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MiR-1b up-regulation inhibits rat neuron proliferation and regeneration yet promotes apoptosis *via* targeting KLF7

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Abstract

Introduction: *MicroRNA (miRNA) is known to be involved in nerve injury. Our study aimed to identify the role and mechanism of miR-1b in rat neuron proliferation, regeneration and apoptosis.*

Material and methods: *Neurons were successfully separated and identified using a microscope and immunofluorescence staining of microtubule-associated protein 2 (MAP-2). The expressions of miR-1b and Krüppel-like factor 7 (KLF7) were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Neuron viability and apoptosis were detected by MTT assay and flow cytometry, respectively. Neuron regeneration states were observed using a microscope and analysed by the ImageJ software. Expressions of C-caspase-3 and cell regeneration-related proteins (nerve growth factor [NGF], ciliary neurotrophic factor [CNTF] and brain-derived neurotrophic factor [BDNF]) were measured by Western blot. Target genes and potential binding sites of KLF7 and miR-1b were predicted by TargetScan 7.2 and confirmed by dual luciferase reporter assay.*

Results: *Neurons were identified as MAP-2-positive. Up-regulation of miR-1b reduced neuron viability and regenerative ability, promoted neuron apoptosis and C-caspase-3 expression, and down-regulated the expressions of cell regeneration-related proteins. KLF7 was the target gene of miR-1b. Overexpressed KLF7 rescued the effects of up-regulation of miR-1b on neuron viability, regeneration and apoptosis. Expressions of NGF, CNTF and BDNF were suppressed yet C-caspase-3 expression was up-regulated by miR-1b mimic, which was partially rescued by overexpressed KLF7.*

Conclusions: *Up-regulation of miR-1b promoted rat neuron proliferation and regeneration yet inhibited apoptosis via targeting KLF7.*

Key words: *miR-1b, Krüppel-like factor 7, neuron, cell regeneration.*

Introduction

The main function of neurons is to process and transmit information, which is realized by integrating information across thousands of their synaptic inputs [1]. A large number of neuron deaths take place during development and pathology, especially due to the limited ability of adult neurons to proliferate or be replaced [7]. Currently, although considerable progress has been obtained in curing neuron apoptosis and regeneration, many questions about their mechanisms remain to be answered [23].

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MicroRNAs (miRNAs) are a class of small non-protein coding RNA with 21-25 nucleotides in length, and function as essential regulators of gene expression by inhibiting the degradation of their target mRNAs and repressing translation [24]. MiRNAs that are located in local processes of neurons regulate protein synthesis, and their expression is regulated by synaptic activity [9]. Increasing evidence has shown that miRNAs are involved in neuron apoptosis and regeneration. For example, Chen *et al*. [5] found that rat neuron apoptosis was reduced by up-regulation of miR-23a after hypoxia exposure, suggesting that miR-23a may be a potential target for alleviating neuron apoptosis. In addition, it has been reported that miR-135s promoted central nervous system axon regeneration after nerve injury in adult mice *via* silencing Krüppel-like factor 4 (KLF4) [30]. Furthermore, some miRNAs have been reported to play a positive role in neuron apoptosis and regeneration. For instance, Gaudet *et al*. [8] discovered that miR-155 up-regulation decreased spontaneous axon growth from neurons after spinal cord injury. And it has been identified that down-regulated miR-1b promoted neuron regeneration of peripheral nerve injury by targeting NDRG3 [20]. Nevertheless, the function of miR-1b in neurons is still poorly studied. Therefore, our study focused on further unveiling the role of miR-1b in neuron apoptosis and regeneration.

Krüppel-like factor 7 (KLF7) is a member of the KLF family of zinc finger transcription factors, and has a low expression level in numerous human tissues [6]. Previous studies have shown that KLF7 regulated the lineages of the neuroectoderm and mesoderm and powerfully regulated the development of many other organs [4]. Moreover, Li *et al*. [18] found that KLF7 overexpression in bone marrow stromal stem cells graft transplantation promoted sciatic nerve regeneration, suggesting that KLF7 functioned as a positive role in curing neural injury. In addition, a previous study has revealed that inhibition of miR-146b promotes sciatic nerve regeneration *via* targeting KLF7 [19]. Therefore, as has been previously reported, miRNA-targeting techniques could be used as an alternative therapeutic approach to the exogenous application of KLF7 on neuron regeneration [33]. However, there are only limited studies on the relationship between miR-1b and its potential target KLF7 in neurons.

Taken together, in this study we set out to explore the effect of miR-1b on neurons and discover whether KLF7 is directly targeted by miR-1b and whether down-regulation of miR-1b affects neuron proliferation, regeneration and apoptosis.

