miR-21 is involved in norepinephrine-mediated rat granulosa cell apoptosis by targeting *SMAD7*

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Abstract

Substantive evidence has indicated that the sympathetic innervation contributes to the regulation and development of ovarian functions. Norepinephrine (NE) is one of the major neurotransmitters contained in the extrinsic ovarian sympathetic nerves and is thought to be a potent moderator of ovarian functions such as steroidogenesis and granulosa cell proliferation or apoptosis. However, the mechanisms of NE regulation of granulosa cell apoptosis in the rat ovary are rare. Real-time PCR and Western blot results show that NE regulates the expression of miR-21 in primary granulosa cells in a dose-dependent manner. Additionally, we found that miR-21 is involved in NE-mediated rat granulosa cells apoptosis and blocks granulosa cell apoptosis by targeting *Smad7*, a transforming growth factor-beta-inducible mediator of apoptosis in granulosa cells. In primary granulosa cells, a combined treatment of NE and TGF- β increased apoptosis and decreased miR-21 expression, but increased SMAD7 protein levels. We also demonstrated that NE regulates miR-21 by coupling to α_{1A} -adrenergic receptor (α_{1A} -AR). This is the first demonstration that NE controls the reproductive functions by modulating the expression of miR-21 and promoting TGF- β -induced granulosa cell apoptosis. Such NE-mediated effects could be potentially used for regulating the reproductive processes and for treating reproductive disorders.

Key Words

- ▶ miR-21
- ▶ ovary
- ▶ apoptosis
- ▶ NE
- ▶ *SMAD7*

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Introduction

The rat ovaries receive their sympathetic innervation from two sources. One is the ovarian plexus nerve, which travels along the ovarian artery, and the other is the superior ovarian nerve (SON), which is associated with the suspensory ligament (Mayerhofer *et al.* 1997, D'Albora *et al.* 2002). The extrinsic innervation of the gland has been shown to regulate ovary-specific functions, such as steroidogenesis and early follicular development (Skarzynski 1993, Mayerhofer *et al.* 1997). The regulatory functions are mainly exerted via NE

(Lawrence & Burden 1980). Previous studies have shown that ovarian non-neuronal, endocrine granulosa cells can both take up and release NE in a regulated manner (Greiner *et al.* 2008). Adrenergic innervation can stimulate steroid secretion and promote granulosa cell proliferation by NE binding to the β -adrenergic receptor (β -AR) or α_{1A} -AR (Lawrence & Burden 1980, Lara *et al.* 2002). NE also facilitates follicular development, as denervation of the ovaries inhibits follicular growth (Advis *et al.* 1989). In the rat, the importance of its interrelation on the

ovarian steroidogenesis and apoptosis on dioestrus II has been demonstrated (Bronzi *et al.* 2015). However, the reports about signaling pathways of NE-regulating ovary functions remain few.

MicroRNAs (miRNAs) are 20–24 nt endogenous non-protein encoding RNAs and have emerged as important mediators of cellular differentiation, proliferation and apoptotic events (Otsuka *et al.* 2008, Donadeu *et al.* 2012). There are scant data about the function of microRNA in the ovary. One such miRNA, miR-378, regulates estradiol production by targeting aromatase (Xu *et al.* 2011). miR-224 is involved in TGF- β -induced granulosa cell proliferation and granulosa cell functions by targeting SMAD4 (Yao *et al.* 2010). MicroRNA-21 was shown to regulate granulosa cell apoptosis, and *in vivo* knockdown of miR-21 causes an increase in apoptosis (Carletti *et al.* 2010). Although miR-21's function of blocking granulosa cell apoptosis has been established in the ovary, the signaling pathway of miR-21 in the rat ovary is unknown.

Smad7 is a general signaling antagonist for both the TGF- β and bone morphogenetic protein (BMP) family (Park 2005). An altered expression of *Smad7* is associated with several human disease processes, including cancer, tissue fibrosis and inflammatory diseases (Briones-Orta *et al.* 2011). A previous study has reported that overexpression of *Smad7* in granulosa cells markedly increases apoptosis, and when *Smad7* expression is reduced, TGF- β -induced apoptosis is blocked (Quezada *et al.* 2012). However, whether *Smad7* regulates NE's granulosa cell function is still unknown.

In this study, we initially examined miR-21 expression in the rat ovary and demonstrated that miR-21 is mainly located in granulosa cells. Upon further research, we showed that miR-21 functions as a mediator of the NE signaling pathway by targeting *Smad7* thus regulating granulosa cells apoptosis. These findings suggest that miR-21 plays a key role in regulating granulosa cell apoptosis by targeting *Smad7*. Additionally, NE regulates miR-21 expression by binding to α_{1A} -AR and is involved in TGF β /SMAD signaling.

