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MTIM affects retinal ganglion cells through PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: Retinal ganglion cells (RGCs) are the main cells that form vision in the retina. MT1M is involved in the occurrence and progression of various diseases. However, the role of MTIM in RGCs cells remains unclear.

MATERIALS AND METHODS: RGCs were cultured in vitro and randomly divided into control group, MT1M group (transfected with MT1M-pcDNA3.1 plasmid), and MT1M siRNA group (transfected with MT1M siRNA) followed by measuring MT1M and NT-3 expression by real time PCR and Western blot, cell proliferation by MTT assay, secretion of IL-2 and IL-6 by enzyme-linked immunosorbent assay (ELISA), SOD activity and ROS content. In addition, expression of PI3K/AKT signaling pathway protein was detected by Western blot.

RESULTS: MT1M expression in MT1M group was significantly increased, which promoted cell proliferation, increased NT-3 expression, and decreased Caspase 3 activity and IL-2 and IL-6 secretion. Meanwhile, SOD activity was increased, ROS content was decreased and PI3K/AKT protein phosphorylation was elevated. The differences were statistically significant compared with control group ($p < 0.05$). MT1M siRNA decreased MT1M expression, inhibited cell proliferation, decreased NT-3, and increased Caspase 3 activity and IL-2 and IL-6 secretion. In addition, MT1M siRNA decreased SOD activity, increased ROS content and reduced PI3K/AKT protein phosphorylation. Compared with control group, the differences were statistically significant ($p < 0.05$).

CONCLUSIONS: Up-regulation of MT1M can inhibit RGC cell apoptosis and inflammation, and promote RGC cell proliferation through the PI3K/AKT signaling pathway.

Key Words:
MT1M, Retinal ganglion cells, PI3K/AKT signaling pathway, Proliferation, Apoptosis.

Introduction

Retinal ganglion cells (RGCs) are the main cells that form vision in the retina and play an important role in various ophthalmic diseases[1,2]. Studies on optical nerve axes confirmed that RGCs mainly transmit visual stimulation of the brain. In 50% of human RGCs, at the synaptic end of the lateral geniculate nucleus (LGN), more than 90% of the rodent RGCs intersect within the optic chiasm[3]. When the optic nerve is damaged, leading to structural and functional damage to RGCs, it can cause a series of diseases[4]. Under ischemic or hypoxic conditions of the retina (such as retinal vascular occlusion, ischemic optic neuropathy, diabetic retinopathy), RGCs undergo neurodegeneration[5]. As the earliest neural cells of retinal differentiation, retinal ganglion cells (RGCs) are the main cells that form vision in the retina. Therefore, the death of RGCs is the main cause of irreversible damage of visual function during the occurrence and development of ophthalmic diseases[6,7]. Retinal neuronal damage in retinopathy occurs earlier than microvascular disease. In patients with ophthalmic diseases without retinal microangiopathy, visual function declines with electroretinogram abnormalities, reduced dark adaptation ability, and visual field damage[8,9]. It is well known that in the case of neurodegeneration, damaged RGCs have corresponding resistance actions, including regulation of neurotrophic factors, inflammatory factors, as well as apoptosis and anti-apoptotic genes[10-12]. RGCs apoptosis, inflammation and growth inhibition lead to neurodegeneration, chronic microglial activation and astrocytosis[13]. As the global elderly population continues to increase, the impact of vision damage and blindness caused by RGCs on eye diseases will continue to increase in the future[14].

MT1M is one of the important members of the metallothionein (MT) family. It is a conserved protein structure, but its abnormal expression can lead to impaired physiological functions and in-

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volves in the development and progression of various diseases. Its abnormal expression is related to tumor and autoimmunity disease[15,16]. However, the role of MTIM in retinal ganglion cells has not been elucidated.

