**miR-181c inhibits prostatic epithelial cell proliferation caused by chronic non-bacterial prostatitis through down-regulating COX-2**

**Running title:** The role of miR-181c in chronic nonbacterial prostatitis.

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**Abstract**

**Background** Chronic non-bacterial prostatitis (CNP) is a common disease of the male reproductive system. MiR-181c can be expressed in prostate tissue, but it has not been reported in CNP. This study aims to explore the role of miR-181c in CNP and its mechanism of action on CNP, providing new ideas for the treatment and diagnosis of CNP.

**Methods** Quantitative real-time PCR (qRT-PCR) and western blotting were performed to determine the expression of miR-181c in clinical CP patients and LPS-induced human prostaglandin epithelial cell RWPE-1. Then, the target relationship between miR-181c and COX-2 was verified by luciferase reporter assay. Through cell transfection experiments, the effect of mi-181c on the expression of COX-2 and PGE2 was studied, and the effect of miR-181c/COX-2 on the proliferation of prostate epithelial cells was also explored.

**Results** qRT-PCR and Western blotting analysis revealed that miR-181c was low expressed in prostate tissue and human prostaglandin epithelial cell RWPE-1. The luciferase reporter assay confirmed the targeting relationship between miR-181c and COX-2. And overexpression of miR-181c reduced the expression of COX-2 and PGE2 and inhibited the proliferation of prostate epithelial cells. Up-regulation of COX-2 reversed these effects caused by overexpression of miR-181c.

**Conclusion** miR-181 inhibited the proliferation of prostate epithelial cells through negatively regulating COX-2 to alleviate chronic non-bacterial prostatitis.

**Keywords** chronic non-bacterial prostatitis; miR-181c; COX-2; prostatic epithelial cell; proliferation

**Introduction**

Chronic prostatitis (CP) is the most prevalent disease in andrology. According to the classification of National Institutes of Health (NIH), prostatitis is divided into the following categories: acute bacterial prostatitis (category I), chronic bacterial prostatitis (category II), chronic prostatitis (CP) / chronic pelvic pain syndrome (CPPS) (category III) and asymptomatic prostatitis (category IV). Category III is further divided into IIIA and IIIB. Chronic nonbacterial prostatitis (CNP) belongs to category IIIA [1]. Among them, CNP accounts for around 70%-90% of patients diagnosed with prostatitis. And the proportion is increasing in recent years [2, 3]. The main clinical manifestations of CNP [4, 5] are irritation of the urethra and pain in the perineum. The etiology involves one or more factors such as body inflammation, endocrine, immune, and nerves. They act on prostate tissue alone or together to cause sensitization and neuroendocrine disruptions around the prostate, ultimately leading to uncontrolled regulation of the pelvic nerve by the cerebral cortex and chronic neuropathic pain [6, 7]. To date, the pathogenesis of CNP has not been fully clarified, and the diagnosis and treatment of CNP a particular challenge.

Cyclooxygenase (COX), also known as prostaglandin-endoperoxide H synthase (PGHS), is a rate-limiting enzyme that plays an important role in prostaglandin (PG) biosynthesis from arachidonic acid and participates in diverse pathophysiological processes [8, 9]. There are three COX isoforms: COX-1, COX-2, and COX-3. COX-1 serves an important role in maintaining the normal physiological functions of the human body, such as gastrointestinal mucosal protection and renal blood flow regulation. It is generally believed that COX-3 is a variant of COX-1, mainly in the cerebral cortex and heart. COX-2 is an inducible enzyme, which is either absent or expressed slightly in most cells [10]. However, when cells are stimulated by pro-inflammatory cytokines or cancer-promoting factors, COX-2 shows up-regulation and participates in inflammation as well as the formation and development of tumors [11, 12]. Studies have shown that COX-2 is involved in CP [13, 14], and inhibition of COX-2 suppresses the proliferation of prostatic epithelial cells in CNP [15].

