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Short communication

NTS TARGETED BY miR-182 MODULATES THE APOPTOSIS OF GOAT ENDOMETRIAL EPITHELIAL CELLS

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Neurotensin is a tridecapeptide that functions in prenatal metabolism. It is targeted and downregulated by miR-182 through binding the seed site of miR-182 to the 3' untranslated region and reduced by a candidate tumor suppressor, testin. Considering that apoptosis is critical in the development of mammalian preattachment embryos, this study investigated the modulation of neurotensin to the apoptosis of goat endometrial epithelial cells and apoptosis-related proteins (P38 MAPK and caspase8). Results showed that *Neurotensin* resisted the apoptosis of goat endometrial epithelial cells through the caspase8 pathway and activated the phosphorylation of P38 MAPK, which is involved in blastocyst formation. Thus, miR-182 is likely to promote uterus health by targeting *Neurotensin* and upregulating *Testin*.

Key words: miRNA, neurotensin, testin, apoptosis

INTRODUCTION

Neurotensin (*NTS*) is a tridecapeptide found in the hypothalamus and the gastrointestinal tract [1]. *NTS* participates in prenatal metabolism and in the early establishment of the neuronal system [2], and it has been used to design dipeptide drugs with high effectiveness [3]. The development of mammalian preattachment embryos is closely related to apoptosis because abnormal cells need to be removed [4,5]. In the present research, the apoptotic rate of goat endometrial epithelial cells (gEECs) was detected after *NTS* overexpression, and the apoptotic pathways, including P38 MAPK and caspase-8, were studied. The activation of P38 MAPK induces the apoptosis of human endometrial epithelial cells [6] and participates in the formation of the blastocyst [7]. In addition, caspase-8 is important to initiate the Fas-induced apoptotic cascade [8].

Molecules that regulate the expression of *NTS* were also studied. miRNAs are a class of endogenous non-coding RNAs that regulate gene expression by binding to the 3' untranslated region of genes [9]. This study verified that the expression of *NTS*

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was targeted by miR-182 and regulated in ways of translation repression and mRNA degradation. *Testin* (*TES*), a candidate tumor suppressor [10] that has been studied in various cancer types, such as endometrial carcinoma [11], nasopharyngeal carcinoma [12], and colorectal cancer [13], was proven to be downregulated by *NTS*.

MATERIALS AND METHODS

Cell culture

The gEEC cell line was cultivated at 37 °C in humid atmosphere of 5% CO_2 and was provided with a DMEM/F12 cultivator (Gibco, Shanghai, China) containing 10% fetal bovine serum (Gibco, Shanghai, China). Negative control and miR-182 mimic were transfected into gEEC in a six-well culture plate at 50 nM and plasmid at 4 $\mu g \cdot mL^{-1}$. Cells were harvested at 48 h after being transfected.

Vector acquirement

The 3' untranslated region and coding sequence region of *NTS* were amplified using 2 × SuperStar PCR Mix with Loading Dye (Genstar, Beijing, China) and were linked to psiCHECK-2 and pcDNA3.1(+) with T4 DNA Ligase (Takara, Dalian, China), respectively. pcDNA3.1-TES was acquired previously in our laboratory. To detect whether the expression level of the candidate tumor suppressor *TES* is regulated by *NTS*, we transfected the pcDNA3.1-NTS vector into gEECs. The regulation of *TES* to *NTS* was also observed by transfecting the pcDNA3.1-TES vector into gEECs. The following primers were used to construct the psiCHECK-2-*NTS* vector: GCCTCGAGCACTTAATGGGTTGTTGA (forward) and GCGCGGCCGCTGATGGCTGTTGTCTTTT (reverse). The following primers were used to construct the pcDNA3.1-NTS vector: CCCTCGAGATGATGGCAGGAATGAAAATC (forward) and GGGGTACCTCAGTAGTAGTAAGAACCTCTTTTGAGTATG (reverse).

Dual luciferase assay

Plasmids of wild-type and mutant-type psiCHECK-2-*NTS* were co-transfected with the negative control (NC) or miR-182 mimic in a 24-well plate. PBS was applied to wash the cells after 24 h. Then, the cells were lysed with 1 × passive lysis buffer. A Varioskan flash (Thermo Fisher Scientific, Rockford, USA) was applied to evaluate luciferase activities in accordance with the protocol of the Dual-Luciferase Reporter Assay System (Promega, Madison, USA). The activity of Renilla luciferase (*hRluc*) was normalized by that of firefly luciferase (*hluc*$^+$).