Materials and Methods

Reagents and Instruments

RGC-5 cells were kept in our laboratory and stored in liquid nitrogen. Dulbecco's Modified Eagle's Medium (DMEM) culture solution and cyan chain double antibody were purchased from Hyclone Corporation (San Angelo, TX, USA). B27, CNTF, BDNF, and glutamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), Thiazolyl Blue Tetrazolium Bromide (MTT) powder was purchased from Gibco (Grand Island, NY, USA); trypsin-ethylenediaminetetraacetic acid (EDTA) digest was purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from Pall Life Sciences (Port Washington, NY, USA); EDTA was purchased from Hyclone; Western blot related chemical reagent was purchased from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China); enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK); rabbit anti-human MT1M monoclonal antibody, PI3K/AKT single Anti- and phosphorylated mAb; goat anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). IL-2 and IL-6 ELISA kits were purchased from R&D (Minneapolis, MN, USA). The pcDNA-MT1M plasmid and MT1M siRNA were designed and synthesized by Shanghai Gemma Gene Co., Ltd (Shanghai, China). The RNA extraction kit and the reverse transcription kit were purchased from Axygen (Union City, CA, USA). The reactive oxygen species (ROS) content detection kit and the superoxide dismutase (SOD) activity detection kit were purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd (Shanghai, China). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation (Hercules, CA, USA).

RGC-5 Retinal Ganglion Cell Culture and Grouping

RGC-5 retinal ganglion cells were seeded in culture dishes at 1×10^6 cells/cm^2 in DMEM medium containing fetal bovine serum (containing 100 U/ml penicillin, 100 ug/ml Streptomycin, 50 μg/L CNTF, B27, 40 μg/L CNTF, 25 mmol/L glucose), and cultured in a 37°C, 5% CO_2 incubator. The liquid was changed every other day and passed every 2-3 days. Cultured RGC-5 retinal ganglion cells were randomly divided into three groups, normal control group (cells were cultured under normal conditions); MT1M group and MT1M siRNA group, which was transfected with MT1M-pcDNA3.1 plasmid and MT1M siRNA in RGC-5 cells, respectively.

MT1M-pcDNA3.1 Plasmid and MT1M SiRNA Transfection into RGC-5 Cells

The MT1M-pcDNA3.1 plasmid and MT1M siRNA were transfected into RGC-5 cells. The MT1M siRNA sequence was: the upstream sequence 5'-GCTGAGGATGGACTTCA-3'; the downstream sequence 5'-GCATGGTGACGTCA-3. The cell density was fused to 70-80% in a 6-well plate; the SMT1M-pcDNA3.1 plasmid and the MT1M siRNA liposome were separately added to 200 μl of serum-free medium, mixed well, and incubated at room temperature for 15 min. The mixed lipo2000 was separately mixed with the corresponding dilution and incubated for 30 min at room temperature. The serum of the cells was removed, phosphate-buffered saline (PBS) was gently rinsed, 1.6 ml of serum-free medium was added, and each system was added to each system, cultured in a 5% CO_2 incubator at 37°C for 48 h for experimental research.

MTT Assay Analysis of Growth of RGC-5 Cells

After 48 hours of cell culture, cells were digested, counted, and seeded in 96-well plates at 3000 cells/well. Five replicate wells were designed for each group, and 20 μl of 5 g/L MTT solution was added. After 4 hours of incubation, the supernatant was completely removed, 150 μl/well of DMSO was added, and shaken for 10 min. After the purple crystals were fully dissolved, the absorbance (A) value was measured at a wavelength of 570 nm by a microplate reader to calculate the cell proliferation rate.

Analysis of SOD Activity

Superoxide dismutase (SOD) activity was examined according to the kit instructions. The cell

protein was extracted and washed in a 95°C water bath. After 40 min, it was taken out and rinsed with cold water. After cooling, it was centrifuged at 4000 rpm for 10 min. The ethanol phase in the tissue homogenate was extracted using an ethanol-chloroform mixture (5:3, v/v volume ratio 5:3) followed by detection of lactate dehydrogenase (LDH) and total SOD activity.

Detection of ROS Content

Changes in the levels of reactive oxygen species in each group of cells were examined. The treated cells were bathed in a 95°C water bath, and after 40 min, they were taken out and rinsed with cold water, and after cooling, centrifuged at 4000 rpm for 10 min. The tissue homogenate was incubated with 2', 7'-dichlorofluorescein diacetate (DCF-DA) for 15 min at 37°C, centrifuged at 10,000 rpm for 15 min, and the supernatant was discarded. Pellets were resuspended in sterile PBS phosphate buffer and incubated for 60 min at 37°C followed by measuring the level of ROS by a spectrophotometer.

