Long non-coding RNA NEAT1 deteriorates polycystic ovary syndrome progression via binding microRNA-324-3p to target bromodomain containing 3

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Research Article

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Abstract

Objective: Long non-coding RNA (LncRNA) nuclear-enriched abundant transcript 1 (NEAT1) has been studied in polycystic ovary syndrome (PCOS), while the regulatory mechanism of NEAT1 on PCOS by regulating microRNA (miR)-324-3p remains extensive exploration. Our study aims to investigate the role of NEAT1, miR-324-3p and bromodomain containing 3 (BRD3) in PCOS.

Methods: Firstly, the PCOS rat model was established, and then NEAT1, miR-324-3p and BRD3 levels were detected in PCOS rats. The decreased NEAT1 or augmented miR-324-3p were injected into the PCOS rats to determine the change of biochemical indices and pathology. Also, the rescue experiment was conducted. Thereafter, the binding relations among NEAT1, miR-324-3p and BRD3 were analyzed.

Results: NEAT1 and BRD3 were enriched while miR-324-3p was decreased in PCOS rats. The reduction of NEAT1 or the elevation of miR-324-3p mitigated the obesity and metabolic disorders in PCOS rats. NEAT1 sponged miR-324-3p as a competing endogenous RNA and miR-324-3p targeted BRD3. The elevated miR-324-3p or reduced BRD3 reversed effects of enhanced NEAT1.

Conclusion: NEAT1 exacerbates obesity and metabolic disorders of PCOS rats via regulating miR-324-3p to target BRD3. This study affords a novel direction for PCOS treatment.

Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder that usually affects females of children-bearing age [1]. The major clinical features of PCOS include anovulation, hyperandrogenism and polycystic ovarian morphology, and the inducers are multiple and not fully confirmed at present [2]. PCOS patients have higher risk of afflicting infertility, insulin resistance, obesity, cardiovascular problems and other healthy problems [3]. In addition, the prevalence of PCOS is 8-13% and it is hard to diagnose because of multiple symptoms [4]. Considering the poor diagnosis, it is necessary to further explore the potential treatment strategy for PCOS from the genetic perspective.

Associated with the metabolic process, long non-coding RNAs (lncRNAs) is different expressed in the follicular fluid of mature follicles from the PCOS patients and healthy people [5]. As a member of lncRNAs, NEAT1 has been verified to be enriched in human ovarian granulosa cell line KGN, whose biological functions in PCOS are regulated by NEAT1 expression alternation [6]. Functionally, in ovarian cancer cell lines, NEAT1 induction accelerates proliferation by elevating the proportion of cells in S phase and blocks apoptosis of ovarian cancer cells [7]. Moreover, NEAT1 is associated with tumor stage and lymph node metastasis of ovarian cancer patients [8]. In addition, NEAT1 was predicated to target microRNA (miR)-324-3p through the bioinformatic website. MiRNAs has been clarified to display crucial impacts on the occurrence and development of PCOS, as women with PCOS has been found to exhibit abnormal expression of microRNA in granulosa cells, follicular fluid and serum and peripheral blood leukocytes [9]. Verified as a participant of follicular atresia process and follicular maturation disorder, miR-324-3p has also been revealed to accelerate the apoptosis of granulosa cells in PCOS patients [10].

Furthermore, Zhong *et al.* further confirm that miR-324 is also accountable for triggering KGN cell apoptosis in PCOS [11]. The bioinformatic website also predicated that there was a binding relation between miR-324-3p and target bromodomain containing 3 (BRD3). Encoded by paralogous genes, Belonging to bromodomain and extra-terminal domain family [12], BRD3 also exhibits high expression in ovarian granulosa cells of PCOS [13]. Furthermore, the silence of BRD3 is responsible for impairing ovarian clear cell carcinoma viability [14]. However, the studies for probing the function of NEAT1, miR-324-3p and BRD3 on PCOS development remain insufficient. Therefore, this study aims to further explore the regulatory impacts of NEAT1 on PCOS progression via mediating miR-324-3p to target BRD3, thus to provide a novel direction for the treatment of PCOS.

