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Original Research Article

Prok1 regulates the proliferation and apoptosis of ovarian granulosa cells in polycystic ovary syndrome via Pi3k/Akt/nf-κ B signaling route

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Abstract

Purpose: *To investigate the regulatory influence of Prok1 on apoptotic and proliferative changes in PCOS, and the implication of Pi3k/Akt/nf-κ B signaling pathway in the process.*
Methods: *Ovarian granulosa cells from a rat model of PCOS were assigned to control and si-Prok1 groups, after cell culture. Then, control lentivirus and Prok1 siRNA lentivirus (50 µL each) were added to the cells to the groups, respectively. Cell cycle ratio and apoptosis in the two groups were determined using flow cytometry, while Pi3k/Akt signal route-linked protein levels were assayed by immunoblot method.*
Results: *The proportions of cells at G0/G1 and S phases of the cell cycle in si-Prok1 group were significantly lower than those in the control group, but G2/M phase cell population was significantly higher, relative to the control ($p < 0.01$). There was significant down-regulation of protein expressions of cyclin A2 and cycline1 in si-Prok1 group, relative to control group, but p21 protein level was significantly higher in si-Prok1 group ($p < 0.05$). There was a significantly higher apoptosis in si-Prok1 group. In the si-Prok1 cells, there were significant increases in protein levels of Bcl-2, cleaved caspase-9 and caspase-3, relative to control group, while protein expression levels of Bax, p-Pi3k and p-Akt in si-Prok1 group were significantly lower than the corresponding control values ($p < 0.05$).*
Conclusion: *si-Prok1 arrests cell cycle, induces apoptotic changes, and inhibits the proliferation of ovarian granulosa cells through a mechanism related to the regulation of Pi3k/Akt signaling pathway. Therefore, it might play a potential role in the treatment of polycystic ovary syndrome.*

Keywords: *Pro-dynamic protein 1, Pi3k/Akt signaling pathway, Polycystic ovary syndrome, Apoptosis*

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INTRODUCTION

Polycystic ovary syndrome (PCOS), an endocrine disease prevalent in women of childbearing age, is characterized by hyperandrogenemia, ovulation dysfunction and polycystic ovary morphology, and it usually leads to infertility. The global prevalence of PCOS is approximately 15 % [1]. With the current increasing incidence of obesity, the incidence of

PCOS is also on the rise, but the pathogenesis of PCOS is still unclear. Recent studies have shown that epigenetic changes caused by hormonal dysregulation of the maternal uterine environment may be the intrinsic mechanism underlying etiology of PCOS [2]. The pathogenesis of PCOS involves a complex array of influences such as genetic, epigenetic, environmental, lifestyle and dietary factors. The disease is associated with hyperinsulinemia, insulin resistance, hyperlipidemia, and chronic low-grade inflammation, all of which pre-dispose to metabolic syndrome and cardiovascular disease [3]. Researchers have focused attention on elucidation of the pathogenesis of PCOS and development of new treatment strategies for the disease.

Pro-dynamic protein 1 (Prok1) is a new angiogenic factor known to participate in controlling implantation [4]. Studies have shown that trophoblast, placental, endometrial and ovarian tissues express Prok1 [5]. It binds to 2 receptors, i.e., Prokr-1 and 2. In man, the Prokr-1 mRNA expression in trophoblasts is 80 times higher than that of Prokr-2 [6]. In addition to angiogenesis, Prok1 regulates many biological processes such as inflammation, migration, multiplication and contact between cells [7]. Studies on porcine endometrium have shown that Prok1 functions as an intermediary signal factor in the embryo through prokr1, thereby stimulating implantation-associated angiogenesis [8]. In addition, Prok1 regulates porcine endometrial expression of pregnancy-related genes and proteins, as well as the secretion of prostaglandins and cytokines [9]. However, not much is known about its mechanism of action in PCOS.

In the present research, the influence of Prok1 on multiplication and apoptotic changes of cells of ovarian granulosa in PCOS, and the related mechanism, were investigated.