ELISA Analysis of IL-2 and IL-6 Secretion

The supernatants of each group were collected to detect changes in the secretion of inflammatory factors IL-2 and IL-6. The experimental procedure was followed according to the ELISA kit instructions. The main operation steps included: taking out a 96-well plate and adding 50 ul of the diluted standard in the corresponding reaction well to prepare a standard curve. Add 50 ul of the sample to be tested to the reaction well. Make three duplicate holes for each sample. Wash the plate 5 times, discard the liquid, dry it, fill each well with the diluted washing solution, shake for 30 s, remove the washing solution, and pat dry with absorbent paper. Repeat this 5 times and shoot dry. Add 50 µl of the enzyme labeling reagent to each well, except for blank wells. Mix gently by shaking and incubate for 30 min at 37°C. Wash the plate 5 times. Add 50 µl of the developer A to each well, then add 50 µl of the developer B, gently shake and mix, and develop at 37°C for 10 min in the dark. The enzyme plate was taken out, and 50 µl of the stop solution was added to each well to terminate the reaction (in this case, the blue color turned yellow). The blank value was zeroed, and the absorbance value (OD value) of each well was measured by a microplate reader at a wavelength of 450 nm. The measurement should be carried out within 15 minutes after the addition of the stop solution. The linear regression equation of the standard curve was calculated according to the concentration of the standard product and the corresponding OD value, and the corresponding sample concentration was calculated on the regression equation according to the OD value of the sample.

Real-Time PCR Detection of MT1M and NT-3 MRNA Expression

Under sterile conditions, RGC-5 cells were washed with PBS and total RNA was extracted using TRIzol reagent. cDNA was synthesized by PCR according to the relevant primers (Table I). Real-time PCR was used to detect the expression of the target gene with reaction conditions as follows: 52°C 1 min, 90°C 30 s, 58°C 50 s, 72°C 35 s, a total of 35 cycles. Fluorescence quantitative PCR reactor software was used to collect relevant data. According to the internal reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the standard cycle number (CT) of the standard was calculated, and the standard curve was drawn. The quantitative analysis was analyzed by $2^{-\Delta}Ct$ method.

Western Blot Analysis of MT1M, PI3K/AKT Signaling Protein Expression

Extract RGC-5 cell protein: add lysate, lyse the cells on ice for 15-30 min, disrupt the cells by sonication for 5 s × 4 times, centrifuge at 4°C, 10 000 × g for 15 min, transfer the supernatant to a new EP tube. The protein was quantified and stored at -20°C for Western blot experiments. The isolated protein was electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to

Table I. Primer sequences.

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
MT1M	CTCATCTAAGCGGAACAATGG	GCACATTCTCTCCGTAGCG
NT-3	CTCGTCAACAATACTCTCC	CACACTCTAAGCGGAAAC

a polyvinylidene difluoride (PVDF) membrane by semi-dry transfer, blocked with 5% skim milk powder to remove the non-specific background and incubated with primary antibody (1:2000, 1:1000; 1:1000; 1:1500, 1:2000 dilutions of primary antibody MT1M, p-PI3K, PI3K monoclonal antibody, p-AKT monoclonal antibody, AKT monoclonal antibody) at 4°C overnight. After phosphate-buffered saline with tween-20 (PBST) washing, 1:2000 goat anti-rabbit secondary antibody was added and incubated for 30 min followed by washing with PBST, X-ray exposure imaging after addition of enhanced chemiluminescence (ECL) reagent for 1 min. X-film and strip density measurements were separately scanned using protein image processing system software and Quantity one software. The experiment was repeated four times (n=4).

Caspase3 Activity Detection

The changes in Caspase3 activity in each group of cells were examined according to the kit instructions. Trypsin digested cells were centrifuged at 600 g at 4°C for 5 min and cell lysate was added, lysed on ice for 15 min followed by centrifugation at 20000 g at 4°C for 5 min. After that, 2 mM Ac-DEVD-pNA was added and OD value was measured at 405 nm to calculate Caspase3 activity.

Statistical Analysis

Data were processed by SPSS 16.0 (Chicago, IL, USA) software. Measurement data were expressed as mean ± standard deviation (SD). Comparison of multiple groups of samples was performed using one-way ANOVA with Bonferroni post-hoc test to validate. $p < 0.05$ indicated a statistically significant difference.

