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The association of RBX1 and BAMBI gene expression with oocyte maturation in PCOS women

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Abstract

Background Polycystic ovarian syndrome (PCOS) is a common endocrine disorder that affects 6–20% of women of reproductive age. One of the symptoms of PCOS is hyperandrogenism, which can impair follicular development. This disruption can cause issues with the development of oocytes and the growth of embryos. Although the exact cause of PCOS is not yet fully understood, studying the gene expression pattern of cumulus cells, which play a crucial role in the maturation and quality of oocytes, could help identify the genes associated with oocyte maturation in PCOS women. Through indirect activation of APC/Cdc20, *RBX1* enables oocytes to bypass the GV (germinal vesicles) stage and advance to the MII (metaphase II) stage. our other gene is the *BAMBI* gene which stimulates WNT signaling, that is a crucial pathway for healthy ovarian function. This study aims to explore the expression level of the *RBX1* and *BAMBI* genes between GV and MII oocytes of PCOS and non-PCOS groups.

Methods In this experiment, we gathered the cumulus cells of MII (38 cases and 33 control) and GV (38 cases and 33 control) oocytes from women with/without PCOS. Besides, quantitative RT-PCR was used to assess the semi-quantitative expression of *BAMBI* and *RBX1*.

Results According to our research, the expression level of *RBX1* and *BAMBI* in MII and GV cumulus cells of PCOS patients was significantly lower than that in non-PCOS ones.

Conclusion This research raises the possibility of *RBX1* and *BAMBI* involvement in oocyte quality in PCOS women.

Keywords *BAMBI*, Cumulus cells, Oocyte maturation, Polycystic ovarian syndrome, *RBX1*

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Introduction

Polycystic ovarian syndrome (PCOS) affects six to twenty percent of reproductive-age females. This syndrome, which is identified by the presence of at least two out of the three distinctive features of Rotterdam criteria including hyperandrogenism, oligo-anovulation, and polycystic ovaries [1], is one of the most common endocrine conditions in women. Additionally, there are clear connections between PCOS and other metabolic illnesses, including cancer, hypertension, diabetes mellitus, and cardiovascular diseases [2]. Metabolic dysfunction in PCOS patients causes issues with oocyte maturation and embryonic development, which is caused by the change in the intrafollicular microenvironment during folliculogenesis [3].

The cumulus cells are located close to the oocyte and maintain communication with it by transzonal and gap junctions [4]. In the cumulus-oocyte complex (COC), there is a bidirectional relationship between the oocyte and the cumulus, leading to oocyte maturation as well as cumulus expansion and differentiation. During the maturation of the oocyte, cumulus cells provide a gateway for the transfer of various substances, including nutrients, regulatory molecules, and paracrine factors [5]. Furthermore, oocytes release substances that promote the differentiation and growth of cumulus cells [6]. Evidence suggests that cumulus cells have an important role in oocyte quality, fertilization, as well as early embryonic development, and pregnancy. Therefore, investigating the expression profile of cumulus cells is a non-invasive way to evaluate the quality of oocytes [7, 8]. There have been several attempts to figure out the gene expression profile of cumulus cells by microarray and RNA sequencing.

The Wnt/ β -catenin signaling can influence the activation of the female reproductive system by altering the hormonal activity in ovarian granulosa cells [22]. Among the genes that have the ability to favorably activate this pathway is *BAMBI* [10]. Numerous investigations have demonstrated the critical function the SCF complex plays in oogenesis [9]. The catalytic core of SCF, a multi-subunit proteasome, is formed by *RBX1*, one of its essential constituents [10]. Indirect activation of APC/Cdc20 by *RBX1* can cause an oocyte to bypass the GV stage and advance to the MII stage [11, 12]. This study aimed to determine if these genes are involved in oocyte maturation, taking into account their role in ovulation.

