Original Article

MicroRNA-182 inhibits rat ovarian granulosa cell apoptosis by targeting Smad7 in polycystic ovarian syndrome

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Abstract: Dysfunction of granulosa cells may contribute to the aberrant folliculogenesis observed in polycystic ovary syndrome (PCOS). MicroRNAs (miRNAs) are highly conserved, small RNA molecules with 19-25 nucleotides in length that post transcriptionally regulate gene expression, involved in many developmental processes, including cell differentiation and apoptosis. Recently, miR-182 was reported to be downregulated in dihydrotestosterone (DHT)-induced rat PCOS model. However, the role of miR-182 in PCOS is still unclear. In this study, we aimed to reveal the role and regulating mechanism of miR-182 in granulosa cell apoptosis. We performed flow cytometry and quantitative Real-Time PCR (qRT-PCR) to detect the effects of DHT on apoptosis and miR-182 expression in granulosa cells, and found that DHT promotes granulosa cell apoptosis and high-concentration DHT inhibits miR-182 expression. We also investigated the role of miR-182, and found that miR-182 inhibits granulosa cell apoptosis. Bioinformatics identified Smad7 as a potential miR-182 target. Luciferase assays, mRNA and protein expression analysis, and flow cytometry assays confirmed that Smad7 is the functional target of miR-182. Taken together, these results suggest that miR-182 inhibits granulosa cell apoptosis by targeting Smad7, which provides a new insight into the mechanism of PCOS in mammals.

Keywords: Polycystic ovary syndrome, miR-182, apoptosis, granulosa cell, dihydrotestosterone

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders and affects 7-9% or more of all women of reproductive age [1]. PCOS women frequently exhibit reproductive malfunction. PCOS is the primary cause of infertility due to dysfunction of follicular maturation and ovulation, dysregulation of reproductive hormones, and distinctive multicystic ovaries. Aside from being associated with infertility, PCOS is also associated with an increased life time risk of developing non-reproductive metabolic disorders, including type 2 diabetes (T2D), insulin resistance, hypertension, hyperinsulinemia, obesity, oxidative stress, dyslipidemia and cardiovascular diseases [2-6]. Despite its prevalence, the etiology of PCOS is still unclear.

Both genetic and environmental factors were found to be associated with the pathogenesis of PCOS. The alteration of many factors such as hypersecretion of luteinizing hormone and hyperinsulinaemia have been reported to may impair the competence of maturing oocytes through endocrine and local paracrine/autoocrine actions, causing a lower pregnancy rate in PCOS women [7-10]. Hyperandrogenism is a crucial characteristic in patients with PCOS and the levels of androgens including testosterone, androstenedione and dihydrotestosterone (DHT) were upregulated in these people [11]. Previous study reported a DHT-induced PCOS model that was similar to those in human PCOS, allowing one to focus on the ovarian characteristics and metabolic features of PCOS [12].

MicroRNAs (miRNAs) are highly conserved, small RNA molecules with 19-25 nucleotides in length that post transcriptionally regulate gene expression based on interactions with the 3’ untranslated regions (UTRs) of mRNAs [13]. miRNAs are involved in a variety of biological
MicroRNA-182 inhibits rat ovarian granulosa cell apoptosis

1381

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processes, including cell proliferation, differentiation, apoptosis and signal transduction [14]. Recently, conditional deletion of Dicer1 in granulosa cells have showed that miRNAs are essential to normal ovarian function [15, 16]. Female Dicer1 knockdown mice reduce ovulation rates and increase granulosa cell apoptosis, resulting in their infertility [15, 16]. miR-182, as a cancer-related miRNA, is widely studied in various cancers and facilitates the occur and development progress of malignant tumors [17, 18]. In addition, miR-182 was reported to be involved in the release of ovarian progestagen and androgen, and miR-182 could promote progesterone release in granulosa cells [19]. Recently, it was reported that miR-182 was downregulated in dihydrotestosterone (DHT)-induced rat PCOS model [20]. Therefore, we hypothesized that miR-182 may play an important role in PCOS. In this study, taking advantage of DHT-induced PCOS model, we aimed to reveal the role and regulating mechanism of miR-182 in granulosa cell apoptosis.

