• Basic Research •

In vitro protective effect of recombinant prominin-1 combined with microRNA-29b on N-methyl-D-aspartate-induced excitotoxicity in retinal ganglion cells

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Abstract

• **AIM:** To determine the *in vitro* protective effect of recombinant prominin-1 (Prominin-1)+microRNA-29b (P1M29) on N-methyl-D-aspartate (NMDA)-induced excitotoxicity in retinal ganglion cells (RGCs).

• **METHODS:** RGC-5 cells were cultured, and NMDA-induced excitotoxicity at the range of 100-800 μmol/L was assessed using the MTT assay. NMDA (800 μmol/L) was selected as the appropriate concentration for preparing the cell model. To evaluate the protective effect of P1M29 on the cell model, Prominin-1 was added at the concentration of 1-6 ng/mL for 48h, and the cell survival was investigated with/without microRNA-29b. After obtaining the appropriate concentration and time of P1M29 at 48h, real-time polymerase chain reaction (PCR) was utilized to detect the relative mRNA expression of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β2. Western blot detection was applied to measure the phosphorylation levels of protein kinase B (AKT) and extracellular regulated protein kinases (ERK) in RGC-5 cells after treatment with Prominin-1. Apoptosis study of the cell model was conducted by flow cytometry for estimating the anti-apoptotic effect of P1M29. Immunofluorescence analysis was used to analyze the expression levels of VEGF and TGF-β2.

• **RESULTS:** MTT cytotoxicity assays demonstrated that P1M29 group had significantly higher cell survival rate than Prominin-1 group (P<0.05). Real-time PCR data indicated that the expression levels of VEGF were significantly increased in both Prominin-1 and P1M29 groups compared NMDA and microRNA-29b group (P<0.05), while TGF-β2 were significantly decreased in both microRNA-29b and P1M29 groups compared NMDA and Prominin-1 group (P<0.05). Western blot results showed that both Prominin-1 and P1M29 groups significantly increased the phosphorylation levels of AKT and ERK compared to NMDA and microRNA-29b groups (P<0.05). Flow cytometry analysis revealed that P1M29 could prevent RGC-5 cell apoptosis in the early stage of apoptosis, while immunofluorescence results showed that P1M29 group had higher expression of VEGF and lower expression of TGF-β2 with a stronger green fluorescence than NMDA group.

• **CONCLUSION:** Prominin-1 combined with microRNA-29b can provide a suitable therapeutic option for ameliorating NMDA-induced excitotoxicity in RGC-5 cells.

• **KEYWORDS:** Prominin-1; microRNA-29b; vascular endothelial growth factor; transforming growth factor-β2
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INTRODUCTION

Glaucoma is an irreversible blinding eye disease, which can lead to the apoptosis of retinal ganglion cells (RGCs) or even permanent blindness. Currently, this disease accounts for approximately 70 million patients worldwide, 8.4 million of whom are blind[1-2]. Glaucoma is a degenerative optic atrophy caused by the programmed cell death of RGCs. Its pathological features mainly involve the degeneration of RGCs, progressive loss of axons and elevation of intraocular pressure (IOP), which can directly cause damage to neurons and optic nerves[3]. Lowering IOP is currently the main way to delay the progression of glaucoma[4]. However, RGC degeneration and axonal loss also occur in individuals with normal IOP. Although IOP can be controlled by medical

therapy within a normal range, some patients experience optic atrophy and visual field deterioration[5]. Therefore, it is necessary to stimulate axonal regeneration for protecting optic nerves and lower IOP during glaucoma treatment[6].

The first strategy is to stimulate axonal regeneration for protecting optic nerves. Neuroprotection refers to any therapeutic modality aimed at preventing or delaying neuronal cell death and maintaining neurological function[7]. Ectopic expression of anti-apoptotic genes enables most RGCs to survive axotomy, but not regenerate axons into optic nerves[8]. Other approaches to improve the survival rate of RGCs include intracellular injection of cAMP stimulants, trophic factors, and caspase inhibitors, but generally these drugs have a weak ability to promote regeneration. Anti-apoptotic and survival factors (*e.g.*, vascular endothelial growth factor, VEGF) is a potent angiogenic signaling protein that can be upregulated locally at the sites of tissue injury[9]. VEGF is essential for vertebrate development and vascular growth of the nervous system, and it also promotes neuronal regeneration, neuroprotection, and glial cell growth. However, current therapeutic approaches to increase VEGF levels by injecting the VEGF gene or protein in some *in vivo* experiments did not yield good results[10-11]. Some proteins have neuroprotective effects and can even prevent neurodegeneration, which are characterized by high specificity and low toxicity. Among them, prominin-1 can promote the growth of vascular endothelial cells and their lumen formation by enhancing the VEGF signaling pathway[12]. Prominin-1 is a 115–120 kDa protein, which belongs to the pentaspan transmembrane protein family in different species such as human, mouse, rat, fly and worm[13-15]. Prominin-1 can bind to plasma membrane cholesterol and is associated with membrane microdomains in a cholesterol-dependent manner[16]. Studies have found that prominin-1 plays a vital role in the retina, and the mutation or deletion of the biallelic gene of prominin-1 can lead to retinal degeneration and abnormal photoreceptor cells[15,17]. Additionally, Xiao *et al*[18] found that prominin-1 was necessary for the correct localization of rhodopsin and opsin in the retina, and played a crucial role in preserving the normal levels of outer segment proteins. Furthermore, prominin-1 was selected, as a crucial regulator of angiogenesis, proliferation, and apoptosis, which can interact with VEGF, stabilize it, and improve the binding of VEGF to its receptors *via* dimerization of VEGF receptors.