EXPERIMENTAL

Animals

Female SD rats were obtained from Jrdun Biotechnology, Shanghai, China. The rats which were aged 3 weeks, with weight range of 50 to 55 g, were kept at a temperature of 22 ^{o}C and 33 % humidity in a room with a 12-h light /12-h dark cycle. This research was approved by the Animal Ethical Committee of The First People's Hospital of Liangshan Yi Autonomous Prefecture (approval no. LSYAP012) in line with revised NIH guidelines [10].

Establishment of PCOS rat model

The PCOS rat model was established via subcutaneous injection of dehydroepiandrosterone (DHEA; Sigma, Shanghai, China) at exposure level of 60 mg/kg. The drug was subcutaneously injected into the back of the neck of each rat every day for 21 days. In the control group, PBS was used instead of DHEA.

Collection and culture of ovarian granulosa cells

Following the successful establishment of PCOS rat model, rats were immediately sacrificed, and ovaries were excised. Then, cystic follicles were stripped and punctured to obtain ovarian granulosa cells which were thereafter re-suspended in PBS and subjected to centrifugation. The resultant supernatant cells were taken as ovarian granulosa cells. Subsequently, cells were incubated in PBS containing 10 % FBS and antibiotics (penicillin (100 U/mL) and streptomycin (100 µg/mL)) in DMEM/f12 at 37 ^{o}C in a saturated humidity incubator containing 5 % CO_2. Ovarian granulosa cells were identified using immunocytochemistry.

Cell transfection

Cells were assigned to control and si-Prok1 groups. Ovarian granulosa cells in logarithmic growth phase were inoculated in 96-well plates (I x 10^5 cells per well) and subjected to a 12-h incubation at 37 ^{o}C and 5 % CO_2. A complete culture medium was added and the virus concentration was adjusted to 1 $\times$ 10^7 TU/mL, followed by addition of 50 µL of Prok1 shRNA lentivirus or control lentivirus. After 12 h of transfection, the culture medium was replaced with complete culture medium, and culturing was continued for 48 h. Puromycin was used to screen the transfected cells and to expand the cell culture for subsequent experiments.

Indices measured

Determination of cell cycle

Two groups of trypsin-digested cells were plated in 6-well plates, with each well containing 1 x 10^5 cells. Serum culture solution (1 mL) was added to each well. After 12 h, the cells were washed thrice with PBS, followed by addition of complete culture medium. After 48 h, the cells were digested with trypsin and washed with PBS. Then, the cells were fixed in 70 % ethanol at 4 ^{o}C overnight, followed by staining with PI cell cycle

dye for 30 min. The red fluorescence was measured at 488 nm using flow cytometry.

Measurement of apoptosis

Cells in the two groups were digested with trypsin, and seeded as indicated above. After 48-h, tryptic digestion was done, and the cells were taken up in EP tubes, followed by rinsing in PBS and binding solution. This was followed by 10 min-staining with 5 µL of annexin V-PE reagent away from light, and staining with l7-add staining solution for 5 min, also in the dark. The cells were subjected to flow cytometric analysis to determine the apoptosis rate.

Western blot assay for protein expressions

The cells in each group were trypsin-digested, centrifuged at 1000 rpm for 3 min, and lysed with RIPA containing 150 µL of protease inhibitors. The plasma cells were transferred to sterile EP tubes, lysed on ice for 30 min, and centrifuged in a high-speed centrifuge at 4 ^{o}C for 30 min. The supernatant protein content was measured using BCA protein concentration kit. Equal amounts of protein (10 - 50 µg) were resolved with SDS-PAGE, followed by electro-transfer to PVDF membranes. The membranes were sealed by incubation with non-fat milk solution. Then, the membranes were subjected to 12-h incubation at 4 ^{o}C with 1:1000 diluted 1^{o} antibodies, i.e., rabbit anti-mouse Bcl-2, Bax, pro-caspase 9, cleaved Caspase 9, pro-caspase 3, and cleaved caspase 3 (Wuhan Saiwei Biotechnology Co. Ltd., China).

Thereafter, the membranes were washed thrice with PBST buffer, followed by incubation at room temperature for 60 min with rabbit anti-mouse IgG secondary antibody (1:2000 dilution; Agilent Technologies Co. Ltd, Santa Clara, California, USA; product no. 559763). The bands were photographed with iBright FL1500 gel imaging analysis system, and gray value of each protein band, relative to GAPDH standard, was analyzed with Image J software.