MicroRNA (miRNA) is a class of endogenous non-coding single-stranded small RNA with a length of 19-25 nt that is relatively conserved during evolution. Lee et al. [16] found it for the first time in the genetic analysis of mutants of C. elegans (Clekgans) in 1993. Since miRNA was first discovered, the role of miRNA in disease has been a research hotspot in recent years. And many investigators have explored the association between miRNAs and male urogenital system diseases [17-19]. miR-181c is one of the important members of miRNA family. Overexpression of miR-181c can lead to mitochondrial complex IV remodeling and dysfunction [20], and increase the production of reactive oxygen species. There is a study indicating that miR-181c is down-regulated in prostate cancer cells [21]. However, miR-181c has not been reported in CNP. Interestingly, we found that miR-181c has a binding site with COX-2 through the bioinformatics software microRNA.org. Therefore, we speculate that miR-181c may be one of the possible molecules regulating COX-2 in CNP.

In this study, we first evaluated the expression of miR-181c in patients with CP and LPS-induced human prostate epithelial cell line RWPE-1. Further studies verified the relationship between miR-181c and COX-2, and its effect on the proliferation of prostate epithelial cells. These findings will provide a potential candidate target for the therapeutic intervention of CNP, and an understanding of miR-181c in CNP by regulating COX-2.

**Materials and methods**

*Clinical sample*

Prostate tissue samples were obtained from Department of Urology of The First Affiliated Hospital of Soochow University, where 15 are from patients with CNP and 15 are from healthy male volunteers. The study was conducted in accordance with the rules of Declaration of Helsinki of 1975. All men underwent prostate ultrasound-guided biopsy (transrectal ultrasound-guided biopsy TRUSBx) [22]. Prostate tissue samples were homogenized with a tissue homogenizer according to the manufacturer's protocol. In simple, tissues were weighed and minced with a scalpel blade. Then, the minced tissue samples were homogenized with cold homogenization buffer (100 mM potassium phosphate, 0.25 M sucrose, PH 7.4) at 6000 rpm. Finally, the homogenized solution was transferred to a pre-cooled Eppendorf (EP) tube and stored at -80°C for further use.

Quantitative real-time PCR (qRT-PCR)

The total RNA in prostate tissues or RWPE-1 cells was extracted according to the TRIZOL method. miR-181c specific primers were used for the reverse transcription reaction of miRNA, and U6 was used as the internal reference. Simultaneously, β-actin was used as the internal reference for the reverse transcription reaction of COX-2. RNA was converted to cDNA using the reverse transcription kit (Promega). PCR amplification (based on each detected gene) was performed with SYBR RT-PCR Kit (TaKaRa). Three specimens were set for each sample and the detected gene. The expression level of miR-181c and COX-2 was calculated according to the formula 2-△△CT method.

*Western blotting*

Prostate tissues or RWPE-1 cells were lysed with RIPA lysis buffer (Beyotime, Beijing, China). The protein concentration was determined using a BCA protein quantification kit (Boster, China). The protein sample was denatured with loading buffer, and then 20 μg protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Then, the protein on the gel was transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% bovine serum albumin (BSA), the membrane was incubated with primary antibodies, including anti-COX-2 and anti-β-actin (diluted 1:1,000) overnight at 4°C. The next day, the membrane was incubated with goat anti-rabbit/mouse secondary antibody for 1 h at room temperature. Finally, ECL chemiluminescence reagent was used to quantify the relative expression of target protein, and Image J software (National Institutes of Health) was applied to analyze the protein bands.

*Cell culture*

Immortalized human prostate epithelial cell line RWPE-1 cells were purchased from American type culture specimens (ATCC). The cells were incubated in keratinocyte serum-free medium (K-SFM) containing 50 μg/mL of bovine pituitary extract (BPE), 1% of penicillin/streptomycin/amphotericin B, and 5 ng/mL of epidermal growth factor. The cell culture flask is placed at 37°C with 5% carbon dioxide. The culture medium was changed every 3 days. Cells were routinely passaged at 80-90% confluence. RWPE-1 cells were stimulated with Lipopolysaccharide (LPS) solution (10μg/mL) for 24h and then tested for corresponding indicators.