Materials and methods

Cell culture

Granulosa cells were collected from rat ovarian follicles as described previously [21], and incubated in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco), 100 mg/ml streptomycin and 100 units/ml penicillin. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Henan Provincial People’s Hospital and performed according to the Guiding Principles for the Care and Use of Laboratory Animals. To detect the effects of DHT on the granulosa cells, granulosa cells were cultured in DMEM/F-12 with or without various concentrations of DHT (0, 10, 20, 40, 80 and 160 ng/ml). All cells were maintained in a humidified incubator containing 5% CO₂/95% air at 37°C.

Cell transfection

Total RNA of granulosa cells were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcribed into cDNA. Smad7 cDNA was amplified and cloned into pcDNA3.1 vectors (pcDNA-Smad7). The miR-182 and negative control (NC) mimics, miR-182 inhibitor and inhibitor NC and small interfering RNA for Smad7 (si-Smad7) and siRNA NC (si-control) were bought from GenePharma (Shanghai, China). The cells were seeded into 6-well plates, incubated for 24 h, and then were transfected by using Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer’s instructions. At 48 h post-transfection, cells were collected and used in subsequent experiments.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cultured cells using Trizol agent (Invitrogen, USA) according to the manufacturer’s instructions, and inverse transcribed to cDNA by using cDNA Synthesis kit (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). The expression level of miR-182 was determined by using mirVana miRNA Detection Kit (GeneMedScientifics, Arlington, MA, USA), and U6 snRNA was used as an endogenous control. The expression level of Smad7 mRNA was determined by using PrimeScript RT-PCR kits (Takara Biochemicals, Kyoto, Japan), and normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blot assay

Anti-Smad7 and anti-b-actin antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-Bax and anti-Bcl-2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goall anti-mouse/rabbit IgG antibody conjugated to horseradish peroxidase (HRP) was purchased from Gene Tech (Shanghai, China) used as the secondary antibody. All these antibodies employed in this study were monoclonal antibodies. Briefly, cultured cells were transferred to tubes containing RIPA buffer (Invitrogen, USA) and vortexed briefly. After the cells were lysed, the lysate supernatant was collected by centrifugation at 14,000 g for 30 min at 4°C. Proteins were denatured with loading buffer at 100°C for 5 min, and protein concentrations were determined by using Pierce BCA Protein Assay Kit (Amersham, Little Chalfont, UK). Proteins were isolated by 10% SDS-PAGE and then transferred onto the PVDF membranes (BioRad, Hercules, CA, USA). After blocking in 5% skimmed milk, the membrane was incubated with the specific antibody. Finally, the blotted pro-
MicroRNA-182 inhibits rat ovarian granulosa cell apoptosis

**Cell apoptosis assay**

The annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (BD Pharmingen, San Diego, CA, USA) was used to identify cell apoptosis in accordance with the manufacturer’s instructions. Briefly, cells were washed twice with cold PBS and resuspended in 1 × binding buffer at a concentration of 1 × 10^6 cells/mL; then, 5 µL of FITC-conjugated annexin V and 5 µL of PI were added to 100 µL of cell suspension; after the samples stood for 10 min in darkness, 400 µL 1 × binding buffer was added. Then the samples were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA) to determine the relative amount of annexin V-FITC positive and PI negative cells.

**Target prediction analysis**

TargetScan, MicroRNA.org and PicTar were used to perform bioinformatics-based target prediction analysis.

**Luciferase reporter assay**

The fragment of wild-type Smad7 3'UTR (3'UTR-WT) and the mutant 3'UTR fragment (3'UTR-MUT) were inserted into XhoI/NotI-digested psiCHECK-2 vector (Promega, Madison, WI, USA) with firefly and renilla luciferase reporter genes, respectively. The sequence of 3'UTR-WT of Smad7 included the putative binding sites of miR-182, and the sequence of 3'UTR-MUT of Smad7 was generated by site-directed mutagenesis of the miR-182 binding sites using Quick Change-mutagenesis kit (Stratagene, Heidelberg, Germany). Then the psiCHECK-2 vectors with 3'UTR-WT or 3'UTR-MUT regions of Smad7 were transfected into granulosa cells containing miR-182 mimics or mimic NC, respectively. At 48 h post-transfection, cells were collected and lysed for luciferase detection by using Dual-Luciferase Reporter System (Promega, USA).

**Statistical analyses**

Statistical analyses were carried out by using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Values were expressed as mean ± standard error (SE). The differences between two groups were analyzed using the Student’s t-test. Statistical significance was achieved at *P<0.05, **P<0.01 or ***P<0.001.

Teins were detected using enhanced chemiluminescence regents (Perkin-Elmer Life Sciences, Wellesley, MA, USA).