Statistical analysis

The data from this study were analyzed with SPSS 20.0 software package. All measurement data are presented as mean ± standard deviation (SD), and *t*-test was used for two-group comparison. Count data are expressed as numbers and percentages (n (%)), and comparisons between groups were done with chi-squared (χ^2) test. Differences were assumed statistically significant at $p < 0.05$.

RESULTS

Cell cycle distribution

There were significantly lower proportions of cells at G0/G1 and S phases of the cell cycle in si-Prok1 group than in control, while the proportion of cells in G2/M phase was significantly higher in si-Prok1 group ($p < 0.01$; Table 1).

Table 1: Cell cycle distribution in the two groups

Group	G0/G1	G2/M	S-phase
Control	38.56±1.46	24.88±1.21	36.56±1.38
si-Prok1	35.49±1.47	34.51±1.35	30.00±1.21
T	6.626	23.755	15.984
P-value	<0.001	<0.001	<0.001

Relative amounts of cell cycle-related proteins

There was significantly down-regulated protein expression level of cyclin A2 and cyclin1 in si-Prok1 group, relative to control values, while p21 protein expression in si-Prok1 group was significantly higher (Figure 1 and Table 2).

Table 2: Expression levels of cell cycle-related proteins in the two groups

Group	P21	Cyclin A2	Cyclin E1
Control	1.06±0.13	1.01±0.11	1.05±0.14
si-Prok1	1.48±0.26[a]	0.73±0.08[a]	0.75±0.09[a]

[a]$P < 0.05$, compared with the control group

Apoptosis

As shown in Table 3, there was a significantly higher apoptosis rate in si-Prok1 group (14.29±2.17[a]) than in the contro (6.07±0.83)l.

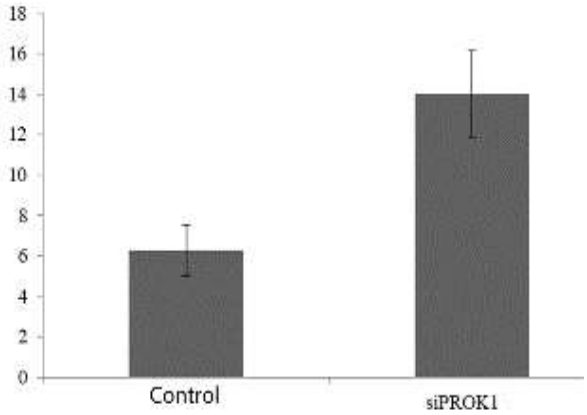

Figure 1: Apoptosis rate in each of the two groups

Table 3: Expression levels of apoptosis-related proteins in the two groups

Group	Bcl-2	Bax	Procaspase-9	Cleaved caspase-9	Procaspase-3	Cleaved caspase-3
Control	1.03±0.09	1.05±0.10	1.01±0.12	0.98±0.08	0.99±0.11	0.98±0.07
si-Prok1	0.73±0.07[a]	1.46±0.16[a]	1.38±0.14[a]	1.36±0.12[a]	1.03±0.15[a]	1.72±0.18[a]

[a]$P < 0.05$, compared with the control group

Apoptotic protein levels

Data presented in Table 3 show that relative to expression levels in control group, protein levels of Bcl-2, Bax, cleaved caspase-9 and caspase-3 were significantly lower in si-Prok1 group ($p < 0.05$).

Expression levels of Pi3k/Akt signaling pathway-related proteins in each group

Table 4 shows significantly down-regulated protein expressions of p-pi3k and p-Akt in si-Prok1 when compared with control values ($p < 0.05$).