Materials And Methods

Ethics statement

The study was approved by the ethics committee of First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, 325000, China. All participants signed the informed consent form. The animal experiment was approved by the experimental animal ethics committee of First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, 325000, China.

Study subjects

A total of 80 PCOS patients and 80 healthy women with regular menstruation were recruited. Diagnosis of patients with PCOS was based on the revised Rotterdam European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine standard (2004): (1) signs of clinical and/or biochemical hyperandrogenism; (2) chronic low ovulation and/or anovulation; (3) polycystic ovaries. All patients had no medical history that affected glucose and lipid metabolism, nor any known medical conditions or diseases, including Cushing's syndrome, congenital adrenal hyperplasia, androgen-secreting tumors and endometriosis.

Clinical and biochemical test

Body mass index (BMI) was calculated by examining the weight and height of patients. After fasting for 10 hours, blood samples were collected and centrifuged, and the gained serum was stored at -20°C. The level of serum hormones, including follicle-stimulating hormone (FSH), sex hormone binding globulin (SHBG), estradiol (E2), progesterone (P4), luteinizing hormone (LH), testosterone (T), fasting plasma glucose (FPG) and fasting insulin (FINS) was determined by Enzyme-Linked Immunosorbent Assay (ELISA) kit (Abcam Inc, Cambridge, MA, USA) [15].

Establishment and grouping of animal model

To establish the animal model of PCOS, 30 day-old female Sprague-Dawley (SD) rats (50-60 g) were purchased from the animal center of Department of Obstetrics and Gynecology, First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, 325000, China. The rats were fed and drank freely at

25°C (50% humidity) in the clean facility with the light cycle of 12 hours per day. SD rats were randomly classified into 9 groups (n = 10): normal group, model group (PCOS modeling), short hairpin (sh)-negative control (NC) group (injected with unrelated plasmid of low-expressed NEAT1), sh-NEAT1 group (injected with low-expressed NEAT1 plasmid), agomir NC group (injected unrelated plasmid with up-regulated miR-324-3p), miR-324-3p agomir group (injected with miR-324-3p agomir), overexpression (oe)-NEAT1 group (injected with oe-NEAT1), oe-NEAT1 group + miR-324-3p agomir group (injected with oe-NEAT1 and miR-324-3p agomir), oe-NEAT1 + small interfering (si)-BRD3 group (injected with oe-NEAT1 and silenced BRD3). These lentivirus vectors above were synthesized by GenePharma Ltd. Company (Shanghai, China).

Except the normal group, the other rats were injected with dehydroepiandrosterone (Santa Cruz Biotechnology, Inc, CA, USA) (60 mg/kg) into subcutaneous tissues once a day for 35 consecutive days to establish PCOS model [16]. On the second day after modeling, rats were treated by tail vein injection for 7 days (8 mg/kg) and the weight change and blood glucose of the rats were measured.

Biochemical index test

After the last injection, all rats were deprived of food (water could be supplied) and serum was isolated after cardiac blood collection. FSH, LH, T, P4 and E2 were determined by ELISA kit (Abcam). The rats were euthanized by cervical dislocation, and the ovaries were removed. Hematoxylin and eosin (HE) staining and the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling Nick-End Labeling (TUNEL) staining were performed [17].

The serum samples of the rats were analyzed by biochemical analysis kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Total cholesterol (TC), triglycerides (TG), high-density lipoproteinhigh cholesterol (HDL-C) and low-density lipoproteinhigh cholesterol (LDL-C) were determined [18].

HE staining

One side of ovary was fixed with 10% formaldehyde for more than 24 hours. The ovary was dehydrated in gradient ethanol, infiltrated with xylene, immersed in wax and embedded with paraffin. Then the ovary was cut into slices of 4 µm, dewaxed, hydrated and processed with HE staining. The dyed part was processed with gradient ethanol treatment, permeabilized with xylene and fixed in neutral gum. A light microscope was used to observe the morphological structure of ovarian follicle [19].