RNA isolation and RT-qPCR

Cell RNA was isolated using Trizol (Invitrogen, Shanghai, China), and complementary DNA was acquired using the StarScript II First-strand cDNA Synthesis Kit (Genstar, Beijing, China). Quantitative real-time Polymerase Chain Reaction (qPCR) was

performed with 2×RealStar Power SYBR Mixture (Genstar, Beijing, China), and the value of Ct was detected by the CFX Connect real-time PCR detection system (Bio-Rad, CA, USA) and was linearized using the $2^{-\Delta\Delta Ct}$ method. The following primers were used to detect *NTS* mRNA level: TCAGTAAGGCAAGTGTTC (forward) and CCATCTAAGGCAGCAGGA (reverse). *GAPDH* primers were GCAAGTTCCACGGCACAG (forward) and GGTTCACGCCCATCACAA (reverse).

Flow cytometry

The gEECs were transfected and cultivated for 24 h in a six-well culture plate. Then, gEECs were harvested by trypsinization. The Annexin V-FITC/PI apoptosis kit (Multi Sciences, Hangzhou, China) was used to evaluate gEEC apoptosis in accordance with the manufacturer's instruction by a flow cytometer (BD FACSCalibur, USA).

Western blot analysis

The gEEC cell protein was extracted using RIPA buffer (Heart, Xi'an, China). Proteins were separated by SDS-PAGE. NTS primary antibody was obtained from BBI (D121026, Shanghai, China). Caspase-8, P38 MAPK, and β-actin primary antibodies and secondary antibodies were purchased from Beyotime (Shanghai, China).

Statistical analysis

SPSS 22.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Differences were compared by Student's t test, and the results were presented as mean ± SEM. Each experiment was performed three times independently. * represents significance ($p < 0.05$), and ** represents high significance ($p < 0.01$).

RESULTS

miR-182 directly regulates *NTS* via combining to *NTS* in the 3' untranslated region

NTS was predicted as a target of miR-182 at Targetscan (http://www.targetscan.org). To verify whether miR-182 regulates *NTS* by binding sites, we constructed a dual luciferase cloning vector with *NTS* or mutant *NTS* binding sites. The sequence information of miR-182 and the wild-type (Wt) or mutant-type (Mu) psiCHECK-2-*NTS* vector is demonstrated in Fig. 1a. Dual luciferase assay showed that the relative luciferase intensity of the Wt psiCHECK-2-*NTS* vector decreased significantly after co-transfecting with the miR-182 mimic compared with NC, but the Mu vector did not considerably change after co-transfection (Fig. 1b). This result suggests that that *NTS* is directly regulated by miR-182.

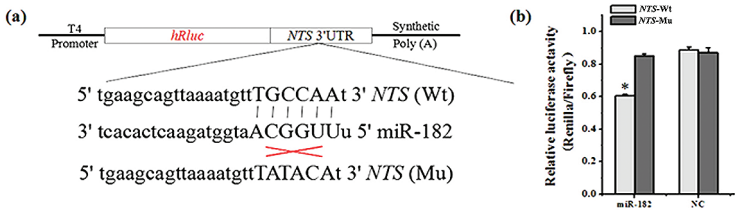

Figure 1. miR-182 decreased the relative luciferase activity of psiCHECK-2-*NTS*. **(a)** Sequence diagram illustrates the sequence details about constructing the wild-type (Wt) and mutant-type (Mu) vectors of psiCHECK-2-*NTS* for luciferase reporter assay. Capital nucleobases of miR-182 are seed sequence, those of *NTS* (Wt) are target sites, and those of *NTS* (Mu) are mutant sites. **(b)** psiCHECK-2-*NTS* Wt and Mu vectors were co-transfected into gEEC cells with miR-182 mimic or NC before luciferase activity was detected at 24 h. Luciferase reporter assay results are shown as relative luciferase activity.

Expression of *NTS* was suppressed after miR-182 transfection

MiR-182 was transfected into gEECs 24 h before gEEC total RNA was isolated. In consideration of the time required for protein synthesis, gEEC total proteins were extracted at 48 h. As shown in Fig. 2, the expression of *NTS* was prominently downregulated at the mRNA and protein levels.