Results

Expression of MT1M In RGC-5 Cells

The expression of MT1M mRNA and protein in RGC-5 cells was detected by Real time PCR and Western blot. The results showed that compared with the control group, transfection of pc DNA3.1 MT1M plasmid in RGC-5 cells promoted the significant increase of MT1M mRNA and protein expression in RGC-5 ($p < 0.05$). The expression of MT1M mRNA was down-regulated after transfection of MT1M siRNA in RGC-5 cells. Compared with control group, the difference was statistically significant ($p < 0.05$) and the expression of MT1M protein was also decreased (Figure 1).

Effect of MT1M on Proliferation of RGC-5 Cells

Transfection of pc DNA3.1 MT1M plasmid promoted proliferation of RGC-5 cells. Compared with the control group, the difference was statistically significant ($p < 0.05$). Transfection of MT1M siRNA to RGC-5 inhibited the proliferation of RGC-5 cells, and the difference was statistically significant ($p < 0.05$; Figure 2).

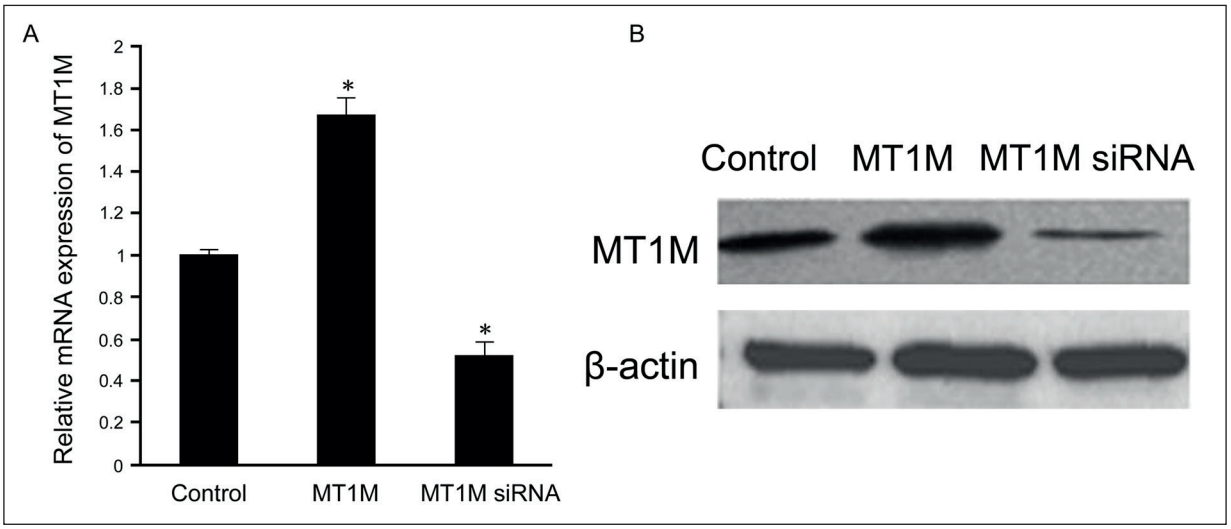

Figure 1. Expression of MT1M in RGC-5 cells. **A**, Real time PCR detection of MT1M mRNA expression changes, compared with the control group, *$p < 0.05$; **B**, Western blot detection of MT1M protein expression.

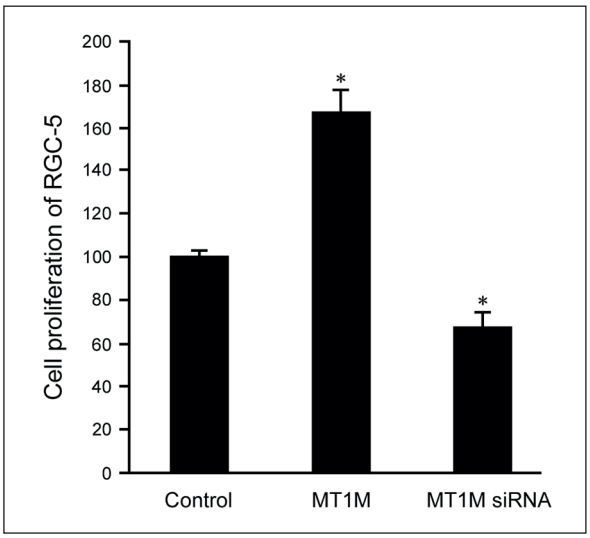

Figure 2. Regulation of MT1M on proliferation of RGC-5 cells. Compared with the control group, * $p < 0.05$.