TUNEL test

The TUNEL staining of paraffin-embedded ovarian tissues was performed by TUNEL apoptosis assay kit (NanJing Vazyme Biotech Co.,Ltd, Nanjing, China). The number of apoptotic cells was counted in 10 fields of views (× 200). Positive cells were represented by brown granules. Apoptosis index (%) = (number of apoptotic cells/total cells) × 100% [19].

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA from ovarian tissue was extracted by RNA extraction kit (Thermo Fisher Scientific, Waltham, MA, USA) and RNA concentration was detected by ultraviolet spectrophotometer. The optical density OD_{260nm}/OD_{280nm} was between 1.8~2.0, which indicated that the sample quality was good. The total RNA was reverse-transcribed into complementary DNA in strict accordance with the reverse transcription kit (Thermo Fisher Scientific). MiR-324-3p, NEAT1, and BRD3 primers sequences were listed in Table 1. U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were set as endogenous controls. The relative quantitative analysis was carried out by $2^{-\Delta\Delta Ct}$ method.

Western blot

The ovarian tissue protein was extracted. Then, the bicinchoninic acid protein detection kit (BOSTER Biological Technology Co. Ltd., Wuhan, Hubei, China) was used to examine the protein concentration. The extracted protein was then separated by 10% polyacrylamide gel electrophoresis (BOSTER Biological Technology Co. Ltd.) and transferred to polyvinylidene fluoride membrane. The membrane was sealed with 5% bovine serum albumin for 1 hour, added with the corresponding proportion of primary antibodies, incubated at 4°C overnight and washed with Tris-buffered saline with Tween 20 (TBST) for three times. The membrane was added with the corresponding second antibody (Shanghai MT-Bio CO., Ltd. Shanghai, China), incubated for 1 hour, and washed with TBST for 3 times. The samples were imaged by chemiluminescence reagent and the gray value of target protein bands was analyzed by ImageJ (National Institutes of Health, Bethesda, MA, USA). The major antibodies included BRD3 and GAPDH (1:1000; Abcam).

Dual luciferase reporter gene assay

The target binding sites of miR-324-3p to NEAT1 and miR-324-3p to BRD3 were predicted by bioinformatic website Starbase. The target relationship of miR-324-3p to NEAT1 and miR-324-3p to BRD3 was verified by dual luciferase reporter gene assay. The NEAT1-wild type (wt), NEAT1-mutant (mut) or BRD3-wt and BRD3-mut were constructed by the insertion of the corresponding sequence to the luciferase reporter vector Glyoxalase pmirGLO (Promega, Madison, WI, USA). The constructed vectors were co-transfected with miR-324-3p-mimic and mimic NC for 48 hours and then the cells were collected and lysed. Finally, the dual luciferase assay system (Promega) was adopted to evaluate the luciferase activity (Promega).

RNA-pull down assay

The cells were transfected with biotin-labeled miR-324-3p-wt and miR-324-3p-mut plasmids (50 nM each) for 48 hours. Then, the cells were collected, and incubated for 10 minutes with a specific cell lysate (Ambion Company, Austin, TX, USA). Thereafter, 50 mL cell lysate was sub-packed while residual lysates were incubated with the M-280 streptavidin magnetic beads (Sigma-Aldrich Chemical Company, St Louis, MO, USA) precoated with RNase-free and yeast tRNA (Sigma-Aldrich Chemical Company) at 4°C for 3

hours. Then the samples were washed twice with the cold lysate, three times with the low buffered saline and once with the high buffered saline. Antagonistic miR-324-3p probes were set as NCs. The expression of NEAT1 was then detected by RT-qPCR [15].

Statistical analysis

Data were statistically analyzed with SPSS 21.0 (IBM Corp. Armonk, NY, USA) statistical software. Measurement data were represented by mean $\pm$ standard deviation. The comparison between two groups of measurement data subjected to normal distribution was performed with t test. The comparison among multiple groups was analyzed by One-way analysis of variance (ANOVA), and pairwise comparison after ANOVA was performed with Tukey's multiple comparisons test. Finally, the dual luciferase assay system (Promega) was adopted to evaluate the luciferase activity.