Another strategy involves reducing IOP as part of the glaucoma treatment. In fact, IOP is determined by the balance of incoming and outlet water, while extracellular matrix (ECM) synthesis or disruption can lead to an alteration of the aqueous balance[19]. Transforming growth factor (TGF)-β is a kind of important regulators of ECM synthesis and wound healing. Compelling evidence has established a connection between TGF-β and ocular hypertension. *In vivo* and *in vitro* studies have shown that elevated IOP is linked to the TGF-β-induced fibrotic response in the eye[20]. One of the isoforms, TGF-β2, can impede the outflow of aqueous humor and consequently elevates IOP. Among the mechanisms involved in aqueous humor outflow may be that TGF-β2 mediates excessive ECM accumulation across the trabecular meshwork (TM) and stimulates the expression of key ECM components (*e.g.*, collagen I, collagen IV, fibronectin, laminin, and thrombospondin-1) in the TM. Recent studies have shown that the microRNA-29 family plays a crucial role in regulating several ECM proteins, such as collagen I, collagen IV, and laminin. The microRNA-29 family has emerged as a significant regulator of ECM homeostasis[20-21]. Moreover, it is worth noting that nearly all the TGF-β members involved in the canonical signaling pathway have been found to be influenced by miRNAs. A previous study has shown that the microRNA-29 family downregulates TGF-β2 and reduces IOP levels, and overexpression of microRNA-29 could affect the expression of TGF-β2 as parts of the components in the ECM[20]. Taking these considerations into account, microRNA-29b was selected as a negative regulator of TGF-β2, which could potentially aid in reducing IOP.

Nowadays, RGC-5 cells have been widely applied to explore the mechanisms related to neuronal injury and death[22-23]. In this study, we assessed the effects of recombinant prominin-1 (Prominin-1) with microRNA-29b (P1M29) on N-methyl-D-aspartate (NMDA)-induced excitotoxicity in RGC-5 cells by measuring the changes in cell survival rates, mRNA expression of VEGF and TGF-β2, protein expression and phosphorylation levels of protein kinase B (AKT) and extracellular regulated protein kinases (ERK), anti-apoptotic activities, and immunofluorescence intensities of VEGF and TGF-β2. This study reveals a potential microRNA-based therapy for improving the neuroprotection activities of Prominin-1 against NMDA-induced excitotoxicity in RGC-5 cells.

MATERIALS AND METHODS

RGC-5 Cell Culture and Treatment by NMDA or Prominin-1 RGC-5 cell line was supplied by Shanghai Aolu Biotechnology Co., Ltd (Shanghai, China) and cultured in RPMI-1640 medium containing 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, and maintained at 37°C and 5% CO_2. After trypsin digestion, a new passage was performed at a ratio of 1:4 every 3–4d. Subsequently, a model of NMDA-induced excitotoxicity in RGC-5 cells was prepared. RGC-5 cells were treated with different concentrations (100–800 μmol/L) of NMDA (MCE, USA) for 48h to determine the appropriate concentration that led to the minimum survival rate. Finally, the cells were cultured in the initial medium for another

6–24h[24]. Besides, RGC-5 cells were also treated with of Prominin-1(1–6 ng/mL; Abcam, Britain) for 48h to investigate its cytotoxic effect, which results could be the basis for the further *in vitro* protective effect of Prominin-1 with/without microRNA-29b on the cell model.

Detection of RGC-5 Cell Survival Rate by MTT Assay The *in vitro* protective activity of Prominin-1 (Abcam, Britain)+ microRNA-29b (Sangon Biotech, China) against NMDA-induced RGC-5 cell excitotoxicity was determined using the MTT assay. Cells in the exponential phase (10^4 cells/well) were grown in a 96-well plate for 24h, and the culture medium was replaced with fresh medium. The cells were exposed to Control group, Control+Negative control (NC, Sangon Biotech, China) group, NMDA group, NMDA+NC group, and Prominin-1 or P1M29 (100 μL) group at a range of Prominin-1 concentrations (1, 3, and 6 ng/mL). In P1M29 group, microRNA-29b was transfected by Lipofectamine 3000. Next, the cells were incubated for 48h, respectively. Then, 20 μL MTT solution (5 mg/mL) was added to each well, followed by incubation for 4h. After carefully removing the supernatant, 100 μL of dimethyl sulfoxide was added to each well. The formazan crystals were allowed to dissolve completely. A Bio-Rad Model 680 Microplate Reader was used to measure optical density at 570 nm (A570). The survival rate of each treatment group was calculated as follows:

$$\text{Survival rate (\%)}=\left[\frac{A_{570}\text{ (treated)}}{A_{570}\text{ (untreated)}}\right]\times 100$$

RNA Isolation and Real-Time Polymerase Chain Reaction Total RNA from all samples was extracted with RNAiso Plus (Takara Bio, Japan). cDNA synthesis was conducted using the PrimeScriptTM RT reagent Kit (Takara Bio). mRNA expression was determined on a Bio-Rad iQ5 polymerase chain reaction (PCR; Bio-Rad, USA) using a SYBR Premix Ex TaqTM II (Takara Bio). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an internal control. The forward and reverse primers of VEGF mRNA and TGF-β2 mRNA were GCACATAGAGAGAATGAGCTTCC (5'-3') and CTCCGCTCTGAACAAGGCT (5'-3'); CTTCGACGTGACAGACGCT (5'-3') and GCAGGGGCAGTGTAAACTTATT (5'-3'), respectively. The reaction conditions were as follows: 10s at 95°C, and 30s at 60°C for 48 cycles. Relative fold changes in VEGF and TGF-β2 expression between treatment and control groups were measured using the $2^{-\Delta\Delta CT}$ method[25].