Methods

Participants' characteristics and experimental design

Participants who met the Rotterdam criteria for polycystic ovarian syndrome were the subjects of this investigation. They were infertile and referred to Yazd Reproductive Sciences Institute for treatment. Clinical

information was gathered for all the study subjects. The exclusion criteria were androgen-secreting tumors, congenital adrenal hyperplasia, and Cushing's syndrome. Cumulus cell samples were collected from the PCOS and non-PCOS women. The non-PCOS group included people who did not have PCOS or any other cause of female infertility and underwent ICSI treatment for male infertility. We considered non-PCOS women as a control group. Based on the examination of the oocyte nucleus, the maturity stage was determined. The oocytes with an extruded polar body were considered mature (MII), but those with a fully developed germinal vesicle were considered immature (GV). The cumulus cells (CCs) were isolated from COCs at the GV (GV-CC) and MII (MII-CC) stages. We collected 38 MII-CC and 38 GV-CC for the PCOS group. Also, 33 MII-CC and 33 GV-CC were gathered from non-PCOS women.

Oocyte retrieval and isolation of CCs

Oocyte retrieval was carried out using ultrasonography 35–36 h following HCG injection, and the COCs used in this investigation were identified with a stereomicroscope. Mechanical and enzyme methods were used to denude the CCs and oocytes from the COCs complex. After the CCs were separated from the COCs, the resulting CCs were washed with PBS. CCs were transferred to -80°C in a TRIzol[®] Reagent (Yekta Tajhiz Azuma, Iran) to preserve RNA until the next step was initiated.

RNA extraction and cDNA synthesis

TRIzol[®] Reagent was used to extract the total RNA from the samples. The concentration and purity of RNA were also determined with a NanoDrop spectrophotometer (DeNovix, USA), the results were verified by agarose gel electrophoresis, and then the extracted RNA was immediately used in the next step. Based on the manufacturer's instructions provided by the Parstous Easy cDNA Synthesis Kit (Parstous, Iran), cDNA was synthesized for both *BAMBI* and *RBX1* genes.

Quantitative real-time PCR (qPCR)

The confirmation of the selected genes was carried out using quantitative-PCR (q-PCR). The sequences of the primers utilized in this study are presented in Table 1. With the use of the SYBR Green master mix (Yekta Tajhiz Azuma, Iran) and the Rotor Gene-Q device (Qiagen, Germany), the q-PCR reaction was carried out to ascertain the relative expression of the genes. To eliminate manual mistakes, all reactions were done in duplicate. The initial denaturation was conducted for RT-PCR at 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 20 s, annealing at 61°C for 20 s, and extension at 72°C for 30 s. As an endogenous control of

Table 1 The list of primers was employed in this research project

Primer	Sequences	Annealing temperature (°C)	Amplification size (bp)
F-RBX1	AACTGTGCCATCTGCAGGAA	59.89	170
R-RBX1	TCCAATGGACACACCTGTCG	59.97	
F-BAMBI	CGCCACTCCAGCTACATCTT	59.82	104
R-BAMBI	CAGTGGGCAGCATCACAGTA	60.04	
F-ACTB	GGGAAATCGTGCGTGACATT	59.20	183
R-ACTB	GGAAGGAAGGCTGGAAGAGT	59.01	

qPCR: Quantitative polymerase chain reaction, *RBX1*: Ring box protein 1, *BAMBI*: Bone morphogenetic protein and activin membrane-bound inhibitor, *ACTB*: β -Actin, bp: Base pairs, F: Forward, R: Reverse

Table 2 Clinical characteristics of women with and without PCOS

Parameters	Non-PCOS	PCOS	P-value
Age (year)	34.50±4.00	32.58±5.48	0.46
BMI (kg/m ²)	26.55±3.94	28.16±5.53	0.38
FSH (IU/l)	6.8±1.2	6.6±1.8	0.25
LH (IU/l)	4.1±1.5	9.1±1.6	<0.05
LH/FSH	0.6±0.4	1.38±0.6	<0.001

Non-PCOS vs PCOS group. BMI: Body Mass Index. FSH: Follicle Stimulating Hormone. LH: Luteinizing Hormone. The values were obtained using a t-test and the results are reported as mean±SD. * p <0.05, ** p <0.001

normalization, we used the *ACTB* housekeeping gene. We used *ACTB* gene as the reference because according to Jesús Cadenas and colleagues (2022), *ACTB* is a suitable reference gene for the study of cumulus cells and oocytes [13].