Results

SHBG and FSH levels are low, and FINS, FPG, LH and T levels are high in PCOS patients

We found no distinct differences in age and BMI between PCOS patients and control donors. (Figure 1A). SHBG, LH, FSH, T, FINS, FPG, E2 and P4 were also measured. The results indicated that there was no distinct differences between E2 and P4 levels in PCOS patients and control donors. In addition, SHBG and FSH levels were low, while FINS, FPG, LH and T levels were high in PCOS patients (Figure 1B-G).

Identification of PCOS rats model

In order to investigate the effects of obesity and metabolic disorders caused by PCOS, we established a rat model of PCOS by intragastric injection of letrozole. The results indicated a significant increase in body weight (Figure 2A) and blood glucose level (Figure 2B) of the model rats.

The biochemical indices of rats mentioned that E2, T, TC and LH level were increased and FSH level was low in model rats (Figure 2C, D). The concentration of HDL-C and LDL-C was enhanced in model rats (Figure 2E).

The morphology of rat ovary was observed by HE staining. The follicles at different development stage and many luteinizing cells were found on the cut surface of ovary in the normal rats. The granulosa cells were arranged evenly, the morphology was complete, and the cell layer was thick (mostly 8-9 layers). While in the PCOS rats model, a large number of cystic dilated follicles were located under the egg membrane; the granular cells were loosely arranged and the cell layer became thinner; proliferating sheath cells and a few luteal cells were observed (Figure 2F). TUNEL test indicated that the number of positive cells in model rats was significantly high (Figure 2G).

The results above indicated that the PCOS rat modeling was successful and could be used in subsequent studies.

Down-regulation of NEAT1 improves obesity and metabolic disorders in PCOS rats

In order to probe NEAT1 level in PCOS rats and clinical sample, RT-qPCR assay was performed, which indicated that NEAT1 displayed high level in both clinical PCOS samples and in ovarian tissue of model rats (Figure 3A, B).

Next, sh-NC and sh-NEAT1 were respectively injected into to PCOS rats to investigate the effect of NEAT1 on the improvement of obesity and metabolic disorders. It was confirmed by RT-qPCR that NEAT1 was reduced in PCOS rats after the sh-NEAT1 treatment (Figure 3C).

The results of physiological and biochemical indices of rats indicated the distinct decreases of the rats weight and the blood glucose level after the reduction of NEAT1 (Figure 3D, E). The level of E2, T and LH decreased and the level of serum FSH increased after the decrease of NEAT1 (Figure 3F, G). In addition, the level of TG, HDL-C and LDL-C level in rats reduced with the descent of NEAT1 (Figure 3H).

To further study NEAT1 impact on obesity and metabolic disorders in PCOS rats, it was found that the down-regulation of NEAT1 effectively inhibited the pathological changes of ovarian tissue in PCOS rats by HE staining and TUNEL assay (Figure 3I, J).

These results suggested that the down-regulation of NEAT1 improved obesity and metabolic disorders of PCOS rats.

NEAT1 binds to miR-324-3p as a competing endogenous RNA (ceRNA)

Previous studies have indicated that miR-324-3p exhibits low level in ovarian tissues of PCOS patients and of PCOS rats [6, 10]. RT-qPCR was adopted to detect miR-324-3p level in PCOS clinical samples and model rats, and it was found miR-324-3p was depleted in clinical samples and PCOS rats (Figure 4A, B). In addition, miR-324-3p was enriched after the reduction of NEAT1 in ovarian tissue of PCOS rats (Figure 4C). The negative correlation between miR-324-3p and NEAT1 was manifested through Pearson test ($P < 0.0001$, $r = -0.7346$) (Figure 4D).