Materials and methods

Animals and tissue collections

Rats (Wistar) for this study were purchased from the Animal Institute of the Chinese Medical Academy (Beijing, China) and raised in standard temperature ($25 \pm 1^\circ\text{C}$) and light (12-h light, 12-h darkness cycle) conditions. All animal procedures were approved by the Chinese

Association for Laboratory Animal Sciences. Female rats at approximately 21 days were injected intraperitoneally with 10 U of pregnant mare serum gonadotropin (PMSG) to stimulate follicular development for 46 h. The rats were killed by cervical dislocation. For *in situ* hybridization and immunohistochemistry, the collected rat ovaries were fixed for 1 h in 4% (paraformaldehyde) PFA at room temperature (RT), and then placed in 30% sucrose at 4°C overnight. The tissues were embedded in 'TissueTek' O.C.T. compound (TaKaRa Biotechnology), and $10\ \mu\text{m}$ thick sections were cut using a cryostat.

In situ hybridization

miR-21 *in situ* hybridization (ISH) was carried out using digoxigenin (DIG)-labeled locked nucleic acid (LNA) probes. Rno-miR-21-5p miRCURY LNA microRNA detection probes (TCAACATCAGTCTGATAAGRANULOSA CELLTA) were purchased from Exiqon (MA, USA). We labeled the LNA probes with digoxigenin using a DIG oligonucleotide tailing kit (Roche Diagnostics) following the manufacturer's instructions. miR-21 ISH assays were carried out as described previously (Obernosterer *et al.* 2007). Briefly, the dried slides were fixed in 4% PFA for 10 min at room temperature and then washed twice for 3 min in $1\times$ phosphate-buffered saline (PBS). The fixed sections were subjected to acetylation for 10 min, followed by PBS washes. The slides were pre-hybridized for 8 h at RT, and the hybridization was carried out at 51°C overnight. After stringency washings using $5\times$ saline sodium citrate (SSC) for 10 min and $0.2\times$ SSC for 1 h at 60°C , the slides were incubated in a blocking solution for 1 h at RT, which was followed by incubation with alkaline phosphatase (AP)-conjugated antibody to digoxigenin at 4°C overnight. After PBS and alkaline phosphates buffer washes, the slides were incubated in nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in the darkness until the prospective intensity of staining was reached. The sections were examined under a DMRB light microscope (Leica).

Immunohistochemistry

For immunohistochemistry (IHC), the ovary sections were dewaxed, rehydrated and treated with 10% normal goat serum in PBS to block nonspecific binding sites. For α_{1A} -AR staining, an antibody against the adrenergic receptor (1:50; sc-1477, Santa Cruz Biotechnology) was incubated at 4°C for 12 h. In the negative control group, the antibody against the adrenergic receptor was replaced by

PBS. The sections were then incubated with a biotinylated goat anti-rabbit IgG (1:200; Zymed Laboratories) and horseradish peroxidase (HRP)-conjugated streptavidin (1:500, Jackson ImmunoResearch Laboratories) at RT for 2 h. Visualization was done using diaminobenzidine (DAB) with hematoxylin counterstaining.

Cell culture

For each individual studies, 10 female rats at approximately 21 days were killed by cervical dislocation after injected intraperitoneally with 10U PMSG to stimulate follicular development for 46 h. Then, the isolation of oocytes and granulosa cells were carried out as described previously (Hanoux *et al.* 2007, Fiedler *et al.* 2008, Teng *et al.* 2015). Under sterile conditions, cumulus-oocyte complexes (COCs) were collected immediately by puncturing follicles from the isolated ovary. The follicular fluid and COCs sank to the bottom of the dishes. The follicular fluid containing COCs was poured into a 90-mm petri dish containing oocyte washing medium consisting of HEPES-buffered TCM 199 supplemented with 10% (v/v) fetal calf serum (FCS). The COCs, with selected under a stereomicroscope (Nikon), then were used for RNA extraction. Meanwhile, granulosa cells were aspirated from the large follicles. This pellet was dispersed, and then filtration and centrifugation were carried out. The granulosa cells were harvested, washed and resuspended in DMEM/F12 media containing 10% FBS (Gibco). The cells were then counted and the viability was assessed. For the assay, cells were plated (1.0×10^5 cells/well) onto eight 35 mm dishes for 24 h at 37°C in a humidified atmosphere of 5% CO₂. After being cultured for 48 h, the primary granulosa cells were transfected.

