miR-142 promotes human ovarian granulosa cell growth by targeting PTEN

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Abstract: Objective: This study aimed to explore the molecular mechanisms of miR-142/PTEN in human ovarian granulosa cells. Methods: Follicular fluid was collected from 62 patients with polycystic ovary syndrome and from 31 normal people, and the granulosa cells were isolated. miR-142 was quantified using qPCR, and the miR-142 down-stream target genes were predicted using the TargetScan database. miR-142 mimic, miR-142 inhibitor, NC mimic, NC inhibitor, PTEN siRNA, and NC siRNA expression vectors were constructed, human ovarian granulosa cells were transfected, and the cell biological function expressions were observed. Western blot was used to measure PTEN, caspase 3, BAX, Bcl-2 and other proteins in the transfected cells, and qPCR was used to quantitatively transfect the miR-142 and PTEN mRNA. Results: miR-142 was highly expressed in granulosa cells, and it has a targeted relationship with PTEN. The overexpression of miR-142 downregulated PTEN and apoptosis and promoted cell proliferation, but the inhibition of miR-142 upregulated PTEN and apoptosis and inhibited cell proliferation. The loss of PTEN can promote cell proliferation and inhibit apoptosis. Conclusion: miR-142 inhibits the apoptosis of human ovarian granulosa cells by targeting PTEN, and miR-142 can be used as a potential target for the treatment of polycystic ovary syndrome.

Keywords: miR-142, polycystic ovary syndrome, human ovarian granulosa cells, PTEN

Introduction

Polycystic ovary syndrome (PCOS) is a common metabolic disease in women [1]. The main symptoms of these patients are irregular ovulation, polycystic ovaries and hyperandrogenism [2]. Polycystic ovary syndrome is more common in women of childbearing age [3, 4]. Since its main pathogenesis is insulin resistance [5], obesity and smoking are risk factors for its occurrence [6, 7]. If polycystic ovary syndrome is not treated in time, it may lead to cardiac diseases and endothelial dysfunction [1, 8]. The genetic regulation of insulin resistance is a potential direction for the treatment of polycystic ovary syndrome.

miR-142 is a non-coding RNA with a length of about 87 bp located on human chromosome 17. Although miR-142 does not participate in the direct coding of genes, it can apparently regulate gene expression by combining with other nucleic acid sequences. In cervical cancer, miR-142 inhibits HMGB1 by binding with HMGB1, which eventually leads to the reduction of cervical cancer metastasis and growth capacity [9]. MiR-142 can promote bladder cancer cell death through the negative regulation of ZEB2 [10]. In addition, miR-142 can be used as a tumor promoter to promote the growth of breast cancer and colorectal cancer [11, 12]. Previous studies [13] have shown that miR-142 is highly expressed in polycystic ovary syndrome and participates in its occurrence.

The PTEN protein is strongly linked to the morphology and growth of cells. The N-terminal phosphatase catalytic domain and the C-terminal domain are important functional domains for the PTEN protein to exert molecular func-
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tions [14]. PTEN is a tumor suppressor in many cancers that works by changing the micro-environment and inducing cell autophagy [15, 16]. Previous studies show that [17] PTEN is low expressed in patients with polycystic ovary syndrome. The loss of PTEN can promote the proliferation of ovarian granulosa cells, and this regulation involves the PI3K/Akt and Wnt pathways [18-20]. The PTEN pathway is closely related to insulin resistance [21, 22], so regulating the PTEN pathway will be helpful in the treatment of PCOS.

At present, the relationship between miR-142 and PTEN in polycystic ovary syndrome is not clear, so this study will take this as its research purpose, and explore the role of miR-142 and PTEN in polycystic ovary syndrome by regulating the expression of miR-142 and PTEN in human ovarian granulosa cells.

Materials and methods

Patients with polycystic ovary syndrome

Follicle samples were collected from 62 patients with polycystic ovary syndrome and from 31 normal people. The inclusion criteria were as follows: patients diagnosed with polycystic ovary syndrome (manifested as polycystic ovaries and hyperandrogenism). The exclusion criteria were as follows: those with mental illness; those who had a previous treatment history such as surgery, chemotherapy, radiotherapy or antibiotic treatment; those with a history of endocrine diseases, ovarian insufficiency or endometriosis; those complicated with other tumors; those who did not cooperate with the treatment. The patients were fully informed and this study was approved by the hospital ethics committee.