Western Blot Analysis The expression levels of AKT, phospho-AKT, ERK, and phospho-ERK in NMDA-induced excitotoxicity RGC-5 cells were determined by Western blotting. Briefly, NMDA-induced excitotoxicity RGC-5 cells treatment with microRNA-29b, Prominin-1 and P1M29 were lysed in a cell lysis buffer (BOSTER, China), respectively. Proteins of the three groups were separated through 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and then transferred onto nitrocellulose membranes (Millipore, USA). Each membrane was incubated with primary antibody against AKT, phospho-AKT, ERK or phospho-ERK (1:1000 dilution, Cell Signaling Technology, USA). After washing with TBS+Tween-20 (TBST) for 5 times, the membrane was incubated again with secondary antibody at room temperature for 2h. Finally, Western blot images were captured using an Odyssey® CLX system (LI-COR, USA), and then washed 5 times with TBST.

Apoptosis Assay Cell apoptosis was investigated using a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, USA). NMDA-induced excitotoxicity RGC-5 cells were exposed to Prominin-1, microRNA-29b, P1M29 or culture medium. After incubation for 48h, the cells were harvested and rinsed with ice-cold PBS at 4ºC for 3 times and resuspended at a density of 1×10^6 cells/mL in 200 μL binding buffer. All samples were incubated with 5 μL Annexin V-FITC and 5 μL PI staining solution at room temperature for 15min in the dark. Finally, Binding Buffer (400 μL) was added and detected by CytoFLEX S flow cytometry (Beckman Coulter, USA) within 1h.

Immunofluorescence Staining After culturing for 48h with Prominin-1, microRNA-29b or P1M29, the NMDA excitotoxicity cell models were fixed in 1 mL 4% paraformaldehyde for 15min before being permeabilized in 0.5% (v/v) Triton X-100 (Sigma-Aldrich, USA) for 15min. The samples were rinsed 3 times with PBS, and then blocked in 10% BSA solution for 1h. The cells were first incubated overnight at 4°C with anti-VEGF (5 μg/mL) or anti-TGF-β2 (1:50 dilution), and then incubated overnight at 4°C with SABC anti-rabbit antibody (BOSTER, China) as the secondary antibody. The samples were rinsed 3 times with PBS, and FITC-SABC (1:100 dilution, BOSTER) was added. After washing thrice in PBS, the cells were stained with 4,6-diamino-2-phenyl indole (0.5 mg/mL) in PBS. All samples were visualized using an Axiovert200 fluorescence inverted microscope (ZEISS, Germany).

Statistical Analysis All data were shown as mean±standard deviation. Statistical difference between groups was compared by one-way ANOVA. *P*-values <0.05 were deemed statistically significant.

RESULTS

P1M29 Improves the Survival Rate of RGC-5 Cells with NMDA-Induced Excitotoxicity *In Vitro* As shown in Figure 1A, 800 μmol/L of NMDA was selected as the appropriate concentration for preparing the NMDA-induced RGC-5 cell excitotoxicity model, due to the minimum survival rate. Furthermore, there was no significant cytotoxic effect of

Prominin-1 (1–6 ng/mL) on RGC-5 cell for 48h compared to RGC-5 cell (Figure 1A). The RGC-5 cells with NMDA-induced excitotoxicity were treated with Prominin-1 and P1M29 with NMDA and NMDA+NC as control for 48h, and the survival rates of these treatment groups were analyzed (Figure 1B). The concentrations of Prominin-1 in the three groups were 1, 3, and 6 ng/mL, respectively. Compared to the NMDA group, both the Prominin-1 and P1M29 groups showed significant improvements in the survival rates of RGC-5 cells subjected to NMDA-induced excitotoxicity when treated with concentrations of 3 and 6 ng/mL (P<0.05). Figure 1B shows that P1M29 group (6 ng/mL) exhibited higher survival rates than Prominin-1 group (P<0.05). As we know, Prominin-1 is a crucial regulator of angiogenesis, proliferation, and apoptosis. This protein can interact with VEGF, stabilize it, and improve the binding of VEGF binding to its receptors *via* dimerization of VEGF receptors. MicroRNA-29b is a negative regulator of TGF-β2. The results demonstrated that Prominin-1 was able to interact with VEGF and improve the survival rate of RGC-5 cells with NMDA-induced excitotoxicity. Moreover, the effect of Prominin-1 on improving the survival rates of RGC-5 cells demonstrated a dose-dependent relationship within the range of 1–6 ng/mL, as shown in Figure 1B. In this study, 6 ng/mL was selected as the most appropriate concentration for Prominin-1, which was subsequently used in the real-time PCR test, Western blot analysis, apoptosis assay, and immunofluorescence staining.