In order to investigate the regulation mechanism of NEAT1 and miR-324-3p, the specific binding site of NEAT1 to miR-324-3p was predicted by Starbase (Figure 4E). The binding relationship between NEAT1 and miR-324-3p was determined by the dual luciferase reporter gene assay. The luciferase activity in cells reduced after the co-transfection with NEAT1-wt and miR-324-3p mimic (Figure 4F), indicating that miR-324-3p was specifically bound with NEAT1. The RNA pull-down assay once again verified that NEAT1 could bind to miR-324-3p as a ceRNA. The enrichment degree of NEAT1 elevated after the transfection with Bio-miR-324-3p-wt (Figure 4G). These results above indicated that NEAT1 could specifically bind to miR-324-3p and thus affect the expression of miR-324-3p.

Up-regulation of miR-324-3p effectively inhibits obesity and metabolic disorders in PCOS rats

In order to further explore the inhibitory effect of miR-324-3p on obesity and metabolic disorders in PCOS rats, agomir NC and miR-324-3p agomir were respectively injected into PCOS rats. It was verified by RT-qPCR that miR-324-3p was enriched in PCOS rats after the induction of miR-324-3p (Figure 5A).

The physiological and biochemical indices test suggested that the weight of the rats decreased significantly after the injection of miR-324-3p agomir (Figure 5B), and the metabolic disturbance and the pathological changes of the ovary improved effectively (Figure 5C-H). These results verified that the up-regulation of miR-324-3p drastically repressed the obesity and metabolic disorders in PCOS rats.

MiR-324-3p targets BRD3

To determine the expression of BRD3 in PCOS, we performed RT-qPCR and Western blot assays, which indicated that BRD3 was elevated in clinical samples of PCOS patients and ovarian tissue of PCOS rats (Figure 6A-D). Thus, we hypothesized that miR-324-3p might target BRD3. Also, Pearson test analyzed a negative correlation between miR-324-3p and BRD3 ($P < 0.0001$, $r = -0.7076$) (Figure 6E).

The targeting relation between miR-324-3p and BRD3 was predicted via Starbase (Figure 6F). It was indicated that the relative luciferase activity of the cells decreased after the co-transfection with BRD3-wt and miR-324-3p mimic as reflected by the outcomes of dual luciferase activity assay; while the relative luciferase activity of the cells exhibited no salient change after the co-transfection with BRD3-mut and miR-324-3p mimic (Figure 6G). These findings above demonstrated that BRD3 was a direct target gene for miR-324-3p.

Then the expression of BRD3 was detected by RT-qPCR and Western blot, and it was found that miR-324-3p enrichment caused the down-regulation of BRD3 (Figure 6H, I), which verified that miR-324-3p targeted BRD3.

NEAT1 as a ceRNA sponges miR-324-3p to target BRD3, thus to deteriorate obesity and metabolic disturbance in PCOS rats

NEAT1 and BRD3 were augmented while miR-324-3p was depleted in PCOS rats. The correlation among miR-324-3p to NEAT1 and BRD3 was negative. NEAT1 knockdown caused significantly decreased BRD3 by RT-qPCR and Western blot assay (Figure 7A, B). Pearson test verified the positive correlation between NEAT1 and BRD3 ($P < 0.0001$, $r = 0.7399$) (Figure 7C).

To further investigate the effects of NEAT1, miR-324-3p and BRD3 on obesity and metabolic disorders in PCOS rats, oe-NEAT1, oe-NEAT1 + miR-324-3p agomir, and oe-NEAT1 + si-BRD3 were injected into PCOS rats, and the transfection efficacy was confirmed by RT-qPCR and Western blot (Figure 7D-G).

Physiological and biochemical tests indicated that miR-324-3p agomir or si-BRD3 reversed multiple effects of enhanced NEAT1 (Figure 7H-M). These results above demonstrated that NEAT1 as a ceRNA binds to miR-324-3p to target BRD3, thus to deteriorate obesity and metabolic disturbance in PCOS rats.

