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Long non-coding RNA GAS5 contributed to the development of glaucoma via regulating the TGF-β signaling pathway

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Abstract. – OBJECTIVE: We aimed at discussing the biological function of lncRNA GAS5 (Growth Arrest Specific 5) on the proliferative, apoptotic and differentiative abilities of retinal ganglion cells.

MATERIALS AND METHODS: GAS5 expression was knocked down by transfection with siRNA targeting GAS5 (siGAS5) in retinal ganglion cells. After transfection, qRT-PCR was utilized to evaluate the mRNA level of GAS5 expression. The protein levels of smad2 and smad3 were detected by Western blot. Proliferative ability was accessed by Cell Counting Kit-8 (CCK-8) assay, and apoptosis and cell cycle changes were tested by flow cytometry. Also, the differentiative ability of RGC-5 cells was evaluated. TGF-β was exogenously administered to test its function on regulation of GAS5.

RESULTS: Knockdown of GAS5 promoted the cell proliferation and differentiation, but negatively regulated cell apoptosis, and had no effect on cell cycle. Exogenous administration of TGF-β could decrease the expression level of GAS5 in a dose-dependent manner. Meanwhile, lowly expressed GAS5 could improve the phosphorylation of smad2 and smad3.

CONCLUSIONS: In our study, we found that down-regulation of lncRNA GAS5 may maintain retinal ganglion cell survival in glaucoma through the activation of TGF-β pathway to promote cell proliferation and differentiation.

Key Words:
Long non-coding RNA, GAS5, Glaucoma, TGF-β signaling pathway.

Introduction

Glaucoma is a group of disease characterized by optic nerve damage and visual field defects; it is also the second most irreversible blinding ophthalmopathy in the world. Therefore, its effective prevention and control has increasingly become the focus of public health[1]. Glaucoma is a common blindness disease including a variety of types, such as primary open-angle glaucoma, primary angle-closure glaucoma and exfoliative glaucoma[2]. At present, the mechanism of optic nerve damage in glaucoma is not yet fully understood. However, studies indicating genetic factors are involved in the pathogenesis of most types of glaucoma, and results from these studies helped to unravel the underlying mechanism of glaucoma[3,4]. Long non-coding RNAs (lncRNA) are transcripts with over 200 nucleotides in length. They are not translated into proteins and play a direct role in the form of RNA. Their functions are influenced by the secondary structure, post-transcriptional modifications and other factors[5,6]. Previous studies[7,8] have shown that abnormally expressed lncRNAs are involved in multiple diseases. A study of glaucoma associated with lncRNA showed that CDKN2B-ASl, located on the short arm of chromosome 9, could encode lncRNA ANRIL. ANRIL is mainly located in the nucleus, which is a kind of antisense lncRNAs and functions in the multi-comb protein pathway. ANRIL can regulate the transcription and translation of CDKN2B and CDNK2A through epigenetic modifications. Meanwhile, the interferon can also regulate the expression levels of CDKN2B and CDNK2B-ASl[9]. The decreased expression of CDNK2A/2B results in a decreased cyclin-dependent kinase activity in glaucoma; therefore, retinal ganglion cells (RGCs) are more susceptible to apoptosis. This theory has also been identified in the mouse model of glaucoma[10,11]. LncRNA GAS5 (Growth Arrest Specific 5) and glucocorticoid response components bind with glucocorticoid receptor competitively, thus inhibiting the expression levels of apoptosis-related genes. The abnormal expression of GAS5 is shown to be associated with numerous types of

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tumors[12,13]. However, it has not yet been explained whether GAS5 is involved in glaucoma. Here, we explore the biological functions and molecular mechanisms of GAS5 in glaucoma.

Materials and Methods

Cell Culture

The cells were obtained from ATCC (Manassas, VA, USA) and maintained in the low-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, Rockville, MD, USA), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C in an atmosphere containing 5% CO_2.