mRNA Expression of VEGF and TGF-β2 in the NMDA-Induced Excitotoxicity Cell Model Previous research has shown that prominin-1 can promote the growth of vascular endothelial cells by enhancing the VEGF signaling pathway, while the microRNA-29 family downregulates TGF-β2 and reduces IOP levels, and overexpression of microRNA-29b can affect the expression of TGF-β2 as parts of the components in the ECM[12,26]. To assess whether the Prominin-1 can interact and potentiate the angiogenic and antiapoptotic properties of VEGF, while microRNA-29b can act as a crucial regulator of TGF-β2, the mRNA expression of VEGF and TGF-β2 was analyzed after a 48h incubation of NMDA-induced excitotoxicity RGC-5 cells treated with either Prominin-1 (6 ng/mL), microRNA-29b or P1M29 (6 ng/mL). Real-time PCR analysis revealed that the expression of VEGF was significantly increased by Prominin-1 in both Prominin-1 (6 ng/mL) and P1M29 (6 ng/mL) groups compared to microRNA-29b group at 48h (P<0.05; Figure 2A), while TGF-β2 was significantly decreased by microRNA-29b in both microRNA-29b and P1M29 (6 ng/mL) groups compared to Prominin-1 group at 48h (P<0.05; Figure 2B). Furthermore, there was no significant difference between the Prominin-1 (6 ng/mL) and P1M29 (6 ng/mL) groups, suggesting that

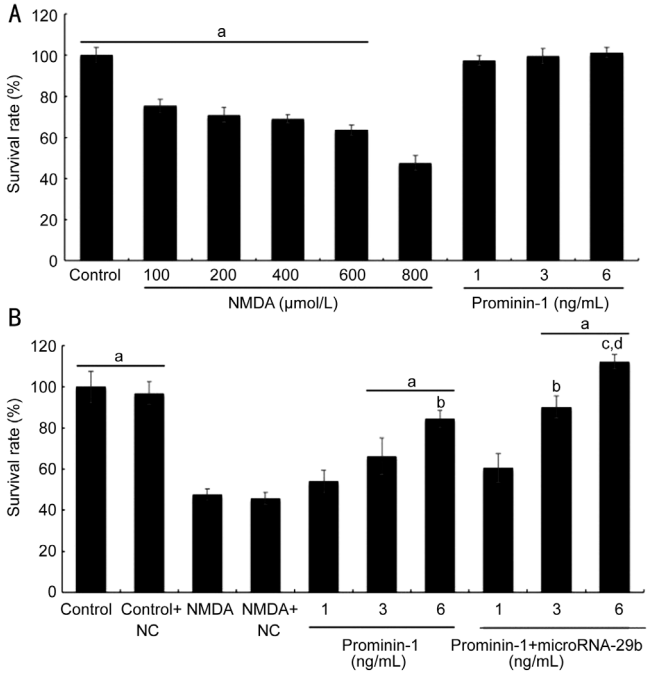

Figure 1 Evaluation of *in vitro* survival rate *In vitro* survival rate of NMDA (100–800 μmol/L Pominin-1 (1 mL) on RGC-5 cells for 48h (n=3). [a]P<0.05 *vs* NMDA (800 μmol/L) group; B: *In vitro* survival rate of Pominin-1 and P1M29 on RGC-5 cells induced by NMDA (800 μmol/L) for 48h (n=3). [a]P<0.05 *vs* NMDA (800 μmol/L) group; [b]P<0.05 *vs* Prominin-1 group (1, 3 ng/mL); [c]P<0.05 *vs* Prominin-1 group (6 ng/mL); [d]P<0.05 *vs* P1M29 group (3 ng/mL). RGC: Retinal ganglion cell; NMDA: N-methyl-D-aspartate; Pominin-1: Recombinant prominin-1; P1M29: Prominin-1+microRNA-29b; NC: Negative control.

VEGF expression is primarily influenced by Prominin-1. However, the microRNA-29b group and the P1M29 (6 ng/mL) group showed no significant difference, indicating that TGF-β2 expression is mainly associated with microRNA-29b.

Effects of Prominin-1 on the Expression and Phosphorylation Levels of AKT and ERK in the NMDA-Induced Excitotoxicity Cell Model It has been reported that VEGF can increase the extent and duration of neurite growth in cortical explants or cultured neurons *via* AKT and ERK signaling pathways. To evaluate the effects of Prominin-1 on the levels of AKT, phospho-AKT, ERK and phospho-ERK, NMDA-induced excitotoxicity RGC-5 cells were treated with Prominin-1 (6 ng/mL), microRNA-29b, or P1M29 (6 ng/mL), and the levels of AKT, phospho-AKT, ERK and phospho-ERK were analyzed by Western blotting (Figure 3A). It was found that Prominin-1 elevated the phosphorylation levels of AKT and ERK in both Prominin-1 and P1M29 (6 ng/mL) groups compared to NMDA and microRNA-29b groups (P<0.05; Figure 3B and 3C). In contrast, microRNA-29b did not significantly affect the ratios of phospho-AKT/AKT and phospho-ERK/ERK.