The 293T cell line was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% (V/V) fetal bovine serum (FBS; Gibco) and 1% penicillin streptomycin. The cells were also incubated at 37°C in a humidified atmosphere of 5% CO₂.

MicroRNA and siRNA transfections

For the assay, cells were plated (1.0×10^5 cells/well) onto 35 mm dishes for 24 h at 37°C in a humidified atmosphere of 5% CO₂. The transfections of the miR-21 inhibitor or mimics were performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Six hours after transfection, the cells were collected for real-time PCR or cell apoptosis analysis. The oligos (nc inhibitor (nc-in), miR-21 inhibitor (miR-21-in),

nc mimics (nc-mi) and miR-21 mimics (miR-21-mi)) were used for the cell transfection were purchased from RibBio (Guangzhou, China).

Real-time PCR

According to the protocol provided by the manufacturer, the total RNA of the granulosa cells was isolated using the TRIzol reagent (TaKaRa). U6 RNA was used for normalization of microRNA expression. Reverse transcriptase reactions contained the purified total RNA (0.3 µg), 50 nM reverse transcription (RT) primer (the RT-miR-21 stem-loop primer: CTCAACTGGTGTCGTGGAGTCGGAATTCAGTTGAGTCAACATC and the RT-U6 stem-loop primer: AACGCTTCACGAATTTGCGT). M-MLV reverse transcriptase (Promega) was used according to the manufacturer's instructions. The 15 µL reactions were incubated in a DNA Thermal Cycler 4800 for 30 min at 16°C, 30 min at 42°C and 5 min at 85°C. Real-time PCR was performed using a standard TaKaRa SYBR Premix Ex Taq protocol on an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems). The primer sequences are listed in Table 1, and the conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative abundance of the genes was determined using the ABI PRISM 7500-equipped software (Applied Biosystems). All the experiments were performed in at least triplicate.

Table 1 Primers used for real-time PCR in this study.

Gene	Sequence of primers (5'→3')
α _{1A} -AR	F: ACTCAGAGCAAGTGACGCTC R: ACGGTTTCCGAAGGCTTGAA
α _{1B} -AR	F: CATGAGGACACCCTCAGCAG R: TGGAGAACAGGGAGCCAAGC
α _{1D} -AR	F: AAGTTTTGGTGGCGTATTGGA R: GAAGTGATGGGCACAGACAGG
β ₂ -AR	F: CTGCCTTCAATCCTCTTATCT R: GTCTGTCTACCGTTGCTGTT
SMAD7	F: TATGATGCGGATTTTGGTTGTG R: TGACTCTTGCTCCCGTTTTTGT
miR-21	RT: CTCAACTGGTGTCGTGGAGTCGG CAATTCAGTTGAGTCAACATC F: GTATGCTCATCCTAGCTTATCAGACT R: GTATGCTCATCCTAGCTTATCAGACT
U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT
Bax	F: TTTTCATCCAGGATCGAGCAGG R: GCAAAGTAGAAGAGGGCAACCAC
Bcl-2	F: CTACCGTCGTGACTTCGCA R: TACCCAGCCTCCGTTATCC

F, forward primer; R, reverse primer.

Western blot

Granulosa cells were lysed with radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The protein concentration of each group was determined using a BCA assay reagent (Vigorous Biotechnology, Beijing, China) according to the manufacturer's recommendations. Equal amounts of protein (50 µg) were electrophoresed on 11% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and the bands were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories). The membrane was blocked with 5% (w/v) non-fat dry milk in 0.05 M pH 7.4 Tris-buffered saline (TBS) for 3 h and incubated with a SMAD7 antibody (1:1000, ab124890, Abcam), and as an internal control, a GAPDH antibody (1:10,000, Ambion) overnight at 4°C. The PVDF membrane was then washed 3 times for 30 min in TBST (0.1% Tween20 in TBS) and incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit IgG. After washing for 30 min with 3 changes of TBST, the membrane was treated with the Pierce ECL 2 Western Blot Substrate (Thermo Scientific). The relative intensity of each blot was assessed and analyzed using the AlphaMager 2200 Software package. The intensity values pertaining to each group were normalized against the optical density of GAPDH corresponding to the same group within a single gel and expressed in terms of the means $\pm$ S.E.M. of 3 independent experiments.