Cell Differentiation

RGC-5 cells transfected with GAS5 siRNA (si-GAS5 group) and negative control (si-control group) were seeded in 6-well plates. Supernatants of HNPE cells incubated in serum-free DMEM medium for 24 h were used to culture RGC-5 cells for 4 days after addition of 0.2% fetal bovine serum (FBS). The differentiation ability was observed. The lengths of axons in 10-12 fields of vision were randomly measured with ImageJ software.

Cell Transfection

After the cells were digested and counted, they were seeded in a 6-well plate to approximately 50% confluency for overnight culture. According to the instructions of Lipofectamine 2000, transfection reagent and siRNA working fluid were prepared with serum-free DMEM medium, and the GAS5 siRNA (si-GAS5 group) and the negative control group (si-control group) were transferred to the cells, respectively. After 6 h, the transfection solution was replaced with DMEM containing 10% FBS for subsequent experiments.

QRT-PCR

The cells were collected after 48 h of transfection; the total RNA of cells was obtained by TRIzol reagent, then reverse transcribed into cDNA as a template. The reaction conditions were as follows: denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s. Each experiment was independently repeated for 3 times. The primer sequences were as follows: Lnc-GAS5 Forward: 5'-TGTGGATTTACCTTATCCCCTCA-3' Reverse: 5'-GTTTGGCTTTGGTCGTTCTGT-3'; GAPDH Forward: 5'-GGAGCGAGATCCCTCCAAAAT-3'; Reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'; Thy1.2 Forward: 5'-ACTCTTGGCACCATGAACCC-3'; Reverse: 5'-GCTGGTCACCTTCTGCCCTC-3'; Tau Forward: 5'-GAAGACGTGACTGCGCCCCTA-3'; Reverse: 5'-CCTGGTTCGGGGTGTCTCCGA-3'; PGP9.5 Forward: 5'-CTTCGCCGACGTGCTAGGG-3'; Reverse: 5'-TTTTCATGCTGGGCCGTGAGGG-3'.

Western Blot

Transfected cells for 48 h were collected and lysed with radioimmunoprecipitation assay (RIPA) lysate. The concentration of total protein was accessed by bicinchoninic acid (BCA) protein kit. The target protein was separated by Bio-Rad (Hercules, CA, USA) vertical electrophoresis system after loading 50 μg of protein into each well. The primary antibodies (1:1000 dilution) were added to specifically bind to target protein. HRP-conjugated antibodies (1:5000 dilution) were used as the secondary antibodies to bind to the primary antibodies. The relative protein expression was detected using the enhanced-chemiluminescence (ECL) method.

Apoptosis Assay

The cells were harvested and washed twice with phosphate-buffered saline (PBS) solution 48 h post-transfection. Binding buffer (1×) was used to re-suspend the cells, the cell density was normalized to 2×10^5/mL according to the instructions of the kit. 195 μL of cell suspension and 5 μL of Annexin V-FITC (fluorescein isothiocyanate) were given. A total of 200 μL of the mixed solution was used after 15 min of incubation in dark. The cells were washed with Binding buffer (1×) and centrifuged at 1000 r/min for 3 min. 10 μL of Propidium Iodide (PI) were added again when the cells were re-suspended with 190 μL of binding buffer (1×). Apoptosis level was analyzed by a flow cytometry after 15-min incubation in the dark.

Cell Counting Kit-8 (CCK-8) Assay

After digesting the cells in logarithmic phase, the cell suspension was mixed into a complete medium and seeded into 96-well plates (1×10^4/well), with 5 replicate wells in each group. Cells were maintained at 37°C, 5% CO_2 incubator and were observed continuously. 10 μL of CCK-8 solution was added into each well, and incubat-