P1M29 Prevents NMDA-Induced Excitotoxicity RGC-5 Cell Death To further verify the protective effect *in vitro* of P1M29 on NMDA-induced RGC-5 excitotoxicity, apoptosis

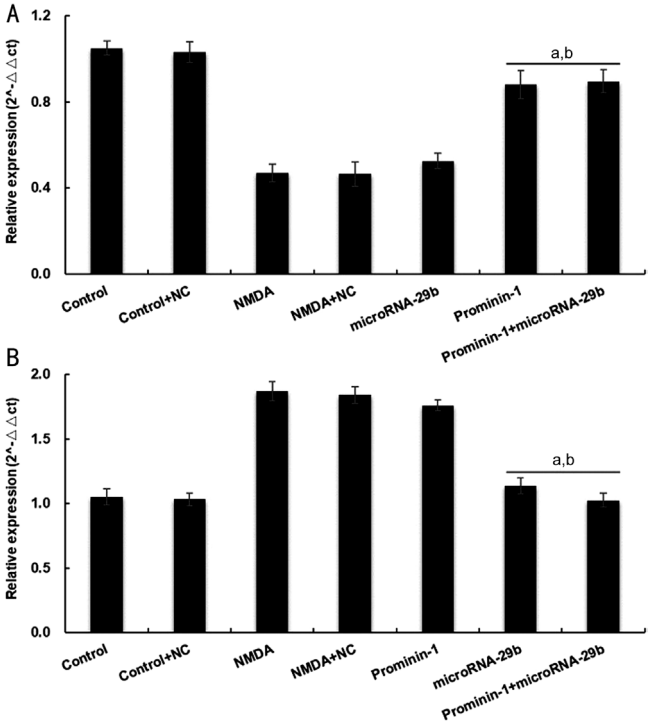

Figure 2 The mRNA expression detection of VEGF and TGF-β2 Expression of VEGF in the Control group, Control+NC group, NMDA group, NMDA+NC group, microRNA-29b group, Prominin-1 group and P1M29 group detected by real-time PCR, and GAPDH was the internal control (n=3). [a]P<0.05 *vs* NMDA (800 μmol/L) group; [b]P<0.05 *vs* microRNA-29b group; B: Expression of TGF-β2 in the Control group, Control+NC group, NMDA group, NMDA+NC group, Prominin-1 group, microRNA-29b group and P1M29 group detected by real-time PCR, and GAPDH was the internal control (n=3). [a]P<0.05 *vs* NMDA (800 μmol/L) group; [b]P<0.05 *vs* Prominin-1 group. NMDA: N-methyl-D-aspartate; Pominin-1: Recombinant prominin-1; P1M29: Prominin-1+microRNA-29b; VEGF: Vascular endothelial growth factor; TGF: Transforming growth factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NC: Negative control; PCR: Polymerase chain reaction.

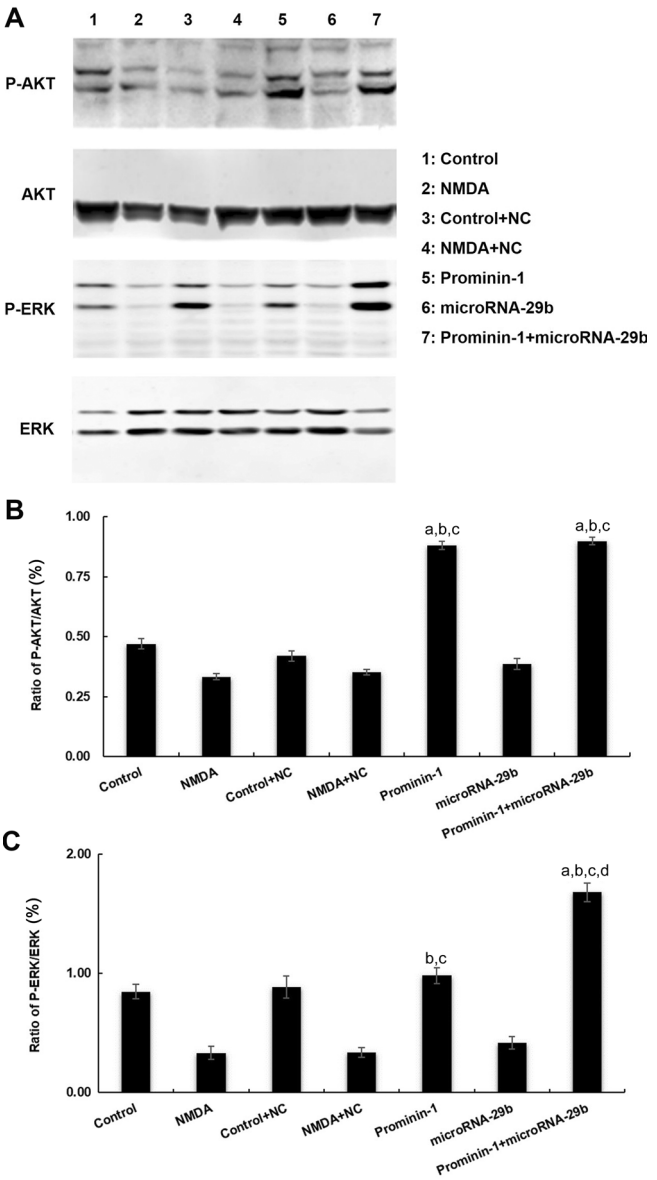

Figure 3 Prominin-1 in P1M29 group activates both AKT and ERK signaling on RGC-5 cells induced by NMDA (800 μmol/L) for 48h A: Western blotting shows the levels of P-AKT, AKT, P-ERK and ERK in the Control group, NMDA group, Control+NC group, NMDA+NC group, Prominin-1 group, microRNA-29b group and P1M29 group. B, C: Histogram shows the ratio of P-AKT/AKT and P-ERK/ERK, respectively. [a]P<0.05 *vs* Control group; [b]P<0.05 *vs* NMDA (800 μmol/L) group; [c]P<0.05 *vs* microRNA-29b group; [d]P<0.05 *vs* Prominin-1 group. NMDA: N-methyl-D-aspartate; Pominin-1: Recombinant prominin-1; P1M29: Prominin-1+microRNA-29b; AKT: Protein kinase B; ERK: Extracellular regulated protein kinases; P-AKT: Phosphor-AKT; P-ERK: Phosphor-ERK; NC: Negative control.

study was conducted by flow cytometry for estimating its anti-apoptotic activity. Annexin V-FITC/PI was used to confirm the anti-apoptotic effect. The results showed that P1M29 could apparently prevent NMDA-induced excitotoxicity RGC-5 cell death by decreasing the early apoptosis regions compared to Prominin-1, microRNA-29b and NMDA group at 48h (P<0.05; Figure 4B).