Luciferase reporter assay

The dual-luciferase reporter genes were constructed using the psiCHECKTM-2 vector (Promega) and the 3'-UTR sequences of rat *Jag1* and *Smad7*. The *Smad7* 3'-UTR fragment cloning was done using an overlap PCR (Table 2), and it was introduced between the NotI and XhoI sites of the Renilla luciferase 3'UTR. The firefly luciferase vector was used for an internal reference. 293T cells were transfected using Lipofectamine 2000 with a mixture containing 200 ng/mL of the dual-luciferase reporter plasmid and 40 nM of miR-21 mimics. Cells transfected with the mut-*Smad7* vector served as controls for normalization. Luciferase activity was measured by a ModulusTMII microplate multimode reader (Promega) 24 h after transfection using a Dual-Lucy Assay Kit (Vigorous Biotechnology Beijing Co.). All transfections were repeated independently at least three times.

Table 2 The *JAG1/SMAD7*-3'UTR sequences containing the miR-21-binding site were constructed into a psi-CHECK 2 vector.

JAG1-3'UTR: attcgtatgtaatgctgaaccactgtagatttgattcattgtt-gtctactgcatttagggagatttctaataagctagatg
aatactgaaccataaaatgtccagtagagcactgttagattggccatagagta-cactgcctgcttaagt
Mut-*JAG1*-3'UTR: attcgtatgtaatgctgaaccactgtagatttgattcatt-gttgtgtctactgcatttagggagatttctaataagctagatg
cataaaatgtccagtagagcactgttagattggccatagagtagactgcctgct-taagt
SMAD7-3'UTR: cttctctcggctcatcacactcgatgatactctgacact-gttcttggtcaatgagcatgctcacacttaataataagctatt
tttctaactacaaggtttaaaatgaacaagagaggcgttctcatcgaaatttag-catcgtagtgctttgagag
Mut-*SMAD7*-3'UTR: cttctctcggctcatcacactcgatgatactctgacact-gttcttggtcaatgagcatgctcacacttaataataagctatt
caaggtttaaaatgaacaagagaggcgttctcatcgaaatttagcatcg-tagtgctttgagag

The sequences in red indicate binding sites of miR-21

Cell counting

For the estimation of granulosa cell apoptosis, we obtained the micrographs of sections for cleaved caspase-3 immunohistochemistry with a Leica DMLB microscope equipped with a Leica DFC320 camera (Leica) under a 20 $\times$ and 40 $\times$ magnification objective, respectively. The cleaved caspase-3 IHC-positive cells and total cells from the micrographs were counted using the manual counter function of Alphamager 2200 software. Cell counting was performed by two investigators, and the results are expressed as the percentages of cleaved caspase-3 immunopositive cells.

Statistical analysis

All experiments were independently performed more than three times. All data were analyzed using SAS 9.0. The values are presented as the means $\pm$ S.E.M. One-way ANOVA was used for statistical comparisons among multiple groups. ANOVA was performed using GraphPad Prism 4.0 (GraphPad Software). For statistical comparisons between the two groups, the Student's two-tailed *t* test was used. A value of $P < 0.05$ or $P < 0.01$ was considered to be statistically significant.

Results

miR-21 and the adrenergic receptor are expressed in the rat ovary

To study whether miR-21 is involved in mediating NE's effects on granulosa cells apoptosis, we initially examined

Figure 1

miR-21 and the adrenergic receptors are expressed in rat ovary. (A) Relative miR-21 expression levels in isolated granulosa cells and oocytes by real-time PCR. The experiments were repeated at least three times and normalized to their respective control. Data are shown as means $\pm$ s.e.m. ($n \geq 3$).

$**P < 0.01$. (B) Localization of miR-21 in the rat ovary using LNA ISH. (C) Localization of miR-21 in the rat ovary which from an animal model for the study of follicular atresia using LNA *in situ* hybridization. (D) Gene expression of adrenergic receptors in granulosa cells. The experiments were repeated at least three times. Data are shown as means $\pm$ s.e.m. ($n \geq 3$). (E) The expression of the α_{1A} -adrenergic receptor in the rat ovary using IHC. All scale bars represent 50 μ m.

the expression of miR-21 and the adrenergic receptors (α_{1A} -AR, α_{1B} -AR, α_{1D} -AR and β_2 -AR) in the rat ovary and granulosa cells by real-time PCR, ISH and IHC. The real-time PCR and ISH results showed that miR-21 was expressed only in the granulosa cells, but not in the oocyte (Fig. 1A and B). In addition, we found that there is less expression of miR-21 in the rat ovaries from the animal model for the study of follicular atresia (Fig. 1C). Real-time PCR and IHC results showed that the adrenergic receptors mainly localized to granulosa cells in the rat ovary (Fig. 1D and E). These data show that miR-21 and adrenergic receptors are co-expressed in rat granulosa cells.