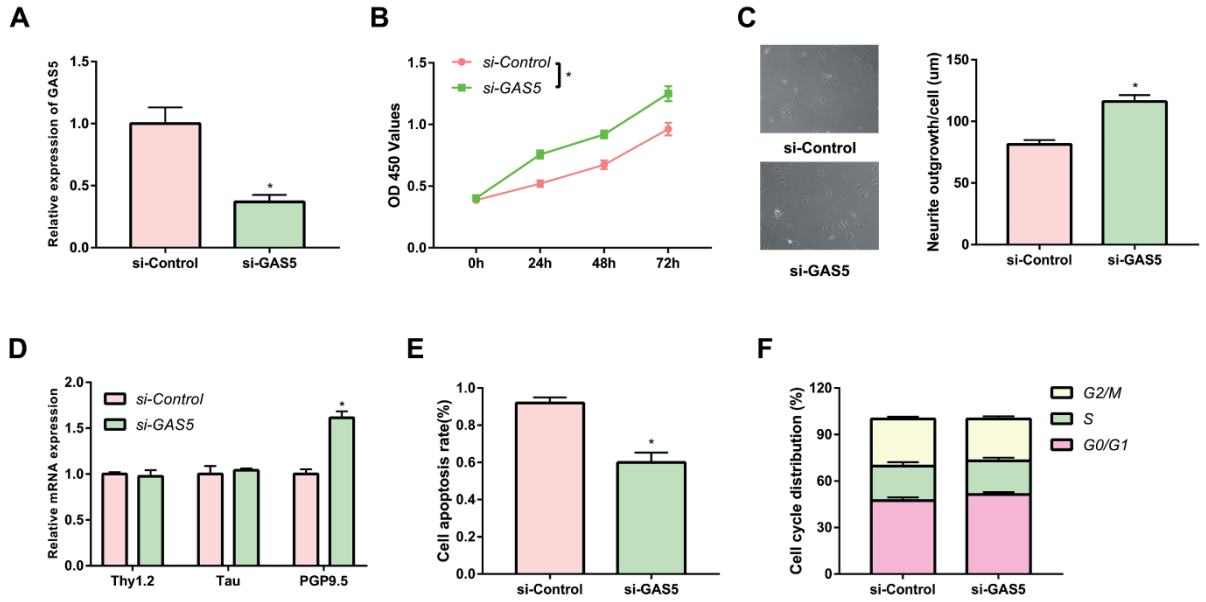

Figure 1. GAS5 regulated cell proliferation, differentiation and apoptosis. ***A,*** GAS5 expression was detected by qRT-PCR 48 h after transfection with GAS5 siRNA in RGC-5 cells. ***B,*** CCK-8 assay was performed to detect the proliferation after GAS5 siRNA transfected into RGC-5 cells and control cells for 0, 24, 48, 72 h. ***C,*** RGC-5 cells were transfected with GAS5 siRNA and control, axon length and cell differentiation were accessed. ***D,*** RGC-5 cells were transfected with GAS5 siRNA and control, expressions of genes relative to the differentiation were detected. E. After 48 h transfection of GAS5 siRNA into RGC-5 cells, the apoptosis was detected by flow cytometry. F. After transfecting RGC-5 cells with GAS5 siRNA and control for 48 h, the cell cycle was assayed by flow cytometry. *p<0.05.

ed for 4 h. The optical density was recorded at the wavelength of 450 nm using a microtiter plate reader. The experiment was repeated three times.

Cell Cycle Analysis

48 h post transfection, the cells were digested, centrifuged, suspended in precooling phosphate-buffered saline (PBS). Transfected cells were rinsed twice. After 3 mL of precooled 75% ethanol were added, cells were collected and stored at 4°C overnight. Cells were stained with 400 μL of PI solution and 100 μL of RNase A (100 mg/L) for 30 min at 4°C. The cells filtered by 300 nylon mesh were detected by the FACScalibur flow cytometry (BD, Franklin Lakes, NJ, USA) at 488 nm.

TGF-β treatment

The effect of TGF-β concentration on GAS5 expression was observed by treating the cells with 0, 5, 10, 20 ng/mL TGF-β for 72 h, respectively. By 20 ng/mL TGF-β treatment for 0, 24, 48, and 72 h, the effect of different treatment times on GAS5 expression was evaluated. The effect of TGF-β on cell proliferation was evaluated by treating cells with 20 ng/mL TGF-β for 24 h.