Figure 1. DHT promotes granulosa cell apoptosis (A) Flow cytometry analysis shows that the apoptosis rate increases with DHT concentration increasing from 20 to 160 ng/ml, with no significant difference at the concentration of 0 and 10 ng/ml. (B) Western blot assay shows that the expression of Bcl-2 decreases and Bax expression increases in granulosa cells treated by 80 ng/ml of DHT compared with the control cells. *P<0.05, **P<0.01, and ***P<0.001.

Figure 2. The miR-182 level decreases with DHT concentration increasing, with no significant difference at the concentration of 0 and 10 ng/ml. *P<0.05 and ***P<0.001.
MicroRNA-182 inhibits rat ovarian granulosa cell apoptosis

Results

DHT promotes granulosa cell apoptosis

To investigate the effect of DHT on granulosa cell apoptosis, we cultured granulosa cells in the medium containing various concentrations of DHT and then detected the apoptosis by flow cytometry. The results showed that the apoptosis rate increased with DHT concentration increasing from 20 to 160 ng/ml, with no significant difference between the concentration of 0 and 10 ng/ml, which indicated that DHT promotes granulosa cell apoptosis (Figure 1A). In addition, we detected the apoptosis-associated proteins Bax and Bcl-2 expression, and found that the expression of Bcl-2 decreases and Bax expression increases in granulosa cells treated by 80 ng/ml of DHT compared with the control cells, which further confirmed that DHT promotes granulosa cell apoptosis (Figure 1B).

DHT inhibits miR-182 expression

We performed qRT-PCR to detect the effect of DHT on miR-182 expression, and found that the miR-182 level decreases with DHT concentration increasing (Figure 2). This result suggests that DHT inhibits miR-182 expression in a dose-dependent manner.

miR-182 inhibits granulosa cell apoptosis

To investigate the effect of miR-182 on granulosa cell apoptosis, we transfected granulosa cells with miR-182 inhibitors, and then cultured in the medium containing 80 ng/ml of DHT. After incubation for 48 h, we detected the apoptosis rate and found the apoptosis rate of the cells transfected with miR-182 inhibitors is significantly higher than that in control cells (Figure 3A). The expression of Bax and Bcl-2 was also detected by western blot assay. As shown in Figure 3B, miR-182 inhibitor group has higher expression of Bax protein and lower expression of Bcl-2 protein than inhibitor NC group. Furthermore, miR-182 mimic group had higher Bcl-2 expression and lower Bax expression than mimic NC group (Figure 3D). Taken together, these results suggest that miR-182 suppresses granulosa cell apoptosis.

Smad7 is the target of miR-182

To understand molecular mechanisms by which miR-182 inhibits granulosa cell apoptosis, we searched for miR-182 targets with TargetScan, MicroRNA.org and PicTar online tools, and identified Smad7 as a potential target of miR-182 (Figure 4A). Then dual luciferase reporter assay was performed on granulosa cells to identify whether the 3'UTR of Smad7 mRNA is the binding sites of miR-182. Granulosa cells co-transfected with plasmid containing 3'UTR-WT regions of Smad7 and miR-182 mimics had less luciferase activity than their controls, while mutation of the putative miR-182 binding sites in the Smad7 3'UTR abol-
MicroRNA-182 inhibits rat ovarian granulosa cell apoptosis

Figure 4. Smad7 is a direct target of miR-182. A. Bioinformatics-based target prediction analysis shows that Smad7 is a potential target gene of miR-182 and the binding site is on the 3’UTR of Smad7. B. Luciferase reporter assay shows that granulosa cells co-transfected with plasmid containing 3’UTR-WT regions of Smad7 and miR-182 mimics have less luciferase activity than their controls, while mutation of the putative miR-182 binding sites in the Smad7 3’UTR abolishes this effect. C and D. The upregulation of miR-182 leads to a dramatic decrease in Smad7 mRNA and protein levels. E and F. The downexpression of miR-182 enhances Smad7 mRNA and protein expression in granulosa cells. **P<0.01 and ***P<0.001.