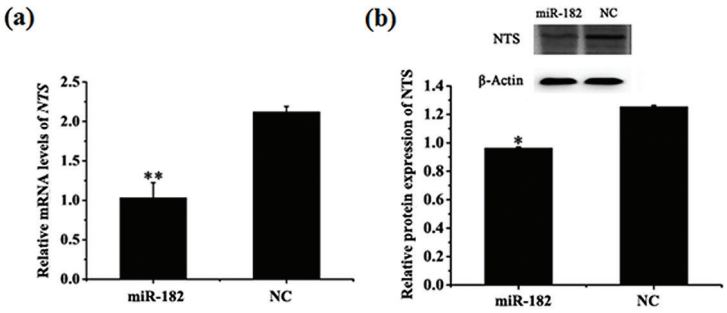

Figure 2. miR-182 downregulated the expression levels of NTS. **(a)** miR-182 decreased the mRNA expression level of *NTS*. RT-qPCR was used to detect *NTS* mRNA level, and *GAPDH* was used to normalize the expression. **(b)** miR-182 downregulated the protein expression level of NTS. Protein levels of NTS were evaluated by Western blot and normalized to β-actin.

gEEC apoptosis was resisted by *NTS*

Overexpression vector pcDNA3.1-NTS was constructed to transfect gEEC, and the efficiency was detected by RT-qPCR and Western blot. The structure of the pcDNA3.1-NTS vector is displayed in Fig. 3a. As shown in Figs. 3b and 3c, pcDNA3.1-NTS efficiently enhanced the expression of *NTS*. To verify how *NTS* regulates the apoptotic rate of gEEC, we performed flow cytometry. Results showed that gEEC apoptosis was suppressed (Fig. 4). The apoptosis-related pathways, including caspase-8 and P38 MAPK, were studied to find the anti-apoptotic pathways involving miR-182.

As shown in Fig. 5, the protein expression of caspase-8 was reduced and that of P38 MAPK was improved when *NTS* was overexpressed in gEEC.

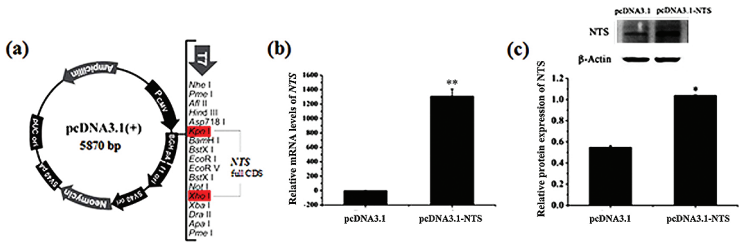

Figure 3. pcDNA3.1-NTS overexpression vector was successfully constructed. **(a)** Structure of pcDNA3.1-NTS vector. *NTS* full CDS sequence was inserted between Kpn I and Xho I. **(b, c)** Efficiency of the pcDNA3.1-NTS vector was ensured at the mRNA and protein levels.

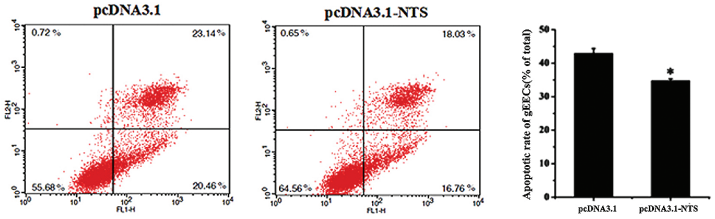

Figure 4. pcDNA3.1-NTS vector was transfected into gEECs, and apoptotic rate was measured by a flow cytometer. Results show that *NTS* suppressed gEEC apoptosis.

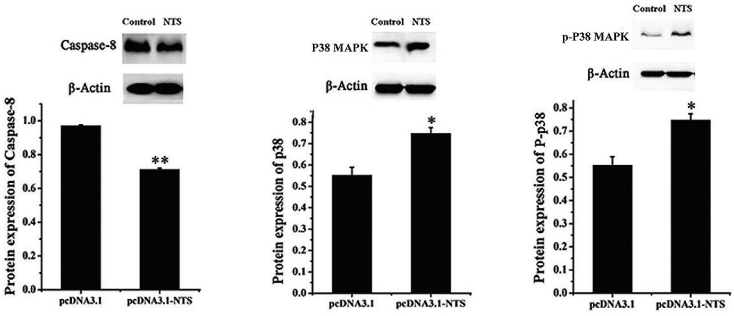

Figure 5. Apoptosis-related proteins (caspase-8, P38 MAPK, and p-P38 MAPK) were detected by Western blot. **(a)** *NTS* reduced the protein expression of caspase-8. **(b)** *NTS* improved the protein expression of P38 MAPK and p-P38 MAPK.

NTS suppressed the expression of *TES*

As shown in Fig. 6, the mRNA and protein expression levels of *TES* were reduced approximately tenfold and twofold, respectively, when *NTS* was overexpressed.