Statistical analysis

GraphPad Prism, version 8.4.3, and IBM SPSS Statistics, version 26 (SPSS Inc., Chicago, USA), were used to analyze the data. The analysis was done through one-way ANOVA and multiple t-tests to check whether there was a significant difference between the groups. The statistical significance was determined by P-values less than 0.05.

Results

Table 2 reports the clinical data of the women with PCOS and those without PCOS who took part in this study. The result, as shown in Table 2, indicates that no significant difference between age, BMI, and FSH ratio in the two groups. The women with PCOS displayed a greater ratio of basic LH (9.1 ± 1.6) and LH/FSH (1.38 ± 0.6) than the non-PCOS women with LH (4.1 ± 1.5) and FSH (0.6 ± 0.4). They achieved these values at P <0.05 and P <0.001, respectively.

When compared to the non-PCOS patients, the PCOS women emerged to have a significantly lower expression of *RBX1* gene in their CCs at the MII stage ($P=0.0019$). Furthermore, the expression of *RBX1* gene in the CCs of the non-PCOS women at the GV stage was significantly lower than that of the non-PCOS women at the MII stage ($P=0.0024$) (Fig. 1a). The expression of *BAMBI* gene significantly was lower in the CCs of the PCOS patients at both the GV ($P=0.02$) and the MII (P <0.0001) stages, compared to that of the non-PCOS women. Additionally, the expression of *BAMBI* gene in the CCs of the PCOS

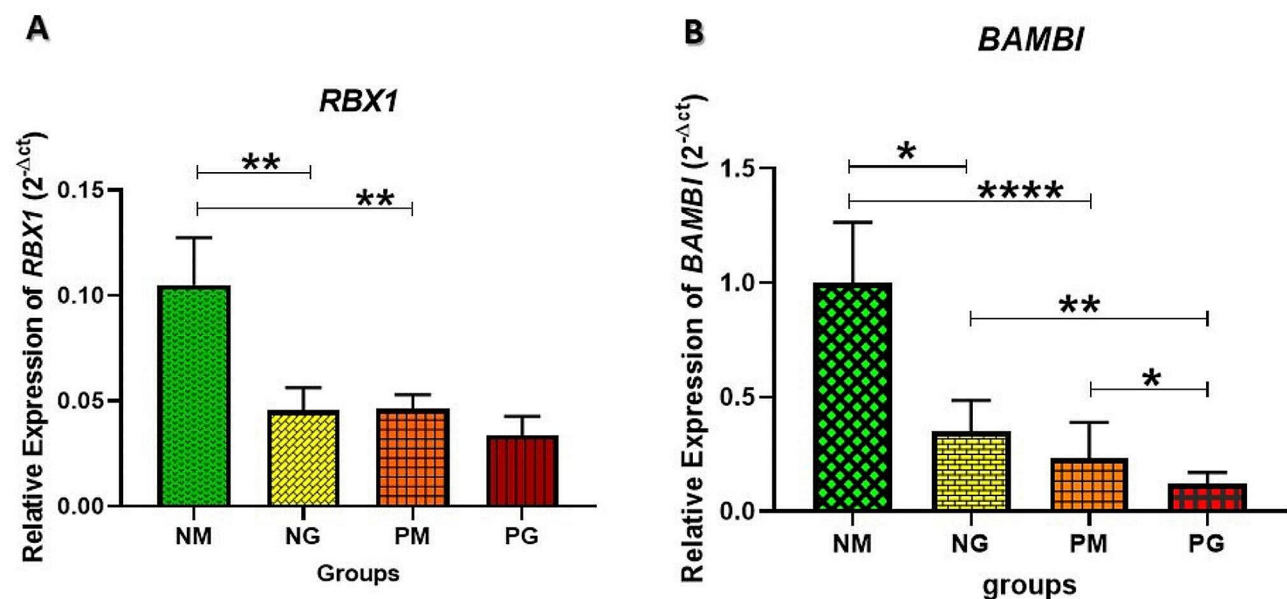


Fig. 1 The gene expressions of *RBX1* and *BAMBI* in CCs with and without PCOS at GV and MII stages. *Indicates a significant difference in gene expression. PM: PCOS at MII stage, PG: PCOS at GV stage, NM: non-PCOS at MII stage, NG: non-PCOS at GV stage. (a) The relative expression of the *RBX1* gene in PCOS and non-PCOS groups based on the developmental stages of the oocyte. (b) The expression of the *BAMBI* gene between groups with different oocyte maturation stages