Expression of VEGF and TGF-β2 in NMDA-Induced Excitotoxicity RGC-5 Cells To determine the expression of VEGF and TGF-β2 in NMDA-induced excitotoxicity RGC-5 cells, immunofluorescent staining was performed. It was found that P1M29 group had higher expression of VEGF with stronger green fluorescence and lower expression of TGF-β2 with weaker green fluorescence than NMDA group at 48h (Figure 5). These findings demonstrate that Prominin-1 interacts and potentiates the angiogenic and antiapoptotic properties of VEGF, and microRNA-29b is a negative regulator of TGF-β2.

DISCUSSION

Glaucoma, one of the leading causes of blindness, refers to a group of irreversible, progressive optic neuropathies. It is characterized by both structural and functional impairment of

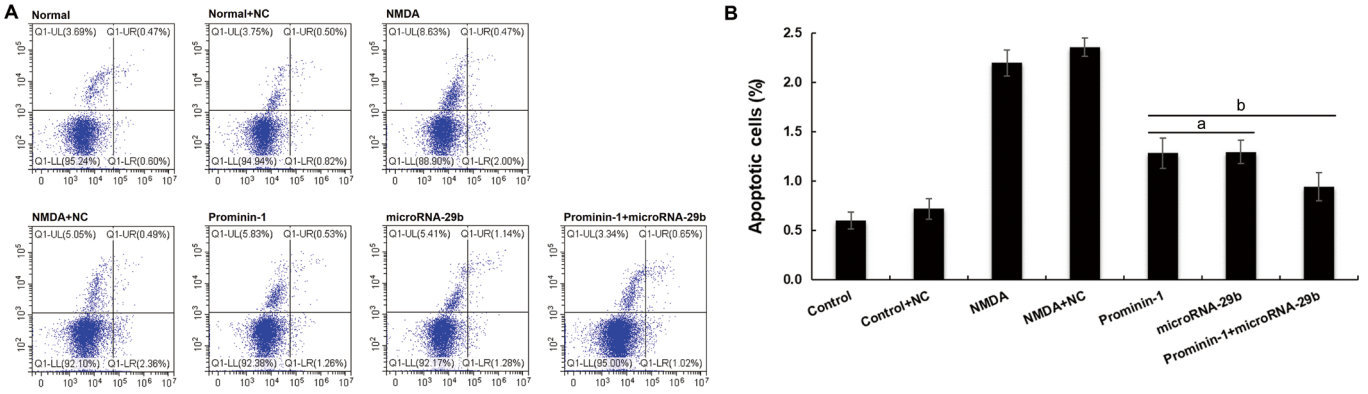

Figure 4 The study of anti-apoptotic effects A: Anti-apoptotic effects of Prominin-1, microRNA-29b, and P1M29 on RGC-5 cells induced by NMDA (800 μmol/L) for 48h; B: Apoptotic rate in the early stage of apoptosis of Pominin-1, microRNA-29b, and P1M29 (n=3). [a]P<0.05 *vs* P1M29 group; [b]P<0.05 *vs* NMDA (800 μmol/L) group. RGC: Retinal ganglion cell; NMDA: N-methyl-D-aspartate; Pominin-1: Recombinant prominin-1; P1M29: Prominin-1+microRNA-29b; NC: Negative control.