*Cell apoptosis*

Cell apoptosis was analyzed by flow cytometry. RWPE-1 (1x106 cells / well) cells were seeded in a 6-well plate. After treatment with LPS, cells were washed with phosphate-buffered saline (PBS). The treated and untreated cells were resuspended in 100 μL of binding buffer and then stained with 100 μL of annexin V reagent and 5 μL of PI for 15 min in the dark. Cell apoptosis was measured using a FACScan flow cytometer according to the manufacturer’ s instructions. Data were analyzed by FlowJo (FlowJo, Ashland, OR, USA).

*Cell transfection*

RWPE-1 cells were incubated in 24-well plates for 24h. miR-181c inhibitor or miR-181c mimic was purchased from RiboBio (Guangzhou, China). The COX-2 overexpression vector (pcDNA-COX-2) and its negative control vector (pcDNA) were constructed by GeneChem (Shanghai, China). LipofectamineTM 2000 (Invitrogen, Carlsbad, California) was applied for cell transfection. Cells were collected for related analysis 48h after transfection.

*Luciferase reporter assay*

COX-2 wild-type (WT) and mutant (Mut) 3'-UTR were constructed and cloned into the pcDNA empty vector. RWPE-1 cells were co-transfected with COX-2 WT or Mut 3'-UTR vector and miR-181c inhibitor or miR-181c mimic using LipofectamineTM 2000 reagent (Invitrogen). Firefly luciferase activity and Renilla luciferase activity were measured using the Dual-Luciferase Reporter Analysis Kit (Promega) according to the manufacturer’s instruction. The relative luciferase activity of each transfected group was tested 3 times. The ratio of firefly luciferase to Renilla luciferase was used as the relative luciferase activity.

*Enzyme-linked immunosorbent assay (ELISA)*

The supernatant of RWPE-1 cells was collected and centrifuged (3000r/min) at 4 ℃. The protein level of PEG2 was determined by an ELISA kit according to the manufacture's instructions. The detection was carried out by a multifunctional enzyme marker. The absorbance was determined at a wavelength of 450 nm.

*Cell proliferation*

Cell proliferation followed the instructions of the MTT detection kit (Fluda). RWPE-1 cells in the logarithmic growth phase were seeded in a 96-well plate, with 2×104 cells per well. The culture plate was placed in the cell incubator for 24 hours. The medium in each well was aspirated before measuring cell viability. And 200 μl of fresh RPMI medium containing 10% FBS and 10 μl of 0.5% MTT was added. Cells were incubated for another 4 h. DMSO (150 μl) was added to each well. And the plate oscillated in a shaker for 10 min. The absorbance was detected using a microplate reader at 490 nm.

*Carrageenan-Induced CNP rats*

A total of 24 male SD rats (4-6 weeks old, 240-260 g) were purchased from Shanghai Experimental Animal Center. The rats were randomly divided into 4 groups (n=6): Sham, CNP, CNP+pre-NC, CNP+miR-181c mimic group. The experimental protocol was approved by the Animal Ethics Committee of The First Affiliated Hospital of Soochow University, and all procedures were performed following the ethical guidelines on animal use.

Each rat was injected with 1% carrageenan (20 μL) and lentivirus (5×107 TU/mL) carrying miR-181c mimic into the prostate under anesthesia. The Sham and CNP+pre-NC group were given the same amount of normal saline or lentivirus carrying pre-NC. Seven days after the operation, the locomotion score was performed. During the observation period, 5 points were assigned when the hind limbs were completely bent or immobile. 1 point was assigned when there was no significant change in exercise capacity from the situation before the injection. All experiments were conducted by blind observers.

