·Basic Research·

Endothelial nitric oxide synthase deficiency influences normal cell cycle progression and apoptosis in trabecular meshwork cells

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Abstract

• **AIM: To clarify how the endothelial nitric oxide synthase (eNOS, NOS3) make effect on outflow facility through the trabecular meshwork (TM).**

• **METHODS: Inhibition of NOS3 gene expression in human TM cells were conducted by three siRNAs. Then the mRNA and protein levels of NOS3 in siRNA-treated and negative control (NC) cells were determined, still were the collagen, type IV, alpha 1 (COL4A1) and fibronectin 1 by real-time PCR and Western blot analysis. In addition, NOS3 concentrations in culture supernatant fluids of TM cells were measured. Cell cycle and cell apoptosis analysis were performed using flow cytometry.**

• **RESULTS: The mRNA level of NOS3 was decreased by three different siRNA interference, similar results were obtained not only of the relative levels of NOS3 protein, but also the expression levels of COL4A1 and fibronectin 1. The number of cells in S phase was decreased, while contrary result was obtained in G2 phase. The number of apoptotic cells in siRNA-treated groups were significant increased compared to the NC samples.**

• **CONCLUSION: Abnormal NOS3 expression can make effects on the proteins levels of extracellular matrix component (*e.g.* fibronectin 1 and COL4A1). Reduced NOS3 restrains the TM cell cycle progression at the G2/M-phase transition and induced cell apoptosis.**

• **KEYWORDS:** endothelial nitric oxide synthase; cell cycle; cell apoptosis; trabecular meshwork
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INTRODUCTION

Ophthalmic diseases associated with various ocular disorders and multifactorial etiology, glaucoma in particular is one of the leading causes of visual field loss[1]. If uncontrolled or untreated, the entire vision will be lost eventually[2]. Glaucoma has became the second leading cause of blindness in the world, second only to cataracts [3]. In addition, visual field defects are related to driving performance[4], a higher risk of falling[5] and fractures[6].

Previous study suggest that the increased fluid pressure within the eyeball is a well-known risk factor for the development of glaucoma [7]. Previous studies suggest that trabecular meshwork (TM) could modify intraocular pressure (IOP) by allowing aqueous humour outflow through the drainage angle. Oxidative stress is involved in the pathogenesis of glaucoma *via* inducing human TM degeneration to lead to an IOP increase [8]. Despite the abnormal IOP of glaucoma and other ocular diseases, pathological mechanism and the optimal treatment of it is still under exploration.

Nitric oxide (NO) involved in the regulation of IOP and cell apoptosis to lead to retinal ganglion cell loss in glaucoma has certain important roles in the pathogenesis of glaucoma [9]. Three nitric oxide synthase (NOS) isoforms including neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3) and inducible NOS (iNOS, NOS2) are described previously [10]. Enhanced NO levels facilitate outflow of aqueous humor in the TM to contribute to the normalization of the IOP accompanied by an up-regulation of iNOS gene expression [11]. Moreover, retinal ganglion cell (RGC) apoptosis is associated with IOP-induced effects on extracellular matrix (ECM). In TM tissue, transforming growth factor (TGF)-β, fibronectin and collagen (*e.g.*

COL4A1), *etc*, are major stimulators of the production of ECM proteins. The effects of various ECM proteins of TM cells on glaucoma were investigated[12-13]. However, how NOS involved in the pathomechanism of glaucoma and others in combination with ECM proteins were unclear.

To identify the functional mechanism of NOS in TM cells, endothelial NOS3, which is the major part of NOS [14] was studied in our current study by RNAi-mediated gene silencing. In addition, correlations between NOS3 expression and COL4A1, as well as fibronectin 1 were evaluated. Moreover, cell cycle arrest and apoptosis in TM cells were observed and examined.

MATERIALS AND METHODS

Cell Culture Human TM cells purchased from Shanghai Laifei Biotech Co., Ltd. (Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM, supplemented with 10% FBS, 1% penicillin/streptomycin) in a CO_2 incubator (5% CO_2/95% O_2) at 37℃ [15]. Cells were dissociated and seeded onto 6-well (35-mm) plates, for the following day transfection.