Effect of MT1M on the Activity of Caspase3 In RGC-5 Cells

Transfection of PC DNA3.1 MT1M plasmid inhibited Caspase3 activity in RGC-5 cells, and the difference was statistically significant ($p < 0.05$). Transfection of MT1M siRNA into RGC-5 cells promoted the activity of Caspase3 in RGC-5 cells. Compared with the control group, the difference was statistically significant ($p < 0.05$; Figure 3).

Effect of MT1M on the Expression of Neurotrophic Factor NT-3

Transfection of pc DNA3.1 MT1M plasmid promoted NT-3 expression in RGC-5 cells. The difference was statistically significant ($p < 0.05$). Transfection of MT1M siRNA inhibited NT-3 expression in RGC-5 cells, and the difference was statistically significant ($p < 0.05$; Figure 4).

Effect of MT1M on Inflammatory Factors In RGC-5 Cells

The effect of MT1M on the inflammatory factors of RGC-5 cells was analyzed by ELISA. Transfection of pc DNA3.1 MT1M plasmid inhibited the secretion of IL-2 and IL-6 in RGC-5 cells. Compared with the control group, the difference was statistically significant ($p < 0.05$). Transfection of MT1M siRNA promoted the secretion of inflammatory cytokines IL-2 and IL-6 in RGC-5 cells. Compared with the control group, the difference was statistically significant ($p < 0.05$; Figure 5).

Effect of MT1M on ROS and SOD Expression In RGC-5 Cells

MT1M plasmid transfection inhibited ROS content in RGC-5 cells and promoted SOD activity. Compared with the control group, the difference was statistically significant ($p < 0.05$). Transfection of MT1M siRNA promoted ROS

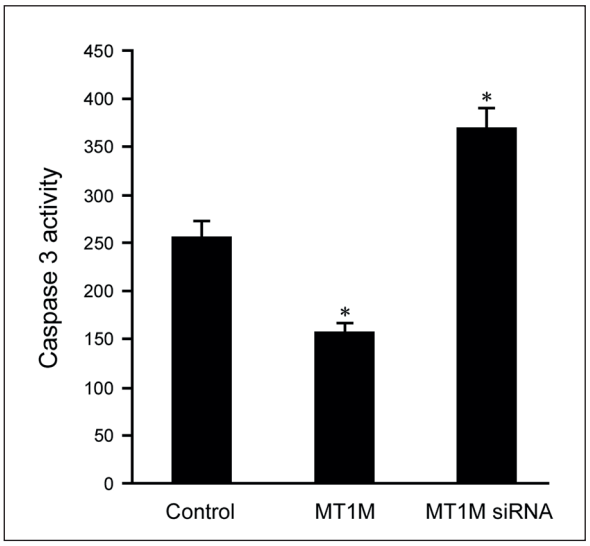

Figure 3. Effect of MT1M on the activity of Caspase3 in RGC-5 cells. Compared with the control group, * $p < 0.05$.

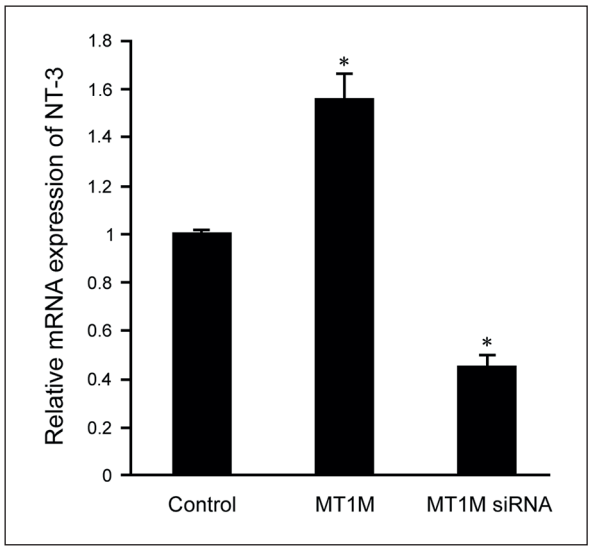

Figure 4. Regulation of MT1M on the expression of neurotrophic factor NT-3 in RGC-5 cells. Compared with the control group, * $p < 0.05$.