After that, the rats were sacrificed and prostate tissue was taken. One part was used to measure prostate volume, the rest was saved for later use. Six points were randomly counted in a high power field at 400 times magnification. And the inflammatory cells were quantified as the density per unit area. Two different histological sections of each rat were randomly selected from 20 microscope fields under a 6400-fold microscope to observe PCNA-positive cells in the rat ventral prostate epithelium. The calculation of cell proliferation index was based on the number of positive nuclei divided by the total nucleus count and expressed as a percentage. At least 2000 cells were counted in each group [23].

*Statistical analysis*

Student's *t*-test was applied to compare the difference (*p*<0.05) of the data between two groups. A one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test was conducted to evaluate the statistical significance (*p*<0.05) between different groups.

**Results**

*The expression of miR-181c was down-regulated in patients with chronic prostatitis*

To explore the expression of miR-181c in CP, we selected healthy male volunteers and clinical CP patients. The expression of miR-181c and COX-2 was detected respectively, and the correlation between the two was analyzed. Our results showed that miR-181c was lowly expressed in the prostate tissues of CP patients, while COX-2 was highly expressed in the prostate tissues (Fig. 1A). Person correlation analysis showed that miR-181c and COX-2 had a negative correlation (Fig. 1B).

*miR-181c was down-regulated in LPS induced RWPE-1 cells*

Later, to further explore the expression of miR-181c in CP, we used LPS to induce RWPE-1 cells *in vitro* and the expression of miR-181c and COX-2 was examined. The expression of miR-181c in RWPE-1cells in the LPS group was decreased compared with the control group (Fig. 2A), while the expression of COX-2 mRNA and protein was increased (Fig. 2B). We next performed flow cytometry to detect cell apoptosis. The results showed that RWPE-1 cell apoptosis was reduced after LPS treatment (Fig. 2C).

*Targeting relationship between miR-181c and COX-2*

Bioinformatics software microRNA.org was applied to predict the potential binding site between miR-181c and COX-2 (Fig. 3C). To further explore the interaction between miR-181c and COX-2, a luciferase reporter assay was performed. COX-2 3’UTR-wild type (WT), COX-2 3’UTR-mutant type (Mut), miR-181c inhibitor, or miR-181c mimic were co-transfected into RWPE-1 cells. The results found that the expression of miR-181c was decreased after interference with miR-181c in COX-2 3'UTR-WT transfected RWPE-1 cells, while the luciferase activity of cells was increased, and the mRNA and protein level of COX-2 was also increased (Fig. 3A). miR-181c was up-regulated in RWPE-1 cells transfected with COX-2 3’UTR-WT and miR-181c mimic, while the luciferase activity was decreased, and the mRNA and protein level of COX-2 were down-regulated (Fig. 3B).

*Overexpression of miR-181c downregulated the expression of COX-2 in LPS induced RWPE-1 cells*

To further confirm the regulatory effect of miR-181c on COX-2, miR-181c mimic or pre-NC was transfected in RWPE-1 cells. The results showed that the expression of miR-181c was increased in the LPS+miR-181c mimic group compared with the LPS group, indicating successful transfection (Fig. 4A). The mRNA and protein levels of COX-2 were down-regulated after transfection of miR-181c mimic (Fig. 4B), and the expression of its downstream synthetic product PGE2 was also decreased (Fig. 4C).

*Overexpression of COX-2 reversed miR-181c induced proliferation inhibition of prostate epithelial cells RWPE-1*

miR-181c mimic or pcDNA-COX-2 was transfected into RWPE-1 cells to explore the effect of miR-181c/COX-2 on the proliferation of prostate epithelial cells. The results of cell proliferation showed that the level of RWPE-1 cell proliferation was significantly increased after LPS induction. While miR-181c mimic suppressed the proliferation of RWPE-1 cells. However, the level of cell proliferation was increased when miR-181c and pcDNA-COX-2 were transfected simultaneously in RWPE-1 cells (Fig. 5).