Discussion

PCOS is an endocrine disorder of unclear pathogenesis that leads to low-quality embryos or even pregnant loss to afflicted patients [20]. This study focused on the regulatory mechanism of NEAT1 in obesity and metabolic disorder of PCOS rats via regulating miR-324-3p and BRD3. Collectively, it was validated that the induction of NEAT1 could aggravate obesity and metabolic disorder of PCOS rats via mediating miR-324-3p to target BRD3.

As previous validation, NEAT1 is robustly expressed in PCOS tissues and cells, and the amplification of NEAT1 facilitates the proliferation and constrains the apoptosis of KGN cells, inducing morbidity of PCOS [6]. Similarly, Luo *et al.* have also elucidated that NEAT1 is enriched in ovarian cancer cell lines, the ovarian cell activities and epithelial-mesenchymal transition are triggered by NEAT1 induction [21]. Clinically, the elevated NEAT1 is also implicated to correlate with deteriorated tumor grade and distant metastasis as well as low survival rate of ovarian cancer patients [22]. Furthermore, in cervical cancer cells, NEAT1 also exhibits a high level, whereas NEAT1 knockdown represses cervical cancer progression [23]. The identically high expression of NEAT1 also exists in endometrial cancer cells and tissues, while the amplification of NEAT1 activates the biological functions of endometrial cancer cells [24]. These preceding findings offered theory references for the study. In line with these conclusions, this study validated that NEAT1 was augmented in PCOS rats, the metabolic disturbances and obesity induced by PCOS could be relieved by NEAT1 ablation.

It was also revealed that NEAT1 targeted miR-324-3p through the bioinformatic website. In a similar study about PCOS, it has also been clarified that miR-324-3p is depleted in granulosa cells, while the elevation of miR-324-3p effectively represses the biological activities of granulosa cells through mediating its downstream gene [10]. Similarly, Liu *et al.* also have implied that miR-324-3p is ablated in ovarian cancer cells SKOV3, whose viability is restrained through the elevation of miR-324-3p [25]. Meanwhile, in cervical cancer cells, the induction of miR-324-3p also exhibits inhibitory impacts on the aggressive behavior of cervical cancer cells, thus repressing cervical cancer development [26]. Furthermore, miR-324-3p augmentation also makes contribution to restrains the malignant phenotypes in triple-negative breast [27]. However, the studies for investigating the regulatory mechanism of miR-324-3p in PCOS are inadequate. Anchored in these findings above, this study further deciphered that miR-324-3p was low-expressed in PCOS rats, while the augmented miR-324-3p blocks the development of obesity and metabolic disorder in PCOS rats.

As predicated by the bioinformatic website, BRD3 was a target gene of miR-324-3p. BRD3 has been clarified to be enriched in ovarian granulosa cells in PCOS [13]. Functionally, the ablation of BRD3 can decelerate the viability of ovarian clear cell carcinoma [14]. Furthermore, Xu *et al.* have elucidated that the elevation of BRD3 is prevalent in human glioblastoma samples, the induction of BRD3 and other bromodomain and extraterminal proteins can co-activate the transcriptional program of glioblastoma cells [28]. In hepatocellular carcinoma, BRD3 also displays a high level, leading to unfavorable overall survival and disease-free survival of patients with hepatocellular carcinoma as well as malignant tumor

grade [29]. The existed literatures for clarifying BRD3 regulatory mechanism during PCOS development were insufficient. Drawing related theoretical references from the preceding findings, this study further validated that BRD3 was high-expressed in PCOS patients, whereas the decrement of BRD3 effectively repressed the progression of the obesity and metabolic disorder of PCOS patients.

In summary, this study concludes that NEAT1 and BRD3 are augmented while miR-324-3p is depleted in PCOS rats. The ablation of NEAT1 effectively attenuated the metabolic disorders and obesity of PCOS patients through mediating miR-324-3p to target BRD3. Our study provides a novel angle and distinct theory references for the treatment of PCOS patients by highlighting the regulatory function of NEAT1, miR-324-3p and BRD3. However, the sample quantity of this study is limited, the study samples still necessitate further supplement to obtain more precise data for a convincing conclusion.