NE suppresses miR-21 expression and induces rat granulosa cell apoptosis

To investigate a potential functional relationship between NE and miR-21 in regulating rat granulosa cells apoptosis, we assessed the effects of NE on miR-21 expression and apoptosis in rat granulosa cells. Cultured rat granulosa cells were treated with 0 (control), 0.1, 1 and 10 μ mol/L NE for 24h, and miR-21 levels were, respectively, detected by using real-time PCR. The results showed that 10 μ mol/L NE significantly decreased miR-21 expression, but 0.1 and 1 μ mol/L NE had no obvious effects on

miR-21 expression (Fig. 2A). Additionally, miR-21 levels were, respectively, assayed after 0-h (control), 12-h and 24-h treatment, and the effect of NE on miR-21 expression was measured. The NE treatment decreased miR-21 levels about 58% and 49%, respectively (Fig. 2B). These data demonstrate that NE regulates the expression of miR-21 in a dose- and time-dependent manner. It is known that there are two kinds of commonly used adrenergic receptors (AR), AR- $\alpha 1$ and AR- $\beta 2$, by NE in rat ovary. To determine which AR couples with NE, we separately added 10 μ mol/L of two AR inhibitors to granulosa cells. The inhibitors are prazosin for AR- $\alpha 1$ and butoxamine for AR- $\beta 2$. The cells were then additionally treated with NE for 24h. The results show that miR-21 repression was not only relieved but also highly stimulated by prazosin (Fig. 2C).

To investigate whether NE has a role in regulating granulosa cells apoptosis, we tested the effect of NE treatment on granulosa cells apoptosis by counting the cleaved caspase3-positive cells. We counted cleaved caspase3-positive cells in more than 5000 cells per treatment. There was roughly 18% cleaved caspase3-positive cell in 10 μ mol/L NE more than that in control group (Fig. 2E) ($P < 0.05$). These data demonstrate that 10 μ mol/L NE induces rat granulosa cells apoptosis.

Figure 2

NE regulates the expression of miR-21 and induces granulosa cells apoptosis. (A) Quantification of miR-21 level after treatment (for 24h) with different levels of NE (0, 0.1, 1 and 10 $\mu\text{mol/L}$). con: control. The experiments were repeated at least three times and normalized to their respective control. Data are shown as means $\pm$ S.E.M. ($n \geq 3$). * $P < 0.05$; ** $P < 0.01$. (B) miR-21 levels after being treated with 10 $\mu\text{mol/L}$ NE for 0, 12 and 24 h. The experiments were repeated at least three times and normalized to their respective control. Data are shown as means $\pm$ S.E.M. ($n \geq 3$). * $P < 0.05$. (C) The relative level of miR-21 in granulosa cells treated with 10 $\mu\text{mol/L}$ NE and two adrenergic receptor inhibitors. The control (con) was a vehicle control (PBS). The inhibitors for AR- $\alpha 1$ and AR- $\beta 2$ are prazosin (Pra) and butoxamine (Buto) respectively. The data are shown as means $\pm$ S.E.M. For multiple separate transfections ($n = 3$); * $P < 0.05$. (D) Granulosa cells apoptosis was measured by using cleaved caspase3 analysis after treatment with NE for 24 h. (E) Percentages of cleaved caspase3-positive cells accounting for the total granulosa cells. All scale bars represent 50 μm .

miR-21 mediates the NE signaling pathway influencing granulosa cell apoptosis

To confirm that miR-21 is involved in the NE signaling pathway, the endogenous miR-21 in cultured granulosa cells was inhibited and also overexpressed by a miR-21 inhibitor and miR-21 mimics, respectively. The results show that the miR-21 inhibitor downregulated the endogenous miR-21 by 71.6%, whereas the mimics upregulated miR-21 expression 8.9-fold (Fig. 3A). Cultured granulosa cells were transfected with miR-21-mi and miR-21-in for 24h and then treated with 10 $\mu\text{mol/L}$ NE for 24h, and then the relative gene expressions of *Bax*/*Bcl-2* were determined (Fig. 3B and C) and the cleaved caspase3-positive cells were counted. The results show that miR-21-in increased both basal and NE-enhanced granulosa cell apoptosis (Fig. 3D and F). We also found that, as expected, NE increased granulosa cell apoptosis in cells transfected with negative control mimics, but this effect vanished in the cells that overexpressed miR-21 mimics (Fig. 3E and G). These results demonstrate that

miR-21 negatively regulates the NE stimulatory action on granulosa cell apoptosis.