siRNA Transfection Three different siRNAs targeting different sequences of NOS3 were designed: fw (forward), 5'-TCAGTGGCTGGTACATGAGC -3' and rev (reverse), 5'-TATCCAGGTCCATGCAGACA-3' for siRNA 1; fw, 5'-GAGACUUCCGAAUCUGGAAdTdT-3′ and rev, 5'-UUCCAGAUUCGGAAGUCUCdTdT-3′ for siRNA 2; fw, 5'-CGGUACUACUCAGUCAGCUdTdT-3' and rev, 5'-AGCUGACUGAGUAGUACCGdTdT-3' for siRNA 3. Negative control (NC) siRNA were synthesized: fw, 5'-UUCUCCGAACGUGUCACGUdTdT-3' and rev, 5'-ACGUGACACGUUCGGAGAAdTdT-3'. About 1.5×10^5 cell/well cells are subcultured in preparation for transfection. On the day of transfection, 10 μL siRNA and 10 μL Lipofectamine 2000 (Life Technologies, NY, USA) were incubated separately in 250 μL opti-MEM (Life Technologies) and mixed. The above mixture was added to each well containing the cells and incubated for 6h at 37℃ in incubator[16].

Real−time Polymerase Chain Reaction Total RNA of TM cells was extracted with Trizol and subjected to reverse transcription using TakaraEx Taq R-polymerase chain reaction (PCR) kit (Takara Bio Inc., Otsu, Japan) after 48h siRNA treatment. PCR primers used to assay gene expression by RT-PCR were: NOS3-fw, 5'-TCAGTGGCTGGTACATGAGC-3', and rev, 5'-TATCCAGGTCCATGCAGACA-3'; fibronectin 1-fw, 5'-GAGATGGACAGGAAAGAGATG-3', and rev, 5'-CGTTTGTAGGGGTTGTGGTAAT-3'; COL4A1-fw, 5'-GCCAGCAAGGTGTTACAGGATT-3', and rev, 5'-AGAAGGACACTGTGGGTCATCTATT-3'; GAPDH-fw, 5'-TGACTCTACCCACGGCAAGTT-3', and rev, 5'-TGATGGGTTTCCCGTTGATGA-3'.

Western Blot TM cells after 48h siRNA treatment were harvested and were lysed in RIPA buffer (Beyotime, Beijing, China), and then protein concentrations were determined by bicinchoninic acid assay (BCA protein kit, Sangon Company, China). For Western blots, proteins were separated on an 12% SDS-PAGE gel (each lane 40 μg) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Then the membrane was incubated with primary antibodies for NOS3 (1:800), fibronectin 1 (1:1000), COL4A1 (1:1000), and β-actin-HRP (1:1000) (Santa Cruz, CA, USA), separately, and a secondary antibody Goat anti-rabbit IgG (H+L)-HRP (1:5000)/Goat anti-mouse IgG (H+L)-HRP (1:5000) (Jackson Immunoresearch Labs, West Grove, PA). The proteins were visualized by enhanced chemiluminescence (ECL) in accordance with the manufacturer's instructions.

Measurement of NOS3 Concentration The NOS3 concentrations in culture supernatant fluids of TM cells were measured by an enzyme-linked immunosorbent assay (ELISA) kit (Uscn Life Science Inc., Wuhan, China) according to the manufacturer's instructions.

Cell Cycle and Cell Apoptosis Analysis Cells after 48h treatment with siRNAs were determined by propidium iodide (PI) staining for cell-cycle analysis using flow cytometry (FCM) (BD Company, USA). Cell were subsequently washed and double stained by FITC-annexin V-PI (BD Company, USA) for cell apoptosis analysis with FCM[17].

Statistical Analysis All statistical analyses were evaluated using a one-way ANOVA and the SPSS18.0 software package. Statistical difference was considered at $P<0.05$ and significant statistical difference was considered at $P<0.01$.

RESULTS

RNA Expression Analysis Quantitation of mRNA by real-time PCR approach was shown in Figure 1. mRNA level of NOS3 (Figure 1A) was decreased in three different siRNA construct-treated samples, especially that of siRNA2 and siRNA3. Moreover, the expression of fibronectin 1 (Figure 1B) and COL4A1 (Figure 1C) were decreased in NOS3-siRNA treated cells compared to the NC group.

Protein Expression Analysis NOS3 protein expression in three siRNA-treated groups were lower than in the NC group (Figure 2A), especially that of siRNA2 and siRNA3, which implied that siRNAs was effective at inhibiting the expression of NOS3. In addition, fibronectin 1 and COL4A1 expression levels were lower in the siRNAs-treated cells compared to those in the normal cells (Figure 2B).