Statistical Analysis

We used statistical product and service solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) for statistical analysis. Data were presented as mean ± standard deviation and were analyzed using *t-t*est to compare the differences among groups. The difference was statistically significant when p<0.05.

Results

GAS5 Regulated Cell Proliferation and Apoptosis

The expression of GAS5 in RGC-5 was remarkably decreased after siRNA transfection (Figure 1A). The cell proliferation was enhanced in the lowly expressed GAS5 group in comparison with control group. The proliferation ability was constantly improved with extended time and can last for at least 72 h (Figure 1B). Lowly expressed GAS5 can significantly promote the differentiation of RGC-5, and axon length in this group was significantly higher than the control group (Figure 1C). Further, we examined the expression of genes related to RGC-5. Only the expression level

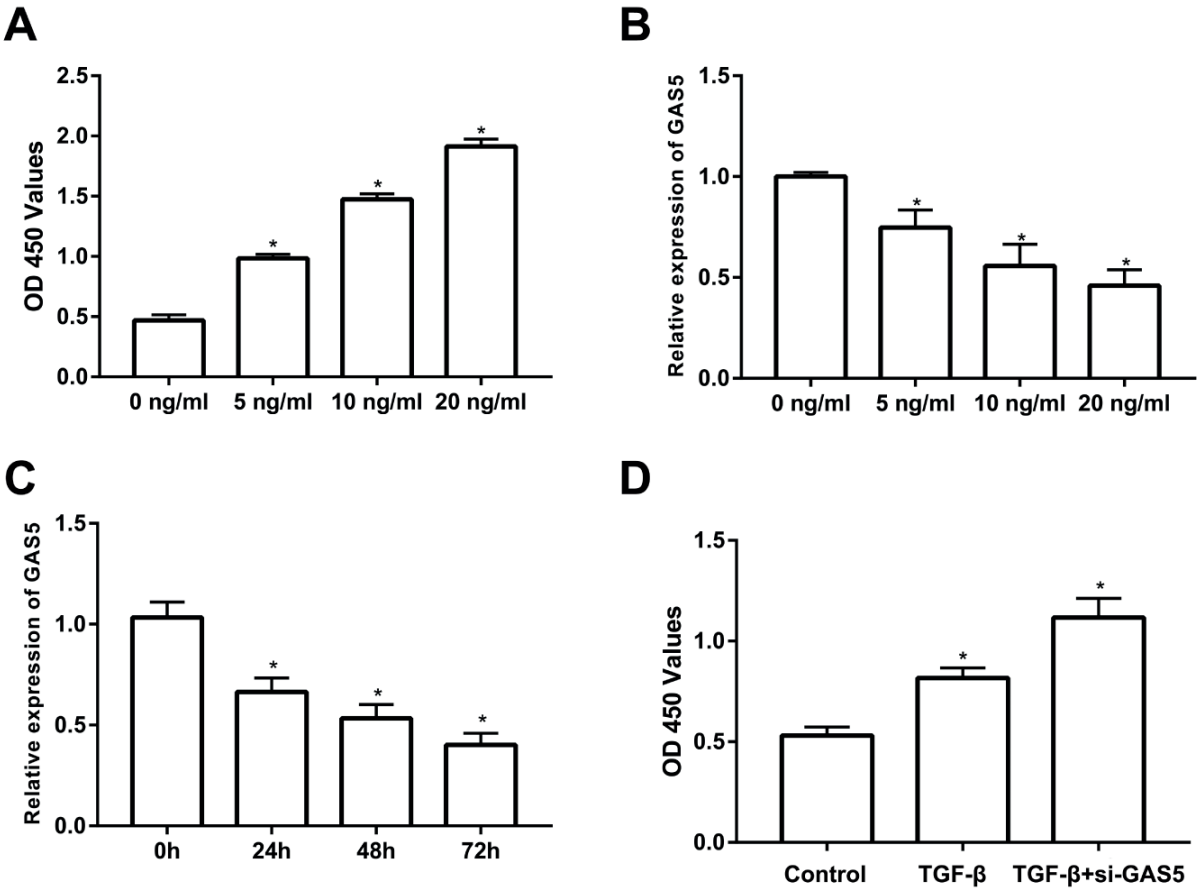

Figure 2. TGF-β regulated GAS5 expression. ***A,*** After 72 h incubation of RGC-5 cells with different concentrations of TGF-β (0, 5, 10 and 20 ng/mL), CCK-8 was used to detect cell proliferation. B. GAS5 expression was detected by qRT-PCR 72 h after RGC-5 cells were incubated with different concentrations of TGF-β (0, 5, 10 and 20 ng/mL). C. RGC-5 cells were incubated with 20 ng/mL of TGF-β at different times (0, 24, 48 and 72 h), and the expression of GAS5 was detected by qRT-PCR. D. CCK-8 was used for accessing cell proliferation 24 h after administration of 20 ng/mL TGF-β exogenously or 20 ng/mL TGF-β and GAS5 siRNA simultaneously. *$p<0.05$.