Smad7 is the direct target gene of miR-21 in rat granulosa cells

To find the target molecules of miR-21 affecting cell apoptosis, we used miRBase Targets and TargetScan to find targeting candidates of miR-21. We found two putative target genes: *Jag1* and *Smad7*. In addition, *Smad7* had also been shown to function as an apoptosis inducer in granulosa cells and the 3'UTR of the *Smad7* gene contains a highly conserved region that is a putative binding site for miR-21.

We then identified the interactions between miR-21 and its putative target genes, *Jag1* and *Smad7* using the 3'-UTR of *Jag1* and *Smad7* mRNA as the predicted binding site (Fig. 4A). Using psiCHECK-2, we cloned the putative 3'-UTR target site downstream of a luciferase reporter gene. We then co-transfected into 293T cells, the psiCHECK-2

Figure 3

miR-21 mediates the signaling pathway that influences granulosa cell apoptosis. (A) Overexpression and inhibition efficiency of miR-21 mimics or inhibitor, transfected into granulosa cells after 24 h. nc-in/nc-mi, miRNA inhibitor/mimics nonsense control; inhibitor, miR-21 inhibitor; mimics, miR-21 mimics. The data are means $\pm$ S.E.M. for multiple separate transfections ($n=3$). * $P<0.05$. (B) and (C) relative gene expression of *Bax/Bcl-2*. Granulosa cells were transfected with nc-in, miR-21-in, nc-mi and miR-21-mi, respectively, for 6 h. We then added 10 μ mol/L NE for 24 h. Data are presented as means $\pm$ S.E.M. ($n=3$) * $P<0.05$; ** $P<0.01$ (ANOVA). (D) and (E) percentages of cleaved caspase3-positive cells accounting for the total granulosa cells. Data are presented as means $\pm$ S.E.M. ($n=3$) * $P<0.05$; (ANOVA). (F) and (G) light microscopy pictures of granulosa cell apoptosis measured by using cleaved caspase3 analysis. Granulosa cells were transfected with nc-in, miR-21-in, nc-mi and miR-21-mi, respectively, for 6 h. We then added 10 μ mol/L NE for 24 h. All scale bars represent 50 μ m.

Figure 4

miR-21 binds to *Smad7* 3'UTR and downregulates its protein level. (A) The predicted miR-21-binding site is in the *Smad7* and *Jag1* 3'UTRs. The data were taken from Sanger's miRNA target database. (B) and (C) relative luminescence intensity detected by the Modulus TMI microplate multimode reader after miR-21-mi nc mimics (nc-mi) and Dual-Luciferase vectors were co-transfected into 293T cells. The data are mean $\pm$ S.E.M. for multiple separate transfections ($n=4$); $*P<0.05$. (D) Relative quantification of *Smad7* mRNA levels. Primary granulosa cells were transfected with nc inhibitor (nc-in), miR-21 in, nc mimics (nc-mi), and miR-21-mi. These data are means $\pm$ S.E.M. for multiple separate transfections ($n=4$); $*P<0.05$. (E) Analysis of SMAD7 protein levels. Primary granulosa cells were transfected as shown previously and protein extracts were analyzed by Western blotting. Results are mean $\pm$ S.E.M. for multiple separate transfections ($n=4$). (F) Quantification of SMAD7 protein levels. The data are means $\pm$ S.E.M., $*P<0.05$; (SAS 9.0).

(wild type or mutant) together with miR-21-mi or negative control (nc) mimics. The results showed that the luciferase activity of the transfected cells with miR-21-mi and 3'-UTR-*Smad7* decreased by ~39% compared with the cells co-transfected with the nc mimic and 3'-UTR-*Smad7*. The negative control construct of mutations in the 3'-UTR of *Smad7* (3'-UTR-MUT-*Smad7*) showed no obvious change in luciferase activity (Fig. 4C). 3'-UTR-JAG1 construct of luciferase activity did not change compared with mutations in the 3'-UTR of *Jag1* (3'-UTR-MUT-*Jag1*) (Fig. 4B). Further functional analysis indicated that miR-21 overexpression in rat granulosa cells resulted in a reduction of *Smad7* mRNA and protein levels, whereas the inhibition of miR-21 significantly increased *Smad7* mRNA and protein levels (Fig. 4D, E and F). These results confirm that the *Smad7* gene is a miR-21 target and that miR-21 regulates *Smad7* gene expression at the transcriptional level (Fig. 4D).