Discussion

In mammalian ovaries, over 99% of the follicles undergo degenerative atresia, and only a few follicles ovulate during ovarian follicular development. Granulosa cells play a critical role in follicular development and atresia, and the apoptosis of granulosa cells is closely related to follicular atresia [22]. In 2007, Murchison et al [23] and Lei et al [24] reported that miRNAs play a significant role in ovarian function and granulosa cell apoptosis by specific knockout of the enzyme Dicer, which regulates the biogenesis of miRNAs. Since then, the role of miRNAs in the regulation of GC apoptosis has attracted growing interest. For example, the expression of miR-92a was downregulated during porcine ovarian follicular atresia and upregulation of miR-92a inhibited apoptosis of in vitro cultured follicular granulosa cells [25]. miR-26b was upregulated during follicular atresia, increased the number of DNA breaks and promoted granulosa cell apoptosis by targeting the ataxia telangiectasia mutated gene (ATM) directly in vitro [26]. miR-23a was significantly upregulated in the plasma of premature ovarian failure patients, and essential for apoptosis.
MicroRNA-182 inhibits rat ovarian granulosa cell apoptosis

induction in human granulosa cells by targeting XIAP and the caspase signaling cascade [27].

DHT, a 5α reduced metabolite of testosterone, was observed to function as a competitive suppressor of aromatase activity in the ovary [28]. It was reported that DHT inhibits the granulosa cell proliferation and induces cycle arrest at the G1 phase by inhibiting cyclin D2 mRNA expression [29]. Moreover, the expression level of miR-182 is decreased in dihydrotestosterone (DHT)-induced rat PCOS model [20]. In this study, we also confirmed that miR-182 as an antiapoptotic DHT-induced factor was a key regulator of rat granulosa cell apoptosis. We determined the effects of DHT on granulosa cell apoptosis and miR-182 expression, and found DHT promotes granulosa cell apoptosis in a concentration dependent manner, and DHT inhibits miR-182 expression. These results were in line with previous studies [20, 29]. Then we detected the effect of miR-182 on granulosa cell apoptosis, and found that miR-182 suppresses granulosa cell apoptosis. We further investigate the molecular mechanism by which miR-182 exerts regulatory effects on granulosa cell apoptosis. We searched TargetScan, MicroRNA.org and PicTar database, and identified Smad7 as a potential target of miR-182.

Smad7 as a member of Smad proteins is an intracellular effector of the transforming growth factor β (TGF-β) signaling pathway. TGF-β signaling pathway plays a critical role in cell proliferation, apoptosis, differentiation, and various aspects of female reproduction [30-32]. Dysregulation of the TGF-β signaling can cause reproductive disorders such as female infertility and PCOS [33, 34]. Li et al [35] reported that mice homozygous for a Smad7 hypomorphic allele reduces body weight and size. Several recent studies showed that Smad7 is upregulated in both oocytes and granulosa cells from preantral and antral follicles [36, 37]. Gao et al [37] reported that Smad7 antagonizes key TGF-β superfamily signaling in mouse granulosa cells in vitro, which is involved in the regulation of oocyte-somatic cell interaction and granulosa cell function during follicular development. Quezada et al [36] reported that overexpression of Smad7 leads to granulosa cell apoptosis and knockdown of Smad7 blocks TGF-β-induced apoptosis of primary mouse granulosa cells. Liu et al [25] reported that miR-92a inhibits porcine ovarian granulosa cell apoptosis by targeting Smad7. These findings are the reasons why we selected Smad7 for further analysis. We performed luciferase assay and confirmed that miR-182 targets Smad7 directly. The expression of Smad7 at both mRNA and protein levels was regulated by miR-182. Flow cytometry analysis showed that Smad7 promotes granulosa cell apoptosis, which is consistent with previous reports [25, 36]. These findings implied that miR-182 represses granulosa cell apoptosis by targeting Smad7.

In conclusion, DHT inhibits miR-182 expression, and miR-182 suppresses granulosa cell apoptosis. Smad7, a major inhibitory regulator of the TGF-β signaling, was confirmed as a functional target of miR-182. The results provide a new insight into the mechanism of PCOS in mammals.

**P<0.01.

Figure 5. Smad7 promotes granulosa cell apoptosis. A and B. pcDNA-Smad7 group has higher apoptosis rate than pcDNA-control group, and si-Smad7 group has lower apoptosis rate than si-control group. C and D. pcDNA-Smad7 group has a marked increase in Bax expression and an obvious decrease in Bcl-2 expression compared with pcDNA-control group, while si-Smad7 group has lower Bax expression and higher Bcl-2 expression than si-control group. **P<0.01.
MicroRNA-182 inhibits rat ovarian granulosa cell apoptosis

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Disclosure of conflict of interest

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