The participants’ follicle fluid was collected, and altogether 300× g of follicular fluid was centrifuged, and the supernatant was removed after 5 min, leaving the bottom pellet layer. The white mucinous substance on the surface of the sediment was carefully vacuumed off. The red blood cells were removed using Percoll separation solution (Solarbio Company). After that, the cell mass was gently buffered with PBS buffer (Solarbio Company), and it was to be subjected to subsequent qPCR quantitative experiments.

Cell culture and transfection

Human ovarian granulosa KGN cells were purchased from the ATCC cell bank. The cells were cultured in an animal cell incubator at 37°C/5% CO₂. The culture medium system used was DMEM medium (HyClone Company) +10% fetal bovine serum solution (Gibco Company) +1% penicillin/streptomycin solution (100X, Solarbio Company). Subsequent experiments could be carried out after the cell culture reached an 80-90% coverage rate.

Before the transfection, the culture medium was replaced with a fetal bovine serum-free culture medium. On the day of the transfection, 1105 cells per well were inoculated into 6-well plates. MiR-142 mimic, miR-142 inhibitor, NC mimic, NC inhibitor, and NC siRNA vectors were purchased from the Shanghai Sangon Biotechnology Company. The cell lines were transfected with a Lipofectamine 2000 transfection kit (Invitrogen, USA). The procedures referred to the kit’s instructions. After transfection for 8 h, the fresh culture was replaced based on 37°C/5% CO₂.

qPCR

Total RNA was extracted using the Trizol method. The OD value of total RNA at 260-280 nm was determined using an ultraviolet spectrophotometer, and OD260/OD280 >1.8 was taken for the subsequent qPCR detection. The RNA was reverse transcribed and the PCR was amplified and quantified using a FastKing one-step reverse transcription-fluorescence quantitative kit (Beijing Tiangen Company, FP314) and the ABI PRISM 7000 (Applied Biosystems, USA). MiR-142, and PTEN mRNA primers were designed and synthesized by the Shanghai Sangon Biotechnology Co., Ltd. More details are shown in Table 1. The qPCR reaction system was referenced from the kit specification (total system 50 L): upstream primer 1.25 L, downstream primer 1.25 L, probe 1.0 uL, RNA template 10 pg/g, 50× ROX Reference Dye ROX 5 μL, and RNase-Free ddH₂O added to the total reaction system. The reaction process was as follows: reverse transcription at 50°C for 30 min, circulating once; pre-denaturation at 95°C for 3 min, circulating once; denaturation at 95°C for 15 s, annealing at 60°C for 30 s, cycling 40 times. The results were analyzed using an ABI PRISM 7000 instrument.
The internal reference genes were U6 and GAPDH, which were standardized using the 2\(^-\Delta\Delta C_{\text{t}}\) method.

**Western blot**

We adopted the 1 ml cell protein extracting solution to split the cells, repeatedly blew on the solution until the cells were completely split, and then they were centrifuged at 1,2104 r/min for 15 min. We took the supernatant, separated the proteins using SDS-PAGE electrophoresis, transferred the proteins to the NC membrane, and left them standing at room temperature for 1 h (closed using 5% skimmed milk-PBS solution). Then PTEN, caspase 3, caspase 9, BAX, Bcl-2, and β-actin primary antibodies were added and it was left to stand at 4°C overnight. The NC membrane was washed with a PBS solution three times, then goat anti-rabbit secondary antibody (HRP cross-linked) was added, and the mixture was allowed to stand to allow the NC film to wash with PBS solution and visualize using the enhanced chemiluminescence method. The internal reference protein was β-actin, and the relative expression level of the protein = gray value of the band to be detected/gray value of the β-actin band.

The PTEN, caspase 3, caspase 9, BAX, Bcl-2, β-actin primary antibody and secondary antibody goat anti-rabbit (HRP cross-linked) were all purchased from the Shanghai Abcam Company.

**Flow cytometry**

After the cells went through enzymolysis, they were used to prepare the cell suspension, and their numbers were controlled to 1106. The cells were immobilized in 70% ethanol ice-cold solution for 30 min, keeping the ambient temperature at 4°C. The ethanol solution was then removed and the cells were incubated in an Annexin V-FITC/7-AAD mixed solution. FACScan flow cytometry (Becton Dickinson Company, USA) was used to analyze the apoptosis.

**MTT**

The transfected cells were under trypsin enzymolysis, centrifuged to remove the enzyme solution, and added into a fresh culture medium, and the solution was aired to prepare the cell suspension. Four 96-well plates were taken and the cells were inoculated into the well plates according to the specification of 5103 cells/100 μL per well, with 3 wells in each group. We took out one well plate every 24 h, added 5 mg/ml MTT solution according to each 10 μL/well, continued to culture them for 1 h, then we removed the culture medium, and measured the OD value at 570 nm with an enzyme reader. The experiment was repeated 3 times to draw the cell activity-time curve.