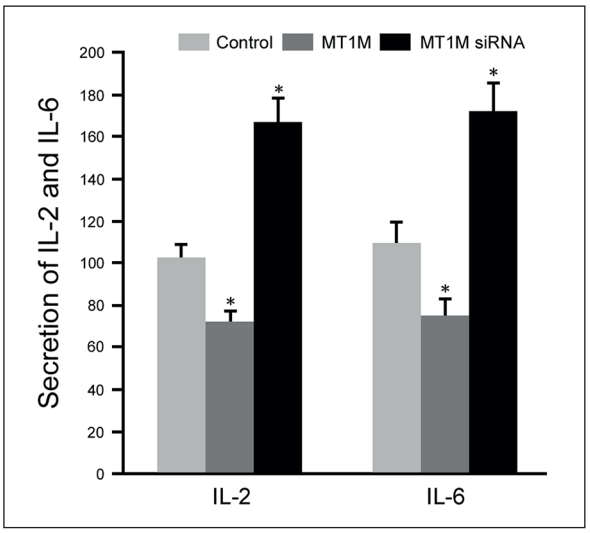

Figure 5. Regulation of MT1M on inflammatory factors in RGC-5 cells. Compared with the control group, * $p < 0.05$.

generation and inhibited SOD activity. Compared with the control group, the difference was statistically significant ($p < 0.05$; Figure 6).

Effect of MT1M on PI3K/AKT Signaling Pathway In RGC-5 Cells

Western blot analysis showed that MT1M plasmid transfection promoted phosphorylation of PI3K/AKT protein, whereas, transfection of MT1M siRNA inhibited PI3K/AKT protein phosphorylation (Figure 7).

Discussion

Retinopathy is one of the common ophthalmic diseases. It is caused by optic nerve damage or microvascular disease, causing progressive damage to the patient's vision, seriously affecting the physical and mental health of patients and the quality of life, bringing heavy weight to society, patients and families. The spiritual and economic burden has become a global concern[17]. Despite advances in medical technology, the therapeutic effects for treating retinopathy have not yet achieved satisfactory outcomes. RGC mainly performs visual signal processing, conduction and processing. However, due to its special structural characteristics, RGC axons can be easily injured, which leads to irreversible damage of the retina and causes abnormalities in the structure and function of the retina and other organs[18,19]. Studies have shown that the metal thiohistidine trimethyl inner salt is a highly conserved metallothionein (MT), a metal-binding protein[20]. MT1M is one of the important members of the MT family and can participate in the regulation of anti-DNA damage, anti-apoptosis, promote cell proliferation and vascular survival and angiogenesis, regulate hormones and cell metabolism, antagonize ionizing radiation. Butyric acid neuronal activity is also an important oxidative scavenger, and its abnormal expression is related to tumors and autoimmune diseases[15,21]. Therefore, this study analyzed the effect of MT1M on RGCs and found that transfection of pc DNA3.1 MT1M plasmid into