Content of NOS3 in Culture Supernatant Comparison of the NOS3 concentration in the culture supernatant among three siRNA-treated groups compared to NC group were shown in Figure 3. There were significant decreases in NOS3 levels in all three siRNA-treated groups than those in the NC group.

Cell Cycle Distribution in Different Groups Cell cycle distribution of cells in four groups were displayed in Figure 4.

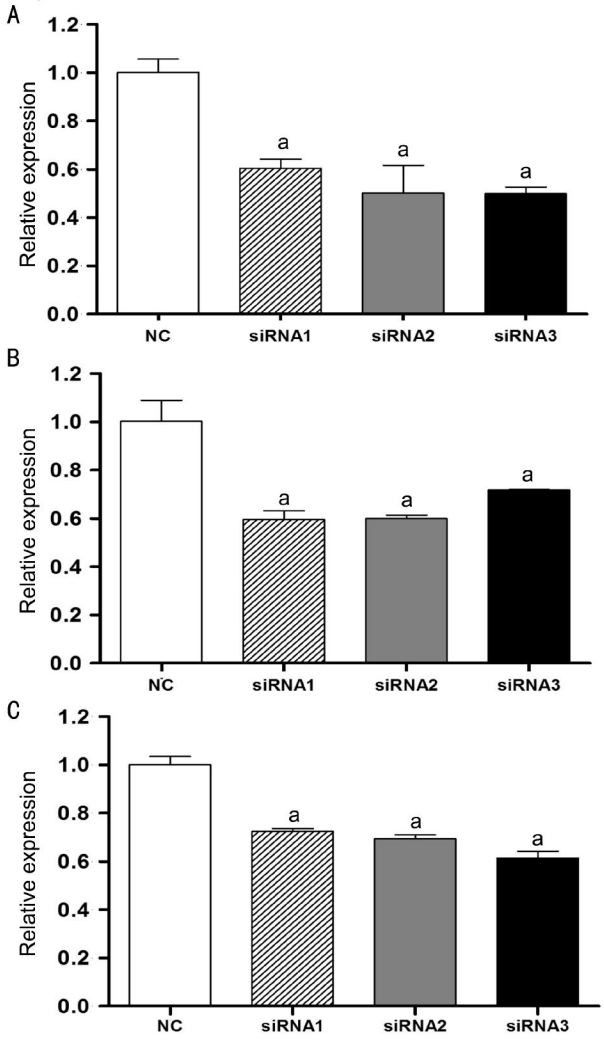

Figure 1 Quantitation of mRNA level of NOS3 (A), fibronectin 1 (B) and COL4A1 (C) by real-time PCR analysis NC: Negative control. [a] $P<0.05$.

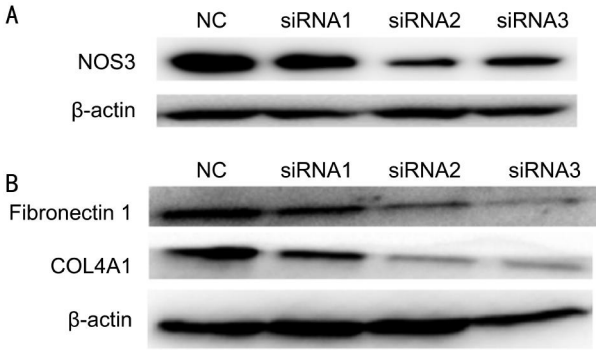

Figure 2 Protein expression level of NOS3 (A), fibronectin 1 (B) and COL4A1 (B) by Western blot analysis after normalization to the β-actin expression NC: Negative control.

There were no significant difference of cell population in the G1 phase of the cell cycle. Percentage of cells in S phase of siRNA-treated groups (Figure 4B, 4C and 4D) was decreased compared to the NC group (Figure 4A), which was 15.38%, 15.36% , 17.95% for siRNA 1, 2, 3-treated groups, respectively, and 25.75% for NC group. On the contrary, higher percentages of cells in G2 phase were found than the NC group, which was 28.37%, 28.45%, 24.65% and 16.54% for siRNA 1, 2, 3-treated and NC groups, respectively.