women at the GV stage had significantly decreased compared to that in the CCs of the PCOS patients at the MII stage ($P=0.023$). The expression of this gene was significantly lower in the CCs of the non-PCOS women at both stages ($P=0.011$) (Fig. 1b).

Discussion

E3 ligases serve to catalyze the transfer of ubiquitin to a particular substrate, which completes the ubiquitination process [14]. One of the common families of E3 enzymes is the Skp1-cullin 1-F-box (SCF) complex, which is made up of three invariant components including Cullin (*Cul*), Ring box protein 1 (*RBX1*), and S-Phase Kinase Associated Protein 1 (*Skp1*). The catalytic core of the SCF complex is made up of *RBX1* and *Cul1* [15].

Yi and colleagues [16] showed that the inhibition of the proteasome complex keeps oocytes at the GV stage. A different morphology was observed by Kinterova and colleagues [12] for cumulus cells from oocytes matured in culture media containing an inhibitor of the SCF complex, which may cause problems during the expansion of cumulus cells. Cumulus expansion is an essential process involved in ovulation, oocyte maturation, and sperm capacitation. When cumulus cells expand, a change occurs in the junctional communication between them and oocytes, thus contributing to oocyte maturation [17]. Studies have shown that the SCF complex is essential for the proper maturation of oocytes, cumulus cell expansion, spermatogenesis, embryogenesis, and fertilization [12].

RBX1 is an important component of the SCF complex that degrades Early mitotic inhibitor 1 (*EMI*). *EMI* is an inhibitor of the anaphase-promoting complex/cyclosome (*APC/C*). Inhibition of this complex prevents the degradation of cyclin B and securin, which leads to stopping oocytes from progressing to the MII stage. Destruction of *EMI* by *RBX1* activates *APC/cdc20*, allowing for chromosome separation and anaphase onset. Dysregulation of *RBX1* can result in the accumulation of *EMI* and consequent inhibition of *APC/cdc20*, leading to anaphase arrest and keeping the cells in the GV stage [18].

In the present study, through a comparison of the MII-CCs in the PCOS patients were found to have lower gene expression than those of the non-PCOS. The expression of *RBX1* was also discovered to be lower in the CCs of the non-PCOS patients at the GV stage than in the MII stage. However, there was no significant difference in expression between the GV and MII stages in the PCOS group. This finding suggests that *RBX1* may play a role in improving the quality of maturation. As Zhou and colleagues [18] found that *RBX1* has an indispensable role in mouse oocyte meiotic maturation, embryonic development, and cell proliferation. To find further evidence, the outcomes were followed for these groups. In some cases,

the mature oocytes taken from the PCOS women who underwent IVF failed to fertilize successfully and resulted in the loss of embryos, as opposed to the mature oocytes taken from non-PCOS women. This evidence, along with the molecular function of *RBX1*, can explain how this gene plays a role in oocyte quality in PCOS.

The WNT signaling pathway plays an imperative role in normal ovarian function, fertility [9, 19], and development of follicles [9, 20], as well as during embryogenesis [21]. Akino and colleagues [22] showed that the dysregulation of this pathway in cumulus cells can result in follicle atresia, a reduced fertilization rate, and abnormal embryogenesis. The Wnt/ β -catenin pathway is initiated when a ligand binds to the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) and the Frizzled receptor. Without a suitable ligand, β -catenin is degraded by a destructive complex made up of GSK3B, Adenomatous Polyposis Coli (*APC*), and Axin [20]. However, when a Wnt ligand attaches to its receptor, the destructive complex stops and β -catenin accumulates in the cytoplasm and is transported into the nucleus [23].