Declaration

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Ethics approval and consent to participate

The study was approved by the ethics committee of Department of Obstetrics and Gynecology, First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, 325000, China. All participants signed the informed consent form. The animal experiment was approved by the experimental animal ethics committee of Department of Obstetrics and Gynecology, First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, 325000, China.

Conflict of interest

The authors declare that they have no conflicts of interest.

Consent for participate and publish

Informed consent was obtained from all individual participants included in the study. The participant has consented to the submission of the case report to the journal.

Availability of data and material

The raw/processed data required to reproduce these findings cannot be shared at this time due to legal or ethical reasons.

Authors' contributions

Li Xu Jin contributed to study design; Le Wu contributed to manuscript editing; Zhi Jian Tu and Ying Bao contributed to experimental studies; Qi Zhai contributed to data analysis. All authors read and approved the final manuscript.

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Tables

Table 1 Primer sequences

Genes	Primer Sequences(5'– 3')
miR-324-3p	F: ACTGCCCCAGGTGCTGCTGG
	R: GCGAGCACAGAATTAATACGAC
NEAT1	F: ACTGAGAGTGATTGAGAGTGGAC
	R: TCTCCTTGCCAAGCTTCCTT
BRD3	F: ACTGAGAGTGATTGAGAGTGGAC
	R: AACCTCT GCACCCAGTTTTTC
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
GAPDH	F: CGGAGTCAACGGATTTGGTCGTAT
	R: AGCCTTCTCCATGGTGGTGAAGAC

Note: F, forward; R, reverse; miR-324-3p, microRNA-324-3p; NEAT1, nuclear-enriched abundant transcript 1; BRD3, bromodomain containing 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Figures

Figure 1

SHBG and FSH levels are low, and FINS, FPG, LH and T levels are high in PCOS. A. BMI of PCOS group and control group; B. FSH and LH levels in PCOS group and control group were detected by ELISA; C. SHBG and T levels in PCOS group and control group were detected by ELISA; D. E2 level in PCOS group and control group were detected by ELISA; E. P4 level in PCOS group and control group were detected by ELISA; F. FINS level in PCOS group and control group were detected by ELISA; G. FPG level in PCOS group and control group were detected by ELISA. The data in the figure were all measurement data, and the values were represented by mean $\pm$ standard deviation, * $P < 0.05$ vs. the control group.

Figure 2

Identification of PCOS rats model. A. Body weight in normal group and model group; B. blood glucose concentration in normal group and model group was detected by automatic analyzer detected ; C/D. E2, P4, T, LH and FSH levels in normal group and model group were examined by ELISA; E. TC, TG, HDL-C and LDL-C levels in normal group and model group was assessed by Biochemical kit; F. Histological changes in normal group and model group were detected by HE staining; G. Cell apoptosis in normal group and

model group was examined by TUNEL staining. All the data in the figure were measurement data, and the values were represented by mean $\pm$ standard deviation, * $P < 0.05$ vs. the normal group.

Figure 3

Down-regulation of NEAT1 improves obesity and metabolic disorders in PCOS rats. A. Clinical expression level of NEAT1; B. Expression of NEAT1 in PCOS rats; C. Expression of NEAT1 after down-regulation of NEAT1; D. Weight of rats after down-regulation of NEAT1; E. blood glucose concentration after down-regulation of NEAT1 was analyzed by Automatic analyzer ; F/G. E2 P4, T, LH and FSH levels after down-regulation of NEAT1 was detected by ELISA ; H. TC, TG, HDL-C and LDL-C levels after down-regulation of NEAT1 were examined by biochemical kit; I. Histological changes in PCOS rats after down-regulation of NEAT1 was assessed by HE staining; J. Cell apoptosis in PCOS rats after down-regulation of NEAT1 was detected by TUNEL staining. All the data in the figure were measurement data, and the values were represented by mean $\pm$ standard deviation, * $P < 0.05$ vs. the sh-NC group.