*Overexpression of miR-181c inhibited inflammatory response in prostate tissue of rats with CNP*

Finally, the rats were injected with lentiviruses carrying miR-181c mimic, and the role of miR-181c in CNP rat was then explored. Results showed that the locomotion score and prostate volume of rats were increased after modeling, while the locomotion score and prostate volume of rats injected with miR-181c mimic were decreased (Fig. 6A, B). And overexpression of miR-181c reduced the average inflammatory cell count at high-power fields and the relative frequency of PCNA-positive cells in prostate tissues (Fig. 6C, D). qRT-PCR and western blotting showed that the expression of miR-181c was up-regulated and the mRNA and protein expression of COX-2 was down-regulated after injection of lentivirus carrying miR-181c mimic (Fig. 6E).

**Discussion**

CP/CPPS is the most common type of prostatitis. Patients with CP/CPPS will be accompanied by urinary tract symptoms and decreased quality of life. To date, some related mechanisms have been reported to be involved in the occurrence and development of CNP [24-26]. COX-2 is an "early and immediate" gene [27], its main role is to catalyze the production of a group of prostaglandins (PGs) products with different chemical properties, including prostaglandin E2 (PGE2), prostaglandin I2 (PGI2), Prostaglandin F2α (PGF2α), etc. PGE2 is the main catalytic product. The massive synthesis of PGE2 promotes the degranulation of neutrophils to release lysosomes, which intensifies the infiltration of local inflammatory cells and promotes inflammation [28]. In our study, COX-2 was highly expressed in the prostate tissues of CP patients. Simultaneously, the expression of COX-2 was up-regulated in human prostate cells RWPE-1 and CNP rats induced by LPS, which is consistent with previous studies [15, 29]. Therefore, elevated COX-2 was a key feature of CNP, which led to an increase in the synthesis of PGE2 and aggravated the inflammatory response in CNP rats.

The prostate is an important accessory gland in men. The prostate includes basal epithelial cells and luminal epithelial cells, as well as rare neuroendocrine cells. The function of prostate epithelium is to secrete prostatic fluid, prostate-specific protein PSA (also known as γ semen protein), prostatic acid phosphatase (PAP), etc [30]. Prostatic epithelial cell proliferation plays an important role in prostate-related diseases. The level of prostatic epithelial cell proliferation is increased in prostate-related diseases such as CNP [31]. Moreover, inhibiting the expression level of COX-2 protein can suppress the proliferation of prostate epithelial cells [15]. Our studies have also confirmed this point. Overexpression of COX-2 promoted the proliferation of human prostaglandin epithelial cell RWPE-1. On the contrary, inhibition of COX-2 led to a decrease in the expression of its synthetic product PGE2, simultaneously suppressed prostatic epithelial cell proliferation and reduced carrageenan-induced damage to the prostate tissue of CNP rats. These results show that suppression of prostate epithelial cell proliferation improves chronic non-bacterial prostatitis.

miRNAs can regulate gene expression through a complete or incomplete pairing of their seed sequences with the 3 'untranslated region (3’UTR) of their target genes, which can degrade the mRNA of target genes or inhibit the translation of target genes [32, 33]. For example, Ding et al. [34] found that inhibiting miR-146a up-regulated NOS1, which reduced the incidence of erectile dysfunction in patients with CP. In addition, Wang et al. [29] found that up-regulation of miR-141 regulated Keap/Nrf2 signaling pathway, and reduced the expression of COX-2, the value of eye score, locomotion score, average inflammatory cell count, improving prostatitis. In this study, we found that miR-181c targeted 3’UTR of COX2 to negatively regulate COX-2 mRNA and protein levels. Also, overexpression of miR-181c inhibited the proliferation of prostatic epithelial cells, reduced the locomotion score of CNP rats, and inflammatory damage of prostate tissue. These indicate that miR-181c/COX-2 plays a role in CNP.