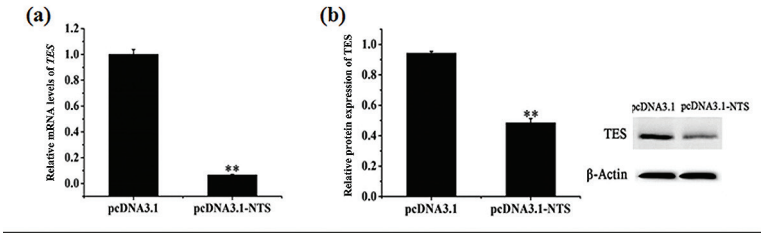

Figure 6. *NTS* decreased the expression of *TES*. **(a)** pcDNA3.1-NTS vector was transfected into gEECs; after 24 h, *TES* mRNA levels were detected by RT-qPCR. *NTS* decreased the mRNA level of *TES*. **(b)** At 48 h after the pcDNA3.1-NTS vector was transfected into gEECs, *TES* protein levels were detected by Western blot. *NTS* decreased the protein expression of *TES*.

TES reduced the protein expression of *NTS*

As shown in Fig. 7, *TES* did not affect the mRNA level of *NTS* even though pcDNA3.1-TES was transfected into gEECs. However, *TES* significantly reduced the protein expression of NTS.

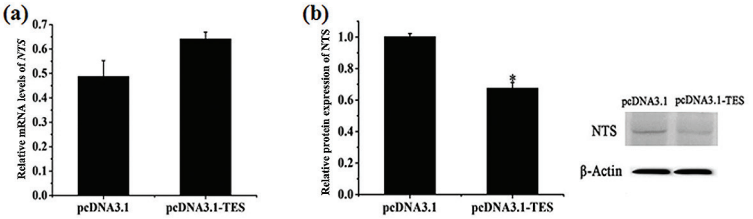

Figure 7. *TES* decreased the protein expression of *NTS*. **(a)** *NTS* mRNA levels were detected by RT-qPCR at 24 h after transfecting pcDNA3.1-NTS. *NTS* exerted no significant effect on the mRNA level of *TES*. **(b)** *NTS* decreased the protein expression of *TES* at 48 h after the pcDNA3.1-NTS vector was transfected into gEECs.

DISCUSSION

NTS is a 13-amino-acid peptide that participates in prenatal metabolism and in the early establishment of the neuronal system [2]. Its 3' untranslated region is bound by miR-182 and leads to the expression of downregulation in gEECs. The expression of *NTS* is regulated by miR-182 in gEECs. To determine the effects of *NTS* on gEECs, we transfected the overexpression vector of *NTS* into Geec and found that *NTS* alleviated the apoptotic rate of gEECs and suppressed the caspase-8 pathway. However, the expression and activation of P38 MAPK are increased by *NTS*. The increase might indicate a promotion of blastocyst formation, considering that P38 MAPK participates in blastocyst formation [7].

In this experiment, *TES* was prominently downregulated by *NTS*. We speculated that *TES* is upregulated when *NTS* is reduced by miR-182. In consideration that *TES* is a

possible tumor suppressor [14], the increase in *TES* expression might be beneficial for uterus development.

Therefore, miR-182 may promote uterus health and then improve embryo attachment by targeting NTS and upregulating TES. In addition, the existence of NTS could resist the apoptosis of gEECs through the caspase8 pathway and activate the phosphorylation of P38 MAPK, thereby improving blastocyst formation [7].

Acknowledgments

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Authors' contributions

XPA and YZ contributed equally to this manuscript, they designed the study and wrote the manuscript. JDL and XNM conducted the experiments and performed the statistic analysis. ZYG and BYC applied funds to support the study.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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EFEKAT miR-182 NA NTS MODULIRA APOPTOZU EPITELNIH ĆELIJA ENDOMETRIJUMA KOZA

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Neurotenzin je tridekapeptid koji je uključen u prenatalni metabolizam. Ciljan je i regulisan preko miR-182 vezivanjem mesta miR-182 za 3' region koji nije poretrpeo translaciju i koji je redukovan putem tumorskog supresora, testina. Uzimajući u obzir da je apoptoza kritična u nastanku embriona pre pripajanja, ova studija je imala za cilj ispitivanje modulacije neurotenzina na endometrijalne epitelne ćelije koze i efekte proteina uključenih u apoptozu (P38 MAPK i caspaza8). Rezultati su pokazali da neurotenzin odoleva apoptozi epitelnih endometrijalnih ćelija posredstvom caspasa 8 puta i aktivira fosforilaciju P38 MAPK-a koji je uključen u nastanak blastociste. miR-182 verovatno promoviše zdravlje uterusa time što cilja neurotenzin i stimuliše testin.