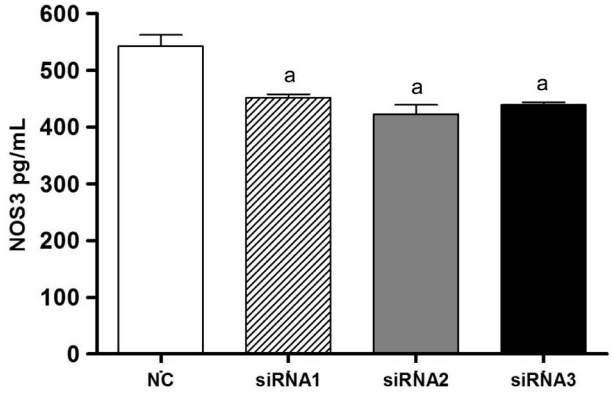

Figure 3 NOS3 concentration in the culture supernatant of siRNA-treated groups and negative control (NC) group NC: Negative control. [a] $P<0.05$.

Apoptosis in Cells As shown in Figure 5, there is little FITC-annexin V negative and PI-positive cells. The upper right quadrant represents the necrotic cells (FITC-annexin V+/PI+). The lower-right quadrant represents the early apoptotic cells which were PI-negative and FITC-annexin V-positive. According to these results, significant increase in the number of apoptotic cells in siRNA-treated groups (Figure 5B, 5C and 5D) were displayed, which were 8.81%, 6.97% and 11.58% for siRNA 1, 2, and 3 group, respectively, *vs*5.28% in the NC group (Figure 5A).

DISCUSSION

The TM which located in the anterior segment of the eye, forms most of the resistance to aqueous humor outflow and modulates IOP [18]. ECM and NO that produced by NOS are suggested involved in the IOP regulation and dysregulation[19]. Potential mechanism behind these effects was revealed in our study.

Lower expression and activity of eNOS in the TM of patients with glaucoma were posed by Fernandez-Durango *et al* [20]. In our current study, three NOS3-specific siRNAs could very efficiently inhibit the NOS3 expression at both the mRNA and protein levels. Moreover, low levels of both fibronectin 1 and COL4A1 were discovered consistent with that of NOS3. Previous study suggest that NO-synthesis induced by NOS2 is involved in enhancing fibronectin production by endothelial cells to regulate ECM protein production [21]. NOS3 associated with NO bioavailibility contributes to the regulation of mobilization and function of endothelial progenitor cells (EPCs) and plays key roles in vascular maintenance and repair [22]. Very little fibronectin was expressed and accumulated in eNOS deficiency mouse model [23]. Therefore, the impact that abnormal NOS3 expression on level of fibronectin 1 and COL4A1 may be accomplished by NO which regulates the synthesis of ECM[24]. RGC apoptosis in glaucoma is significantly linked to IOP-induced and specific ECM proteins changes in the RGC