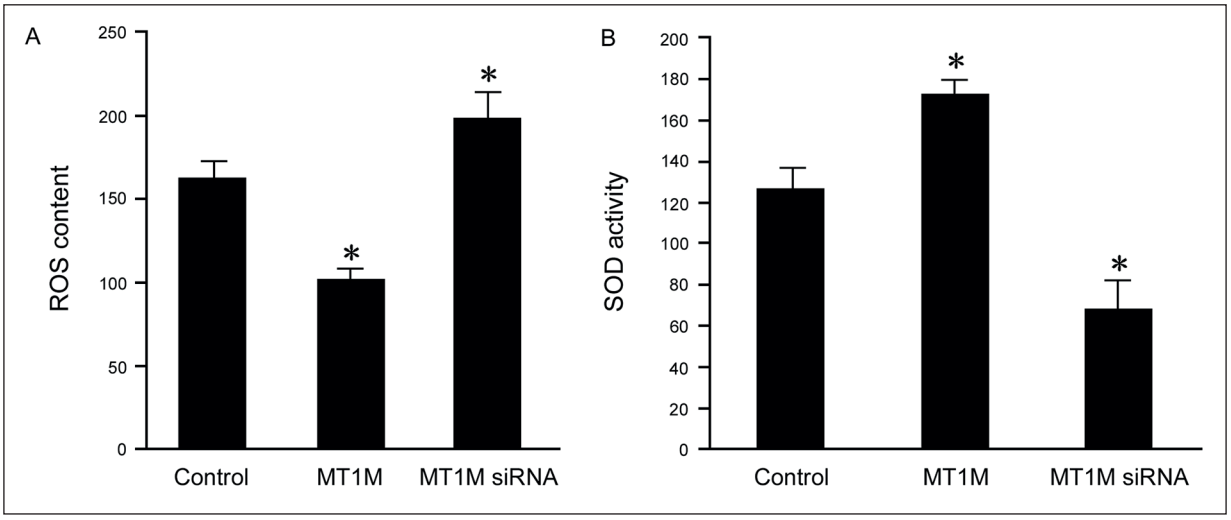

Figure 6. Regulation of MT1M on ROS and SOD expression in RGC-5 cells. **A**, ROS content; **B**, SOD activity, compared with the control group, * $p < 0.05$.

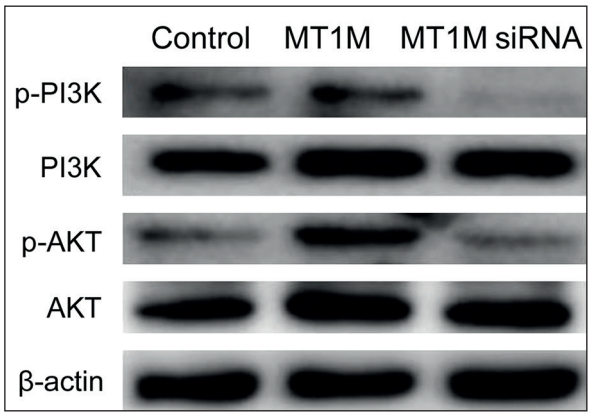

Figure 7. Effect of MT1M regulation on PI3K/AKT signaling pathway in RGC-5 cells.

RGC-5 cells promoted the significant increase of MT1M mRNA and protein expression in RGC-5, promoted the proliferation of RGC-5 cells, and inhibited caspase3 activity. Down-regulation of MT1M expression inhibited cell proliferation and increased Caspase3 activity. This result suggests that MT1M may be involved in the regulation of RGC-5 cell activity and proliferation.

This study further analyzed the mechanism of MT1M on retinal ganglion cells. During the occurrence of RGCs injury, it can be affected by the oxidative stress, resulting in excessive production of free radicals such as reactive oxygen species, dynamic imbalance of oxidation and antioxidant systems, further causing tissue inflammation and damage, as well as RGCs apoptosis and damage[22]. The PI3K/AKT signaling pathway can participate in the regulation of redox balance. After activation, it can inhibit external oxidation and chemical substances, thereby inhibiting oxidative stress and exerting a defense effect. It is one of the most important endogenous antioxidant signaling pathways[23]. The decrease in the secretion of neurotrophic factors can further cause damage to RGCs[24]. This study confirmed that increased expression of MT1M in RGC cells increased phosphorylation of PI3K/AKT protein, promoted the increase of neurotrophic factor NT-3, increased SOD activity, decreased ROS content and secretion of inflammatory factors IL-2 and IL-6. MT1M siRNA down-regulated MT1M expression, inhibited PI3K/AKT protein phosphorylation, decreased NT-3 expression and SOD activity, increased ROS content and secretion of inflammatory factors, suggesting that MT1M is involved in the regulation of RGCs cells through regulating PI3K/AKT signaling pathway.

Conclusions

Summarily, these results indicated that the up-regulation of MT1M can inhibit apoptosis and inflammation and promote RGC cell proliferation through the PI3K/AKT signaling pathway. Down-regulation of MT1M can inhibit the activation of PI3K/AKT signaling pathway, promote apoptosis and inflammation, inhibit the secretion of neurotrophic factors and proliferation of RGC cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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