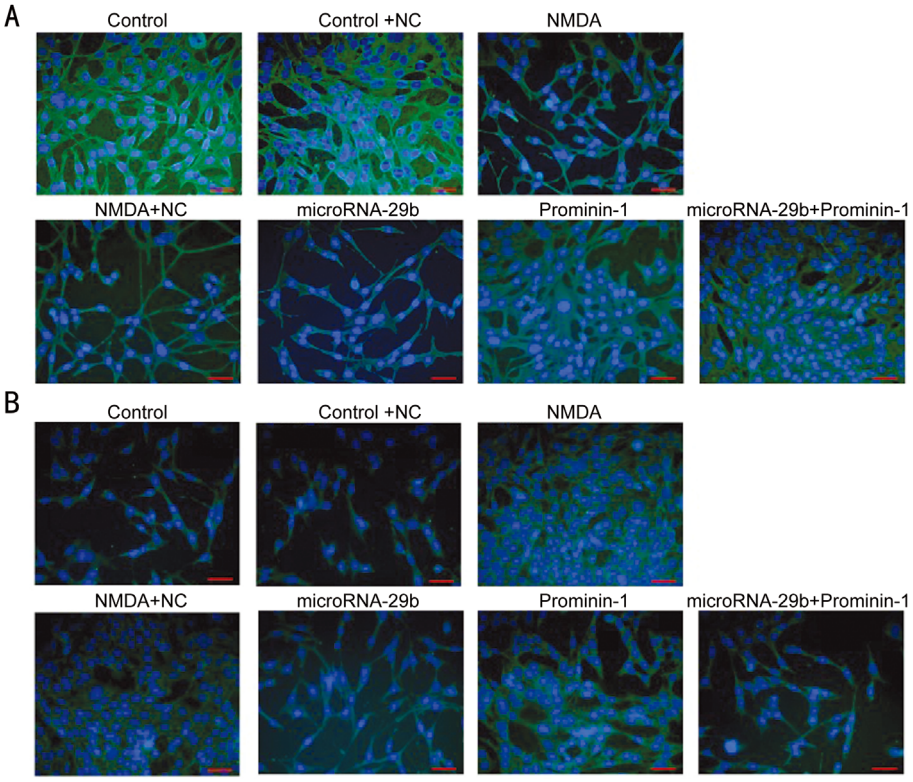

Figure 5 Immunofluorescent staining in the control group, Control+NC group, NMDA group, NMDA+NC group, microRNA-29b group, Prominin-1 group, P1M29 group A: The expression of VEGF in NMDA-induced excitotoxicity RGC-5 cells (bar=50 μm). B: The expression of TGF-β2 in NMDA-induced excitotoxicity RGC-5 cells (Bar=50 μm). RGC: Retinal ganglion cell; NMDA: N-methyl-D-aspartate; Pominin-1: Recombinant prominin-1; P1M29: Prominin-1+microRNA-29b; VEGF: Vascular endothelial growth factor; TGF: Transforming growth factor; NC: Negative control.

RGCs[27]. Increased IOP is a major risk factor of glaucoma. Besides, ischemia is also involved in the occurrence of this disease[28]. The medical treatments for glaucoma are focused on IOP reduction by increasing the uveoscleral outflow of aqueous humor and inhibiting aqueous humor production[29]. However, it is important to note that not all individuals with hypertension have a significant risk of developing glaucoma[30-31]. Therefore, a new therapeutic approach that protects RGCs, targets apoptosis pathways, and does not cause significant side effects is needed for the treatment of patients with glaucoma[6]. Different neuroprotective strategies have been proposed for treating glaucoma patients, including improvement of optic nerve blood flow, attenuation of glutamate excitotoxicity, and prevention or inhibition of apoptosis[32].

Neuronal injury or death is an early event in the pathogenesis of retinal disorders such as glaucoma and diabetic retinopathy[33-34]. Despite that the mechanism of neuronal injury or death is not fully understood, excessive glutamate

receptor activation has been suggested to play a major role[35-36]. The neurotoxic effect of glutamate is largely modulated by NMDA receptors[37]. The survival rate of RGC-5 cells was remarkably decreased after treatment with NMDA in a concentration-dependent manner, and a NMDA receptor antagonist (MK801) effectively blocked this pathway, implying that NMDA can induce RGC-5 cell injury or death[24]. Although the relationships between vascular dysfunction and neural cell death during glaucoma or diabetic retinopathy are still unknown, a recent study has shown that retinal neurodegeneration may precede vascular dysfunction in an experimental model[38].

RGCs are the neurons that integrate incoming visual signals from the retina and convey them to the brain. Loss or dysfunction of RGCs is associated with different optic neuropathies, including glaucoma. RGC apoptosis has been recognized as a common hallmark of primary open-angle glaucoma[39]. The purified primary RGCs have been used as an experimental model to assess physiological processes and apoptosis mechanisms, but this method is labor intensive and the cells can be cultured in vitro only for a limited period[40]. Therefore, it is important to use a transformed cell line for cell proliferation. At present, RGC-5 is the only available immortalized cell line that has been introduced by Krishnamoorthy *et al*[41]. Many studies have demonstrated that RGC-5 can still be used to study the neurobiology of neural cells derived from retinal cell lines[42-44].

Therefore, considering the factors mentioned above, this study chose RGC-5 cells as the subject of investigation and evaluated NMDA-induced excitotoxicity within the range of 100–800 μmol/L using the MTT assay. Subsequently, NMDA at a concentration of 800 μmol/L was selected as the appropriate concentration for constructing the in vitro model. The neuroprotective effects of Prominin-1 combined with microRNA-29b on NMDA-induced excitotoxicity in RGC-5 cells were then evaluated with NMDA at 800 μmol/L. VEGF is a homo dimeric glycoprotein bound by disulfide bonds. Owing to its multiple functions such as induction of angiogenesis, enhancement of vascular permeability, regulation of lymph angiogenesis, promotion of neurogenesis and protection of nerves, VEGF has become a research hotspot in the fields of cardiovascular system, tumor, nervous system and ophthalmology[45]. VEGF can recognize highly specific receptors on vascular endothelial cells, which in turn activate ERK1/2 through MAPK pathway, and promote DNA synthesis and cell proliferation. VEGF can also activate PI3K-AKT pathway through phosphoinositol specific phospholipase C[46]. PI3K-AKT is an important pathway for cell survival, and AKT regulates anti-apoptotic signaling through serine kinase[47]. MAPK/ERK has important anti-apoptotic and pro-proliferation functions in cells, and ERK is a key protein in this pathway[48]. Based on the Western blotting results of AKT, phospho-AKT, ERK, and phospho-ERK, it was observed that Prominin-1 increased the phosphorylation levels of both AKT and ERK in both the Prominin-1 and P1M29 (6 ng/mL) groups when compared to the NMDA and microRNA-29b groups (P<0.05; Figure 3). In recent years, both cell and animal experimental studies have found that VEGF can maintain axonal transport, inhibit inflammatory response, and reduce oxidative stress, thereby inhibiting RGC apoptosis in glaucoma models. This suggests that VEGF may exhibit a neuroprotective effect on the optic nerves.