of PGP9.5 was found to be significantly increased in the GAS5 low expression group, while there was no significant difference in the mRNA expressions of Thy 1.2 and Tau (Figure 1D). Lowly expressed GAS5 can significantly reduce the cell apoptosis (Figure 1E). However, we found that the expression of GAS5 does not affect the cell cycle (Figure 1F). These results suggested that the abnormal expression of GAS5 may participate in the occurrence of glaucoma by regulating cell proliferation, differentiation and apoptosis.

TGF-β Regulated the Expression of GAS5

Studies have shown that TGF-β signal is essential for retinal ganglion cells[14,15]. Therefore, we decided to investigate whether the expression of GAS5 is regulated by TGF-β. In our study, different concentrations of TGF-β were treated exogenously. We found that the proliferative ability of RGC-5 cells was increased as the concentration of TGF-β elevated (Figure 2A). Meanwhile, the expression of GAS5 was decreased with the increased concentration of TGF-β (Figure 2B). When TGF-β was exogenously administrated, the expression of GAS5 was decreased with the extension of incubation time (Figure 2C). Further, we found that TGF-β can promote cell proliferation, and the lowly expressed GAS5 can promote the process of proliferation (Figure 2D). The above results indicated that the expression of GAS5 is regulated by TGF-β, which is presented as the time- and dose-dependent manners. Moreover, GAS5 and TGF-β have a synergistic effect on the regulation of cell proliferation.

GAS5 Regulated Phosphorylation of Smad2 and Smad3

Furthermore, we explored whether GAS5 may participate in the TGF-β signaling pathway. We found no significant change in total smad2/3 expression after downregulation of GAS5, whereas the ratio of phosphorylated smad2/3 to the total smad2/3 increased significantly. The treatment of SB431542, the inhibitor of TGF-β signaling pathway, reversed the ratio of p-smad2/3 to the normal level in the control group (Figure 3A-B) demonstrating that low expression of GAS5 activates the TGF-β signaling pathway.