Figure 4

NEAT1 binds to miR-324-3p as a ceRNA. A. The clinical expression of miR-324-3p of PCOS group and control group; B. The expression of miR-324-3p in rats of PCOS group and normal group; C. The expression of miR-324-3p after sh-NEAT1 injection; D. The correlation between miR-324-3p and NEAT1; E. Starbase predicted the binding site of NEAT1 to miR-324-3p; F. The dual luciferase reporter gene assay verified the targeting relationship between miR-324-3p and NEAT1; G. RNA pull down detected the binding of NEAT1 to miR-324-3p. All the data in the figure were measurement data, and the values were represented by mean $\pm$ standard deviation. In Figure B, * $P < 0.05$ vs. the normal group; in Figure C, * $P < 0.05$ vs. sh-NC group; in Figure F, * $P < 0.05$ vs. the mimic NC group; in Figure G, * $P < 0.05$ vs. the Bio-probe NC group.

Figure 5

Up-regulation of miR-324-3p effectively inhibits obesity and metabolic disorders in PCOS rats. A. Expression of miR-324-3p after up-regulation of miR-324-3p was detected by RT-qPCR; B. Rats weight after up-regulation of miR-324-3p; C. blood glucose concentration after up-regulation of miR-324-3p was detected by automatic analyzer; D/E. E2, P4, T, LH and FSH level after up-regulation of miR-324-3p was examined by ELISA; F. TC, TG, HDL-C and LDL-C levels after up-regulation of miR-324-3p was detected by Biochemical kit; G. Histological changes in PCOS rats after the up-regulation of miR-324-3p was assessed by HE staining; H. Cell apoptosis in PCOS rats after the up-regulation of miR-324-3p was detected by TUNEL staining. All the data in the figure were measurement data, and the values were represented by mean $\pm$ standard deviation, * $P < 0.05$ vs. the agomir NC group .

Figure 6

MiR-324-3p targets BRD3. A/B. BRD3 expression in PCOS patients was detected by RT-qPCR and Western blot assay; C/D. BRD3 expression in PCOS rats was detected by RT-qPCR and Western blot; E. Correlation between miR-324-3p and BRD3 was analyzed by Pearson test; F. The binding sites of miR-324-3p and BRD3 were predicted by Starbase; G. The target relationship between miR-324-3p and BRD3 was verified by dual luciferase reporter gene assay; H/I. BRD3 expression after the up-regulation of miR-324-3p was detected by RT-qPCR and Western blot. All the data in the figure were measurement data, and the values were represented by mean $\pm$ standard deviation. In Figure B, * $P < 0.05$ vs. the control group; in Figure C, D, * $P < 0.05$ vs. the normal group; in Figure G, * $P < 0.05$ vs. the mimic NC group; in Figure H, I, * $P < 0.05$ vs. the agomir NC group.

Figure 7

NEAT1 as a ceRNA sponges miR-324-3p to target BRD3, thus to deteriorate obesity and metabolic disturbance in PCOS rats. A. BRD3 mRNA expression in PCOS rats after sh-NEAT1 injection; B. BRD3 protein expression in PCOS rats after sh-NEAT1 injection; C. Correlation between NEAT1 and BRD3; D-E. Expression of NEAT1, miR-324-3p and BRD3 in rescue experiment; F. Rats weight in rescue experiment; G. The blood glucose concentration in rescue experiment was detected by automatic analyzer; H. E2 level in rescue experiment was detected by ELISA; I. P4, T, LH and FSH levels in rescue experiment was detected by ELISA; J. Biochemical kit detected TC, TG, HDL-C and LDL-C levels in rescue experiment; K/L. Histological changes in rescue experiment was detected by HE staining; M. Cell apoptosis in rescue experiment was detected by TUNEL staining. All the data in the figure were measurement data, and the values were represented by mean $\pm$ standard deviation. In Figure A, B, * $P < 0.05$ vs. the sh-NEAT1 group; in Figure D, G, * $P < 0.05$ vs. the oe-NEAT1 group.