Table 4: Relative expressions of apoptosis-related proteins

Group	p-pi3k	p-Akt
Control	1.05±0.13	1.04±0.11
si-Prok1	0.67±0.11[a]	0.64±0.08[a]

[a]$P < 0.05$, compared with the control group

DISCUSSION

It is known that PCOS is an endocrine disease that may lead to female anovulatory infertility. It may also lead to other complications such as cerebrovascular diseases, type 2 diabetes and hyperandrogenemia [11]. The prevalence of PCOS in adult women worldwide has been increasing steadily. Thus, there is need to elucidate the mechanism underlying the etiology of PCOS and to evolve efficient therapeutic approaches for the disease. It has been established that granulosa cells have crucial roles in oocyte and follicular biogenesis. Granulosa cells enhance the growth and development of primary and secondary follicles via hormonal and nutrient support, thereby providing a conducive atmosphere for maturation and meiosis of oocytes [12]. Therefore, apoptosis of granulosa cells results in reduction of ovarian hormonal production, thereby decreasing follicular growth and inducing apoptosis of oocytes.

It is known that P21 is a cell cycle inhibitor protein which inhibits cyclin and cycle-dependent activity. Cyclin A2 regulates G2/M and G1-S phase transitions. The level of cyclin A2 is highest in G2 phase, and it is a recognized marker molecule of G2/M [13]. Cycline1 regulates the transition from G1 phase to S phase, and its expression level is highest in G1/S phase, which makes it a reliable marker molecule for S phase. In this study, si-Prok1 induced cell arrest of ovarian granulosa cells in G2/M phase. In the si-Prok1 cells, the levels of cyclin A2 and cycline1 were significantly lowered, but p21 expression was significantly up-regulated. These results suggest that si-Prok1 regulated the proliferation of ovarian granulosa cells by regulating the expressions of cell cycle-related proteins.

Apoptosis is an important pathway of programmed cell death which is involved in regulating cellular homeostasis. Changes in the ratio of Bax and Bcl-2 determine the degree of apoptosis in cells. Cleaved caspase-9 and caspase-3 are pro-apoptotic factors which also participate in the apoptotic process [14]. In this study, apoptosis rate in si-Prok1 group was significantly increased. In si-Prok1 group, Bcl-2 expression was significantly decreased, while the expressions of Bax, cleaved caspase-9 and caspase-3 were significantly increased. These results suggest that si-Prok1 induces apoptosis in ovarian granulosa cells by regulating the expression levels of apoptosis-related proteins.

One of the most important features of PCOS is dysfunctional ovulation. The quality of oocytes is crucial for the reproductive potential of women with PCOS [15]. The upregulation of Pi3k/Glut4 signal route seems to be related to improvement of oocyte quality and embryonic development potential. Inhibition of Pi3k/Akt/mTOR route reduces autophagy in granulosa cells and decreases ovulation disorders. Recently, it was shown that electroacupuncture reduced the insulin resistance rating of PCOS patients through upregulation of the Irs-1/Pi3k/Glut4 signaling pathway [16]. Thus, Pi3K-related pathways play key roles in PCOS. The Pi3k/Akt signaling pathway is a dynamic route which plays an important regulatory role in cell cycle progression and proliferation. Phosphorylation of Akt inhibits the activity of cycle inhibitor protein p21, thereby increasing the proliferation potential of cells [17]. The NF- κ B signaling pathway is important for cellular growth and proliferation.

Phosphorylated Akt phosphorylates IKK α, which in turn, regulates the tumor cell cycle and increases the proliferation potential of tumor cells [18]. In this study, the protein expression levels of p-pi3k and p-Akt were significantly lower in si-Prok1 group than in control cells. Therefore, si-Prok1 regulated the multiplication and apoptotic changes in ovarian granulosa cells via a mechanism that may involve Prok1-mediated regulation of Pi3k/Akt signaling route-associated proteins.

CONCLUSION

si-Prok1 inhibits proliferative potential in ovarian granulosa cells, induces cell cycle arrest, and induces apoptosis through a mechanism most likely involving Prok1-mediated regulation of expressions of pi3k/Akt signal pathway-associated proteins. Therefore, it might play a role in the treatment of polycystic ovary syndrome.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors, all authors read and approved the manuscript for publication. Jun Peng, Yanxi Li and Yanxi Li conceived and designed the study. Jun Peng, Yong Huang, Yicun Man, Xin Wen, Xi Yang collected and analysed the data. Yanxi Li and Yanxi Li wrote the manuscript.

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