Discussion

Glaucoma, caused by intraocular pressure, is an ophthalmic disease characterized by optic atrophy and visual field defect. Glaucoma has a complex pathogenesis, and different classifications of glaucoma vary a lot. Symptoms are associated with the long-term intraocular pressure and damage, as well as apoptosis of retinal ganglion cells (RGCs) caused by various pathological factors[1,16]. Glaucoma is mainly treated with drugs, lasers and surgery to reduce further damage to the optic nerve[17]. Glaucoma filtering is a common operation for glaucoma, which is mainly applied for glaucoma that can't be controlled by drugs and lasers. Fibroblastic proliferation after surgery, however, may lead to surgery failure[18]. TGF-β is a member of a growth factor superfamily with five subtypes[19]. The major biological function of TGF-β is to regulate proliferative ability, apoptosis and differentiation. TGF-β is also capable of regulating embryonic development, thus promoting the formation of extracellular matrix and resisting the immune response[20]. Other studies have shown that TGF-β is a chemical stimuli of monocytes and fibroblasts, which can promote the migration and proliferation of fibroblasts to wounds and induce the production of various cytokines, such as bFGF, IL-1, PDGF, etc. In other cases, TGF-β reduces the degradation of extracellular matrix and promotes the extracellular matrix, such as I, III, procollagen type IV, fibronectin and other large amount of synthesis through the inhibition of collagenase activity. It also regulates the extracellular matrix adhesion of protein receptor affinity, increasing matrix adsorption in cells. Therefore, TGF-β functions a lot in the reconstruction and remodeling of post-traumatic tissues[21,22]. TGF-β2 is widely expressed in various eye cells and participates in wound healing and repair. Therefore, more studies have been working on the role of TGF-β2 in anti-scarring after filtering surgery of glaucoma. For example, the usages of TGF-β2 antibody CAT-152, TGF-β2 antagonist Decorin, and TGF-β2 antisense oligonucleotide method in inhibition of scarring have achieved satisfactory results[23-25]. In this study, it is demonstrated that TGF-β has the ability to modulate the expression level of GAS5 in the time- and dose-dependent manners, indicating that TGF-β could regulate the pathogenesis of glaucoma via regulation of GAS5. RGC-5 cell line is ideal for *in vitro* experimental model of glaucoma. Although it has been proved that undifferentiated RGC-5 cells are different from normal RGCs in electrophysiology, changes of the cell membrane surface and intracellular molecules and apoptosis related genes are consistent with the normal RGCs, which is

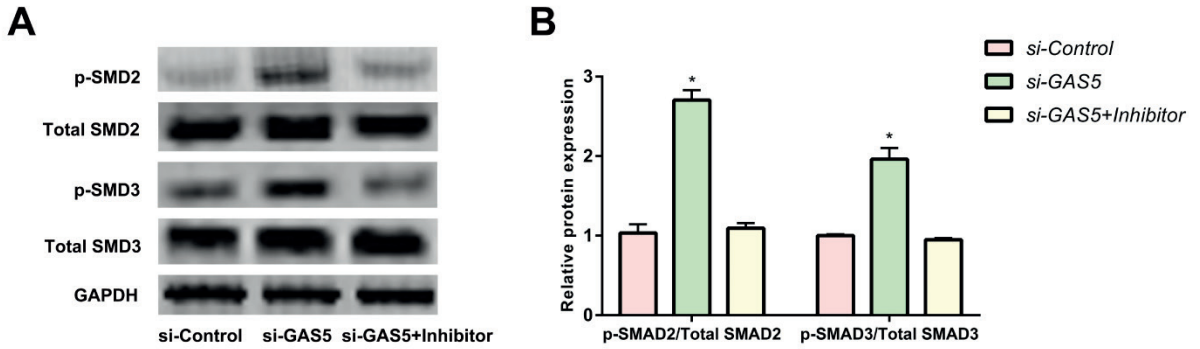

Figure 3. GAS5 regulated the phosphorylation of smad2 and smad3. After transfected with GAS5 siRNA, GAS5 siRNA+ Inhibitor and control for 48 h, the expressions of phosphorylated smad2 and smad3 as well as total smad2 and smad3 were detected by Western blot. *$p<0.05$.

the same as its response to the neuroprotective drugs. Therefore, it is more reliable to use RGC-5 cell line as a model to study ophthalmological diseases, with the advantages of continuous culture and longer survival[26-28]. Using this cell line, we found that GAS5 may participate in the pathogenesis of glaucoma by regulating proliferative and apoptotic ability.

Conclusions

Lowly expressed GAS5 can promote the proliferation of retinal ganglion cells and inhibits its apoptosis in glaucoma by regulating the TGF-β pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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