In conclusion, this study determined that the expression of miR-181c was decreased in prostate tissues and prostatic epithelial cells, while the expression of COX-2 was increased. miR-181c inhibits the proliferation of human prostate epithelial cells by negatively regulating COX-2. *In vivo* experiments, overexpression of miR-181c reduced the damage of CNP. This study reveals the mechanism of prostatic epithelial cell proliferation in CNP, which will offer new ideas for the treatment of CNP.

**Conflict of interest**

The authors declare no conflict of interest.

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**Figure legends**

**Figure1**

**Figure 1** miR-181c was lowly expressed in prostate tissues of patients with CP. A. qRT-PCR was performed to detect the expression of miR-181c and COX-2. And the protein expression of COX-2 was examined by western blotting. B. Person correlation was applied to analyze the correlation between miR-181c and COX-2. \**P*<0.05 *vs.* the normal group.

Figure2

**Figure 2** Human prostate epithelial cells RWPE-1 were isolated and cultured *in vitro*. LPS (10μg/mL) was used to treat RWPE-1 cells. Cells were grouped as follows: control group and the LPS group. A. miR-181c expression was analyzed by qRT-PCR. B. qRT-PCR or western blotting was used to measure the expression of COX-2. \**P*<0.05 *vs.* the control group.

Figure3

**Figure 3** The targeting relationship between miR-181c and COX-2. RWPE-1 cells were transfected with COX-2 3’UTR-wild type (WT), COX-2 3’UTR-mutant type (Mut), miR-181c inhibitor, or miR-181c mimic, respectively. A. The luciferase activity of RWPE-1 cells was measured using luciferase reporter assay and qRT-PCR or western blotting was performed to detect the expression of miR-181c and COX-2 in RWPE-1 cells transfected with miR-181c inhibitor. B. The luciferase activity of RWPE-1 cells was measured using luciferase reporter assay and qRT-PCR or western blotting was performed to detect the expression of miR-181c and COX-2 in RWPE-1 cells transfected with miR-181c mimic. C. The binding site was predicted with microRNA.org. \**P*<0.05 *vs.* the NC group, #*P*<0.05 *vs.* the pre-NC group.

Figure4

**Figure 4** RWPE-1 cells were transfected with miR-181c mimic or pre-NC. Cells were grouped as follows: control, LPS, LPS+pre-NC, and LPS+miR-181c mimic group. A. The expression of miR-181c was measured using qRT-PCR. B. The mRNA and protein expression of COX-2 was detected by western blotting. C. ELISA analysis was used to detect the concentration of PGE2. \**P*<0.05 *vs.* the control group, #*P*<0.05 *vs.* the LPS+pre-NC group.

Figure5

**Figure 5** RWPE-1 cells were transfected with miR-181c mimic, pcDNA-COX-2, and its corresponding negative control (pre-NC and pcDNA). Cells were grouped as follows: control, LPS, LPS+pre-NC, LPS+miR-181c mimic, LPS+miR-181c mimic+pcDNA, LPS+miR-181c mimic+pcDNA-COX-2 group. Cell proliferation was measured by MTT assay. \**P*<0.05 *vs.* the control group, #*P*<0.05 *vs.* the LPS+pre-NC group, &*P*<0.05 *vs.* the LPS+miR-181c mimic +pcDNA group.

Figure6

**Figure 6** Rats were injected with lentivirus carrying miR-181c mimic or pre-NC. Animals were grouped as follows: Sham, CNP, CNP+pre-NC, CNP+miR-181c mimic group. A. Seven days after modeling, the locomotion score was evaluated. B. Seven days after modeling, rats were sacrificed and prostate tissues were taken. Prostate volume was measured. C. The average inflammatory cell count at high-power fields was detected in prostate tissues. D. Prostatic epithelial cell proliferation was measured in prostate tissues. The relative number of PCNA-positive cells (%) represented the level of epithelial cell proliferation. E. qRT-PCR or western blotting was performed to detect the expression of miR-181c and COX-2. \**P*<0.05 *vs.* the Sham group, #*P*<0.05 *vs.* the pre-NC group.