Bone morphogenetic protein and activin membrane-bound inhibitor (*BAMBI*) is shown to interact with Frizzled5 and Dishevelled2 receptors and positively activate the Wnt/ β -catenin pathway. *BAMBI* knock-down impairs the Wnt signaling pathway, whereas its overexpression enhances cell cycle progression and transition from G1 to S phase by activating β -catenin nuclear translocation and stimulating cyclin D1 and c-myc synthesis [24, 25].

Using microarray, Assou and colleagues (2006) showed that the *BAMBI* gene is overexpressed in human cumulus cells. They stated that the overexpression of the genes having a role in cell-to-cell communication helps the maturation of the oocyte-cumulus complex [26]. Xinjian Li and colleagues (2020) performed high-throughput sequencing to identify candidate genes for reproductive traits in pigs. Their findings revealed that the *BAMBI* gene is related to reproductive traits [27]. Long Bai and colleagues (2014) examined the role of *BAMBI* in porcine granulosa cells, finding that this gene can regulate the development of ovarian follicles and the maturation of oocytes [28]. Lankford and colleagues [29] found that the relative expression of *BAMBI* gene was increased in the follicles that were competent to respond to the maturation-inducing hormone and also in mature follicles suggesting that *BAMBI* may contribute to the maturation process.

In the present study, it was discovered that the expression of the *BAMBI* gene was down-regulated in the MII-CC of the PCOS compared to MII-CC of the non-PCOS groups, and this decrease in *BAMBI*

expression in PCOS women can result in an arrested cell cycle in the primary stage, which may be the cause of lower oocyte quality in those patients. The result of the normal groups at various stages of differentiation revealed that this gene might be involved in the maturation process because its expression was downregulated in the GV-CC group compared to the MII-CC group. Furthermore, a comparison of immature cumulus cells to mature cumulus cells showed a downregulation of expression among PCOS women. It seems that this gene may contribute to oocyte maturation.

Conclusion

Gene expression analysis of cumulus cells provides a non-invasive method for examining oocyte conditions. According to our research, *RBX1* and *BAMBI* may be involved in oocyte quality in PCOS patients; however, more research is needed to validate this claim.

Abbreviations

PCOS	polycystic ovarian syndrome
RBX1	Ring box protein 1
APC/Cdc20	anaphase-promoting complex/cyclosome
GV	germinal vesicles
MI	metaphase II
WNT	Wingless/Integrated signaling pathway
BAMBI	Bone morphogenetic protein and activin membrane-bound inhibitor
RT-PCR	Reverse transcription polymerase chain reaction
COC	cumulus-oocyte complex
SCF complex	Skp, Cullin, F-box containing complex
ICSI	Intracytoplasmic sperm injection
CCs	cumulus cells
GV-CC	COCs at the GV
MI-CC	COCs at the MI
HCG	Human chorionic gonadotropin
PBS	Phosphate-buffered saline
ACTB	Actin beta
BMI	Body Mass Index
FSH	Follicle Stimulating Hormone
LH	Luteinizing Hormone
NM	non-PCOS at MI stage
NG	non-PCOS at GV stage
PM	PCOS at MI stage
PG	PCOS at GV stage
IVF	In vitro fertilization

Acknowledgements

We would like to thank our research colleagues from the Yazd Institute of Reproductive Sciences for their assistance to this project.

Author contributions

M.D. designed the study; F.A. and M.K. collected samples; K.M. and M.T. performed the experiments; M.V. and K.M. analysed data; K.M. wrote the manuscript in consultation with M.D.

Funding

No funding has been received for this experiment.

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

The Institutional Ethics Committee of the Shahid Sadoughi University of Medical Sciences of Yazd approved the study design (IR.SSU.MEDICINE.REC.1401.022) and written informed consent was obtained from each participant before collecting samples.

Consent for publication

Patients signed informed consent regarding publishing their data.

Competing interests

The authors declare no competing interests.

Received: 19 November 2023 / Accepted: 9 January 2024

Published online: 18 January 2024

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