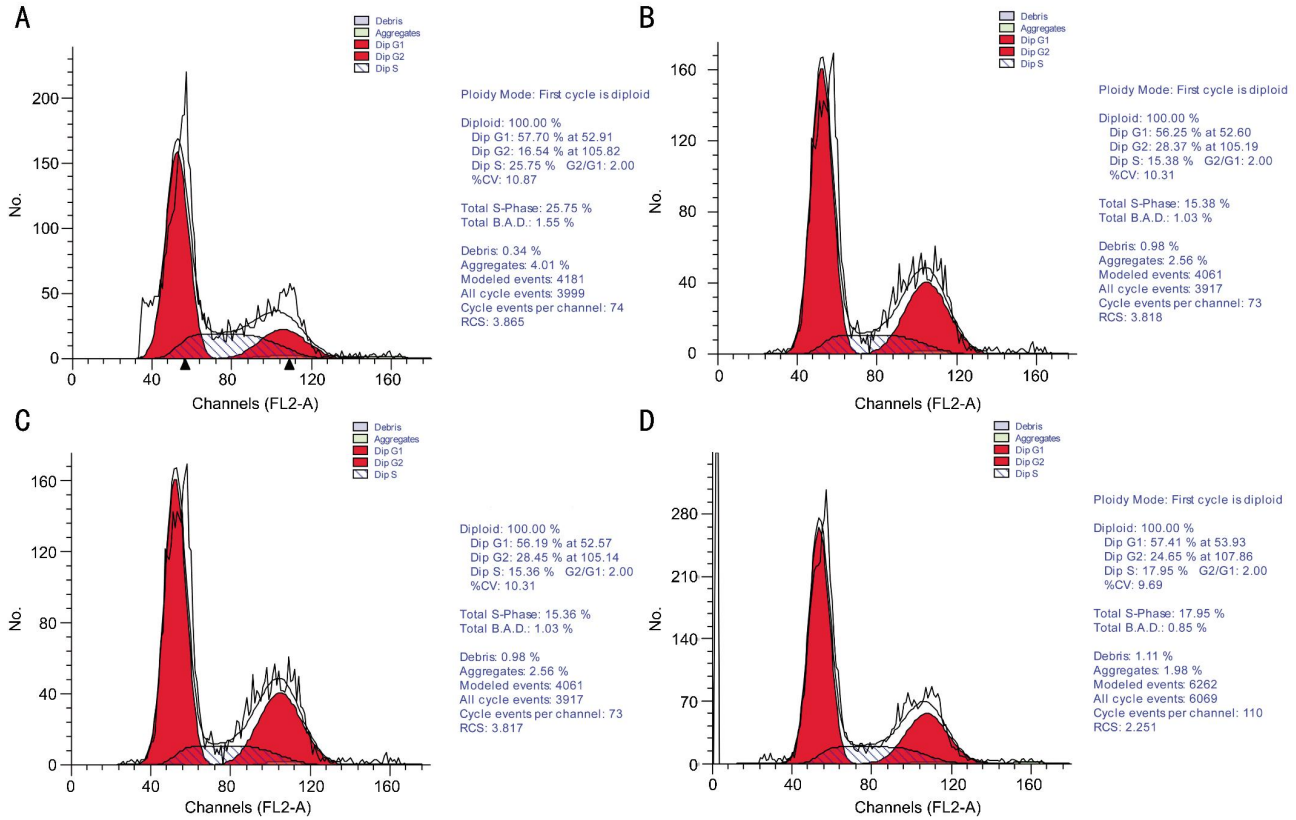

Figure 4 Cell cycle distribution of cells in negative control (NC) (A) and siRNA-treated groups (B, C and D).

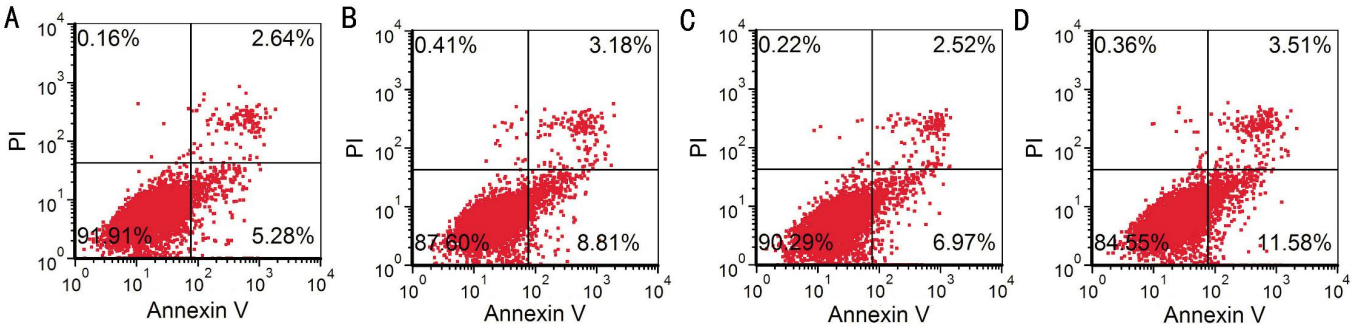

Figure 5 Trabecular meshwork cells in negative control (NC) (A) and siRNA-treated groups (B, C and D) double stained by Annexin V/propidium iodide (PI) and analyzed by flow cytometry.

layer, which is a primary site of injury in glaucoma [12]. In our study, cell cycle distribution and apoptosis of TM cells of different samples were detected and found. It is interesting that, the number of S-phase cells were decreased while the number of cells at the G2 phase were increased, this mined that reduced eNOS resulted in an initial accumulation of S-phase cells in G2 phase, this may be caused by the degradation of ECM components[25] or the abnormal NO level of TM cells[26-27]. Deduced eNOS would induced G2/M phase arrest in TM cell proliferation. In addition, TM cell apoptosis appeared to occur at the G2/M transition and eNOS may involve events that occur at the G2/M checkpoint at this stage of cell cycle[28].

As a consequence, abnormal NOS3 expression could effect the ECM component, especially, fibronectin 1 and COL4A1 concentration in TM cells. Cell cycle were arrested at G2/M phase and cell apoptosis of TM cells were increased. These may be how the endothelial isoform of NOS involved in mediating the outflow facility through the TM.

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Conflicts of Interest: Liao Q, None; **Huang YM,** None; **Fan W,** None; **Li C,** None; **Yang H,** None.

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