***Smad7* is involved in the signaling pathway of NE that regulates granulosa cell apoptosis**

To identify whether *Smad7* is involved in the signaling pathway of NE that regulates granulosa cells apoptosis,

we treated rat granulosa cell with 0 (control), 0.1, 1 and 10 μ mol/L NE for 24 h, and *Smad7* expression was assayed. The results show that 10 μ mol/L NE upregulated *Smad7* mRNA significantly (Fig. 5A). Western blot analysis confirmed that 10 μ mol/L NE significantly upregulates SMAD7 protein levels (Fig. 5B and C). We then used three different *Smad7* siRNAs and chose *Smad7* siRNA3, which had a higher inhibition on cleaved caspase3 protein (Fig. 5D). *Smad7* siRNA also inhibited the effect of NE on cleaved caspase3 protein level by 37% (Fig. 5E and F). These preliminary results demonstrate that *Smad7* plays an important role in mediating the regulatory effect of NE on rat granulosa cell apoptosis.

NE enhances apoptosis mediated by TGF- β in granulosa cells

The previously mentioned data show that *Smad7* is a miR-21 target in rat ovaries. It is also known that SMAD7 is a general antagonist for TGF- β signaling (Yan *et al.* 2009). We hypothesized that NE is involved in TGF- β signaling. To confirm this, primary granulosa cells were treated with NE, TGF- β or a combination of NE and TGF- β . The results show that NE or TGF- β treatment decreased miR-21

Figure 5

SMAD7 is involved in the signaling pathway of NE that regulates granulosa cells apoptosis. (A) Quantification of *SMAD7* mRNA levels after being treated with different levels of NE (0, 0.1, 1 and 10 $\mu\text{mol/L}$). The experiments were repeated at least three times and normalized to their respective control. Data are shown as means $\pm$ S.E.M. ($n \geq 3$). $*P < 0.05$. (B) *SMAD7* protein expression levels were analyzed by Western blotting. (C) Quantification of *SMAD7* protein levels. Data are presented as means $\pm$ S.E.M. ($n = 3$). $*P < 0.05$. (D) Quantification of intracellular *SMAD7* mRNA levels after the granulosa cells were transfected with *SMAD7* siRNA1-3 for 24 h. Data are presented as mean $\pm$ S.E.M. ($n = 3$) ($*P < 0.05$, by ANOVA). (E) Cleaved caspase3 protein expression levels were analyzed by Western blotting. The granulosa cells were transfected with nc siRNA or *SMAD7* siRNA and 24 h later, 10 $\mu\text{mol/L}$ NE was added to granulosa cells and were treated for 24 h. (F) Quantification of cleaved caspase3 protein levels. Data are presented as means $\pm$ S.E.M. ($n = 3$). $*P < 0.05$.

expression 3- and 4-fold, respectively, whereas treatment with a combination of NE and TGF- β decreased miR-21 expression by approximately 10-fold (Fig. 6A), indicating that NE has an additive effect on TGF- β signaling. We then detected *Smad7* mRNA levels and SMAD7 protein levels in these four treatment groups (control, NE, TGF- β and NE+TGF- β). The results showed that both *Smad7* mRNA levels and protein levels increased (Fig. 6B, C and D). These data indicated that NE enhances TGF- β -induced granulosa cell apoptosis.

Discussion

NE has been shown to be involved in many regulatory aspects of ovary functions, including early follicular development (Mayerhofer *et al.* 1997) and the importance of its interrelation on the ovarian steroidogenesis and apoptosis on dioestrus II in rat (Bronzi *et al.* 2015). Several recent reports have shown that NE acts on β 2-adrenergic receptors present in granulosa cells from the rat ovary and stimulates the production of progesterone, but not the secretion of estradiol (Lawrence & Burden 1980, Skarzynski 1993, Lara *et al.* 2002). NE may also facilitate the follicular development, as seen, for example, in the

inhibition of follicular growth after ovarian denervation (Mayerhofer *et al.* 1997, Doganay *et al.* 2010). In this study, we identified miRNA expression profiles of NE-stimulated rat granulosa cells for the first time. Furthermore, miR-21 was shown to play an important role in NE-induced granulosa cell apoptosis by targeting *Smad7*. The findings of this study provide initial evidence that miR-21 participates in regulating rat granulosa cells apoptosis by targeting *Smad7*. A direct link between miRNAs and NE during ovarian follicle development has also been established for the first time.

miRNAs have been shown to play important roles in several biological events. For example, miR-378 is involved in regulating ovarian estradiol production by targeting aromatase (Xu *et al.* 2011) and miR-181a has been reported to play a role in mouse granulosa cell proliferation (Zhang *et al.* 2013). The regulating effects of the miR-21 on granulosa cell apoptosis have been previously reported (Carletti *et al.* 2010). However, a functional relationship between NE and miR-21 has not been established for regulating granulosa cell apoptosis. Our results demonstrate that adrenergic receptors and miR-21 are expressed in rat granulosa cells. And the expression of miR-21 is decreased in the ovary from the

