors' Contributions

F.A., P.A., M. Shi, M. Sha, E.H.; Contributed to conception and design. F.A.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. P.A., M. Shi.; Were responsible for overall supervision. B.M., M. Shi.; Cooperated in the sampling section. M. Shi.; Was responsible for patients assessment and diagnosis, also for patient recruitment to study. F.A.; Collected the sample. Z.H., M. Kh.; Cooperated with some experimental tests. F.A., E.H.; Drafted the initial manuscript and wrote the manuscript. P.A., E.H., M. Shi.; Contributed to revise and edit the manuscript. All authors read and approved the final manuscript.

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