**Dual luciferase report gene**

PmirGLO-PTEN-WT and pmirGLO-PTEN-MUT vectors were constructed and co-transfected into cells with miR-142 mimics and NC mimics respectively. After 48 h of transfection, the luciferase intensity was quantified using the dual luciferase report gene (Promega). Each step in the operation was carried out strictly in accordance with the instructions.

**Statistics and analysis**

The above index data were input into the SPSS 20.0 software package (Asia Analytics Formerly SPSS China) and GraphPad Prism 6.0 for the statistical analysis. The experiment was repeated three times. The measurement data were expressed as the means ± standard deviations. The data comparison method between the healthy group and the PCOS group, or the NC siRNA group and the PTEN siRNA group employed independent-samples t tests. The comparisons among the NC group, the miR-142 mimics group, and the miR-inhibitor group employed one-way analyses of variance, and the post hoc pairwise comparison used an LSD-t test. A Pearson correlation coefficient analysis was applied to the correlation between the miR-142 and PTEN mRNA. All the data adopted a two-tailed test. Ninety-five percent was used as the confidence interval, and the data were considered statistically different when P<0.05.
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**Results**

*MiR-142 was increased in human ovarian granulosa cells*

In this paper, follicular samples of 62 patients confirmed to have polycystic ovary syndrome and 31 healthy people were collected, and granulosa cells were isolated from them. miR-142 and PTEN mRNA were quantified using qPCR. According to Figure 1A and 1B, the levels of miR-142 in the ovarian granulosa cells of patients with polycystic ovary syndrome increased while the PTEN levels decreased. Then a miR-142 mimic and an miR-142 inhibitor were constructed to transfect human ovarian granulosa cells, and the PTEN protein was detected with Western blot. According to Figure 1C and 1D, an increase of miR-142 inhibited PTEN, while a decrease of miR-142 upregulated PTEN. The above results show that miR-142 is highly expressed in polycystic ovary syndrome, while PTEN is poorly expressed therein, and miR-142 might be involved in regulating PTEN.

*MiR-142 inhibited apoptosis and promoted cell proliferation*

In order to investigate the effect of miR-142 on polycystic ovary syndrome, miR-142 mimics and miR-142 inhibitor vectors were constructed and transfected into ovarian granulosa cells. The apoptosis was quantified using flow cytometry, and cell activity was measured using the MTT method, and caspase 3, caspase 9, BAX, and Bcl-2 were detected using Western blot. The specific results are shown in Figure 2. Based on Figure 2A, we discovered that increasing miR-142 could reduce the apoptosis rate, while inhibiting miR-142 could increase the apoptosis rate. Based on Figure 2B, we discovered that increasing miR-142 could downregulate caspase 3, caspase 9 and BAX, upregulate Bcl-2, and inhibiting miR-142 could upregulate caspase 3, caspase 9 and BAX, and downregulate Bcl2. Based on Figure 2C, we discovered that the over-expression of miR-142 could enhance cell activity, while the inhibition of miR-142 could reduce cell activity. In addition, PTEN decreased when miR-142 increased, and PTEN increased when miR-142 decreased. These results indicated that miR-142 might inhibit apoptosis and promote cell proliferation by regulating PTEN.

*PTEN inhibited cell proliferation and promoted apoptosis*

In this study, in order to confirm that miR-142 regulated apoptosis through PTEN, a PTEN siRNA vector was constructed and transfected into ovarian granulosa cells, and apoptosis was...
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The molecular mechanisms of miR-142/PTEN in human ovarian granulosa cells were determined using flow cytometry, and the cell activity was determined using the MTT method, and caspase 3, caspase 9, BAX, and Bcl-2 were detected using Western blot. The specific results are shown in Figure 3. Through Figure 3A, we confirmed that the inhibition of PTEN could reduce the apoptosis rate. Through Figure 3B, we confirmed that the inhibition of PTEN could upregulate caspase 3, caspase 9, and BAX and downregulate Bcl-2. Through Figure 3C, we confirmed that the inhibition of PTEN could enhance cell activity. These results indicate that PTEN inhibits cell proliferation and promotes apoptosis.

miR-142 targeted the inhibition of PTEN

The TargetScan database predicted the miR-142 binding site would be at 2427-2433 bp of PTEN 3’UTR (Figure 4A). The dual luciferase report gene confirmed that PTEN-wt could bind to the miR-142 mimics and caused the down-regulation of the fluorescence activity (Figure 4B). The Pearson analysis showed that the PTEN mRNA was negatively correlated with the miR-142 level (Figure 4C). These results indicate that miR-142 targets PTEN inhibition at the post-transcriptional level.