Figure 6

NE enhances apoptosis mediated by TGF- β in granulosa cells. SMAD7 mRNA and protein levels along with miR-21 expression were detected after primary granulosa cells were treated with NE (10 μ mol/L), TGF- β (5 ng/mL) or combination of 10 μ mol/L NE and TGF β for 24 h. (A) Relative expression of miR-21 levels. Data are presented as means $\pm$ S.E.M. ($n=4$) (* $P<0.05$; ** $P<0.01$, by t -test). (B) Expression levels of SMAD7 mRNA were measured by real-time PCR. Data are presented as means $\pm$ S.E.M. ($n=4$) (* $P<0.05$, ** $P<0.01$, by t -test). (C) SMAD7 protein levels were detected by Western blot. (D) Quantification of SMAD7 protein levels. Data are presented as means $\pm$ S.E.M. ($n=3$) (* $P<0.05$, ** $P<0.01$, by ANOVA).

animal model for study the follicular atresia. Therefore, miR-21 may be associated with follicular atresia. One of the prominent features of follicular atresia is granulosa cell apoptosis. In addition, our functional experiments have demonstrated that addition of 10 μ mol/L NE significantly inhibits miR-21 expression. In addition, NE treatment significantly increased granulosa cell apoptosis.

NE, a known sympathetic neurotransmitter, regulates sympathetic nerve activities. It is also well documented that NE induced cultured rat cardiomyocyte apoptosis (Ma *et al.* 2013). The report (Bronzi *et al.* 2015) has demonstrated the importance of its interrelation on the ovarian steroidogenesis and apoptosis on dioestrus II in rat. However, the apoptotic pathway of NE regulating ovarian granulosa cell is unknown. The previous report (Greiner *et al.* 2008) shows that ovarian non-neuronal, endocrine granulosa cells can take up NE, and then serve as an intrafollicular catecholamine-storing compartment. In this study, NE was shown to regulate ovarian functions by targeting miR-21 and acting on AR- α_{1A} in cultured granulosa cells. To investigate the roles of miR-21 in NE-induced granulosa cell apoptosis, miR-21 was functionally characterized in rat granulosa cells. Our results show that knockdown of miR-21 induces rat granulosa cell apoptosis and enhances NE-induced granulosa cell apoptosis. From the previously mentioned results, we can state that miR-21 controls reproductive functions by blocking rat granulosa cell apoptosis, either alone or in combination with NE.

Smad7, the gene target of miR-21, was identified to account for the previously mentioned phenotype. SMAD7, a critical factor of the TGF- β signaling pathway, has been shown to induce apoptosis in granulosa cells

(Quezada *et al.* 2012). Forced expression of miR-21 and knockdown of miR-21 in granulosa cells decreased and increased *Smad7* mRNA and protein expression levels, respectively. Additionally, *Smad7* siRNA attenuated miR-21 inhibitor-induced rat granulosa cell apoptosis. These results confirm that *Smad7* is a target of miR-21. We identified that *Smad7* is involved in the signaling pathway of NE that regulated granulosa cells apoptosis.

Smad7, a common mediator of TGF- β signaling, has been suggested to play an important role in the regulation of ovarian follicle growth and female fertility. One study reported that *Smad7* expression is upregulated by treating with TGF- β , and the overexpression of *Smad7* induces granulosa cell apoptosis (Quezada *et al.* 2012). This result is consistent with ours. Our results demonstrated that 10 μ mol/L NE increases SMAD7 protein expression by the knockdown of miR-21 and that *Smad7* mediates the roles of miR-21 thus influencing granulosa cell apoptosis. We also demonstrated that miR-21 blocks cell apoptosis by targeting *Smad7*. These results not only validate the functional relevance of *Smad7* (a target gene of miR-21) in NE/miR-21-stimulated rat granulosa cells but also provide a new molecular mechanism of NE's regulation of *Smad7*.

In conclusion, we have revealed that miR-21 is expressed in the rat ovary and is located in the granulosa cells. Additionally, miR-21 acts as a negative mediator to regulate NE-induced granulosa cell apoptosis by targeting *Smad7*. In the light of our study, due to the functions of miR-21 in granulosa cells, it is worth pursuing research into using it as a novel target for physiological or pharmacological interventions in diseases such as polycystic ovarian syndrome (PCOS).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Sheng Cui conceived and designed the experiments; Li Zhang and Jie Gao carried out the experiments; Li Zhang analyzed the data; Jie Gao provided the reagents/materials/analysis tools and Sheng Cui and Li Zhang wrote the manuscript.

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