The research on the neuroprotective mechanism of VEGF is currently focused on promoting the survival of nerve cells, inhibiting cell apoptosis, and promoting nerve regeneration by increasing blood perfusion, especially in the treatment of nervous system diseases and eye diseases. VEGF can directly act on optic neurons, stimulate the proliferation of neural stem cells, and promote neurogenesis[49], while indirectly promote neurogenesis via regulation of angiogenesis. Under the pathological conditions of nerve injury, macrophages can migrate into the axonal space and secrete VEGF to induce angiogenesis, so that Schwann cells can use these vessels to migrate into the axonal space to achieve axonal regeneration[50]. In human eyes, vascular endothelial cells, pericytes, retinal neurons, and other cells can all produce VEGF[45,51]. The current researches suggest that VEGF is generally limited to the part where it is generated to play its role, and will not spread to other parts[52-54]. VEGF can inhibit RGC apoptosis and protect the optic nerve in glaucoma models by maintaining axonal transport, inhibiting inflammatory reaction and reducing oxidative stress. Endogenous VEGF-A can inhibit the apoptosis of RGC in glaucoma[53]. However, current therapeutic approaches to increase VEGF levels by injecting the VEGF gene or protein in some *in vivo* experiments did not yield good results[10-11]. Therefore, it is necessary to find an effective way to increase the level of VEGF. As we know, anti-VEGF drugs have been widely applied in the treatment of ocular neovascular diseases[55]. Although the results of anti-VEGF drugs in the treatment of neovascular ophthalmopathy are encouraging, these drugs can only achieve the purpose of treating neovascular ophthalmopathy by inhibiting VEGF angiogenesis. However, from another point of view, VEGF has neuroprotective effects, and is necessary for the survival of endothelial cells and glial cells in the static state. Therefore, how to inhibit VEGF angiogenesis while preserving its neuroprotective effects is always an important research concern. Our study raises a new question about the potential effects of the currently used anti-VEGF intravitreal injections for proliferative diabetic retinopathy in patients who also

have advanced glaucoma. However, our study was conducted in vitro, and for the same findings to be effective *in vivo*, further research is required in the future. Before any definitive therapeutic benefits can be derived from the use of VEGF molecules as neuroprotective agents for glaucoma, extensive studies in living organisms should be conducted.

Adini *et al*[56] found that prominin-1 is a crucial regulator of angiogenesis, proliferation and apoptosis, which is able to interact with VEGF, stabilize it and improve the binding of VEGF binding to its receptors *via* dimerization of VEGF receptors. Moreover, Almasry *et al*[57] assessed the relationship between prominin-1 and VEGFA in diabetes-induced retinopathy, and found that a positive feedback regulation between them. Specific localization of prominin-1 in endothelial cells supports the hypothesis of Adini *et al*[56] who reported that prominin-1 could interact and potentiate the angiogenic and anti-apoptotic properties of VEGFA and there was a direct interaction between prominin-1 and VEGFA on endothelial cells.

On the other hand, TGF-β is a multifunctional growth factor that can regulate the functions of vascular cells[58]. An increasing number of studies have suggested the potential roles of the TGF-β family members in vascular dysfunction and morphogenesis[59-60]. The expression of soluble endoglin, an inhibitor of TGF-β, could induce retinal vascular abnormalities, including vascular leakage and impaired retinal perfusion[61]. Therefore, constitutive activation of TGF-β pathway is needed for the survival of vascular cells under physiological conditions. Ueda *et al*[62] demonstrated that retinal blood vessels were damaged in the NMDA-induced retinal degeneration model, indicating that neuronal injury can lead to a progressive loss of retinal cells by disrupting circulation. Inhibiting TGF-β signaling pathway could attenuate neuronal loss and prevent vascular damage in NMDA-induced retinal degeneration model. Hence, TGF-β inhibition may serve as a new therapeutic approach for treatment and prevention of retinal disorders, including glaucoma, diabetic retinopathy and retinal ischemia.

The microRNA-29 family is composed of microRNA-29a, microRNA-29b, and microRNA-29c, which share identical seed sequences. These microRNAs are known to suppress the post transcriptional regulation of several genes related to fibrosis and ECM synthesis, including collagen, elastin and fibrillin[63-64]. As a negative regulator of ECM, Luna *et al*[21] investigated the interactions between microRNA-29 and TGF-βs. Notably, TGF-β2 downregulated the expression of microRNA-29 to induce multiple ECM components in TM cells, suggesting that this microRNA may play an important role in regulating TGF-βs *via* the outflow pathway[21,65]. Based on the results obtained from real-time PCR, flow cytometry analysis, and immunofluorescence staining, the Prominin-1 combined with microRNA-29b group exhibited a significant *in vitro* protective effect when compared to the NMDA group.

In summary, our work demonstrates that Prominin-1 combined with microRNA-29b exhibits protective effects on NMDA-induced excitotoxicity in RGC-5 cells. This may be attributed to the fact that Prominin-1 could interact and potentiate the angiogenic and anti-apoptotic properties of VEGF, while microRNA-29b is a negative regulator of TGF-β2. This study is the first to apply the combination therapeutic strategy of Prominin-1 and microRNA-29b for improving their neuroprotection activities. Our *in vitro* data establish the therapeutic potential of this treatment strategy. Nevertheless, the detailed mechanisms and *in vivo* protective effect of this treatment strategy should be further explored in animal retinal degeneration models.

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