Discussion

This article compared the expressions of miR-142 and PTEN in the ovarian granulosa cells of patients with polycystic ovary syndrome and healthy people. The results showed that compared with the healthy people, the miR-142 in patients with polycystic ovary syndrome was highly expressed and the PTEN was poorly expressed. Li [13] verified that the upregulation of miR-142 might lead to the occurrence of polycystic ovary syndrome. Therefore, we speculated that the abnormal expressions of miR-142 and PTEN might be related to the occurrence of polycystic ovary syndrome. In addition, the inhibition of miR-142 or the over-expression of miR-142 lead to the upregulation or down-regulation of PTEN, respectively. The TargetScan database predicted that miR-142 and PTEN had binding sites in the 3’UTR region of PTEN. These results indicate that miR-142 might be involved in regulating PTEN expression in polycystic ovary syndrome.
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Figure 3. PTEN inhibits cell proliferation and promotes apoptosis. A. Downregulating PTEN inhibits apoptosis. B. Downregulating PTEN inhibits caspase 3, caspase 9, and BAX and promotes Bcl-2. C. Downregulating PTEN increases cell activity; *P was lower than 0.05 vs the NC siRNA group.

Figure 4. MiR-142 targets the inhibition of PTEN. A. The miR-142 binding site existed in PTEN 3’UTR. B. PTEN-wt bound to the miR-142 mimics, causing a downregulation of fluorescence activity. C. miR-142 was negatively correlated with PTEN mRNA; *P was lower than 0.05 vs the NC mimics.

In order to understand the functions of miR-142 and PTEN in polycystic ovary syndrome, three expression vectors of an miR-142 mimic, an miR-142 inhibitor, and a PTEN siRNA were constructed in our experiment, and the expressions of miR-142 and PTEN in human ovarian granulosa cells were regulated by the three expression vectors respectively. The results showed that when miR-142 was over-expressed or PTEN was inhibited, caspase 3, caspase 9 and BAX in the human ovarian granulosa cells were downregulated, and Bcl-2 was upregulated, while the apoptosis rate was reduced and cell activity was increased; when miR-142 was inhibited, caspase 3, Cas-
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Polycystic ovary syndrome is a metabolic disease characterized by peripheral insulin resistance [23], so insulin imbalance is very likely to lead to polycystic ovary syndrome. Previous studies [17] have shown that a disorder of the insulin pathway is related to polycystic ovary syndrome, and it is believed that the disorder of this pathway may be related to the downregulation of PTEN. Among the relevant mechanisms of insulin resistance, PTEN regulates insulin by inhibiting the PI3K/Akt pathway, which leads to glucose and lipid metabolism disorders [24, 25]. In addition, PTEN also promotes apoptosis through the PI3K/Akt pathway [26]. The results also confirm that the loss of PTEN leads to the upregulation of apoptosis-promoting proteins such as caspase 3, caspase 9, and BAX. Therefore, the inhibition of PTEN by miR-142 might induce the activation of the insulin and PI3K/Akt pathways, leading to insulin disorder and the downregulation of the apoptosis pathway activity, eventually leading to polycystic ovary syndrome.

This article discussed the regulation mechanism of the miR-142/PTEN pathway in polycystic ovary syndrome. MiR-142 promotes cell proliferation and inhibits apoptosis by inhibiting the PTEN pathway. This regulatory relationship causes the malignant proliferation of human ovarian granulosa cells. Although this article clarified the molecular biological significance of the miR-142/PTEN pathway, the clinical significance of this regulatory relationship still needs to be further explored. In future studies, the therapeutic value and clinical predictive value of miR-142/PTEN should be further explored. In addition, the relationship between miR-142/PTEN pathway, mTOR pathway and NF-B pathway should also be studied, work will greatly promote the development of polycystic ovary syndrome research.

In summary, this article discussed a possible mechanism of polycystic ovary syndrome by studying the effects of the changes of miR-142 and PTEN on human ovarian granulosa cells. It is believed that miR-142 inhibits apoptosis and promotes cell proliferation through the targeted inhibition of PTEN, so the high expression of miR-142 promotes the occurrence and development of polycystic ovary syndrome. Targeted regulation of the miR-142/PTEN pathway will help improve polycystic ovary syndrome.

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Disclosure of conflict of interest

None.

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