Material and methods

Ethics statement

The guidelines of the China Council on Animal Care and Use were strictly followed during animal experiments. This study was approved by the Committee of Experimental Animals of Inner Mongolia Baogang Hospital (approval number IMBH20191106). The animal experiments were carried out in the Inner Mongolia Baogang Hospital. Maximum effort was made to minimize pain to the animals.

Neuron isolation and culture

Male Wistar rats (180-220 g, 18 days) were purchased from the Experimental Animal Centre of the Zhejiang Province. Firstly, the rats were soaked in 75% alcohol for about 3 minutes and then anesthetized with phenobarbital (45 mg/kg). Then the brain of the rats was taken out and placed in 8 ml of phosphate-buffered saline (PBS) with high-glucose on ice, and the meninges and large blood vessels in the brain were removed. Subsequently, the rest of the brain was removed except for the cerebral cortex. The cerebral cortex was cut into small pieces of about 1 cubic millimetre, and then 500 µl of 2.5% trypsin and 300 µl of DNA enzyme was added. The cells were digested at 37° with 5% CO_2 for 30 minutes, followed by addition of 3 ml of Dulbecco's modified Eagle's medium (DMEM, Solarbio, Beijing, China). After precipitation, the supernatant was obtained and filtered through a cell sieve. The cells were then seeded into 6-well plates at an adjusted concentration of 5×10^5/ml, followed by incubation at 37° with 5% CO_2 for 6 h. Afterwards, the DMEM was replaced with Neurobasal-A medium (10888022, Thermo Fisher Scientific, USA), and the neurons were incubated for another 7 days [20].

Neuron morphological identification and immunofluorescence staining

Neuron morphological identification was performed on days 3, 5 and 7 by using an optical microscope (BX53M, Olympus, Japan). After the neurons were incubated for 72 h, they were washed with PBS (0.01 mol/l, pH 7.4) three times, fixed with 4% paraformaldehyde for 20 min, and permeated with 0.1%

TritonX-100 (X100, Sigma-Aldrich, USA) for 30 min. Subsequently, 5% albumin from bovine serum (BSA; #9998, Cell Signaling Technology, USA) was employed for sealing for 20 min. Next, the neurons were incubated with primary antibody anti-MAP-2 antibody (ab183830, 1 : 200, Abcam, UK) overnight at 4%, followed by 1 h incubation with the secondary antibody goat anti-rabbit IgG H&L (ab150077, 1 : 500, Abcam, UK) at 37° without light. Before sealing with glycerine, 1 µg/ml of DAPI staining solution (C1005, Beyotime, China) was added to incubate the neurons for 4 min. The neuron growth states were analysed *via* ImageJ (version 5.0; Bio-Rad, Hercules, CA, USA).

Transfection

The miR-1b mimic (M; sequence: 5'-UGGAAUGU AAAGAAGUAUGUAU-3'), miR-1b inhibitor (I; sequence: 5'-AUACAUACUUCUUUACAUUCCA-3'), and their negative controls (mimic control – MC, inhibitor control – IC) were purchased from GenePharma (Shanghai, China) and then transfected into neurons using Lipofectamine 2000 Transfection Reagent (Invitrogen, USA) in strict accordance with the manufacturer's protocols.

Meanwhile, the small interfering RNA for KLF7 (siKLF7) and negative control siRNA (siNC) were obtained from RiboBio Co., Ltd. (Guangzhou, China) for this study, and pcDNA3.1 plasmid carrying overexpressed KLF7 was purchased (VT1010, YouBio, China). They were transfected into neurons (2.5×10^3 cells per well) using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Neurons transfected with empty plasmid were used as the control group. In this paper, the siRNA sequences used for KLF7 were listed below: KLF7 siRNA: sense 5'-GCCAACCAGCUCUUCUCUATT-3', antisense 5'-UAGAGAAGAGCUGGUUGGCTT-3'; si-negative control (siNC): sense 5'-UUCUCCGAACGUGUCACGUTT-3', antisense 5'-ACGUGACACGUUCGGAGAATT-3'. After transfection for 48 h, cells were collected for subsequent analysis.

RNA extraction and real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from the neurons by using the TRIzol reagent (D9108A, Takara Bio) based on the manufacturer's instructions. RNA concentration was quantified with a spectrophotometer (ND-LITE, Automation Technologies Online, Thermo Fisher Scientific, USA). The mRNA expression of KLF7 was measured with a SYBR Premix Ex Taq™ kit (Takara, Dalian, China). For miR-1b expression detection, a miRcute Plus miRNA qPCR Detection Kit (Tiangen, China) was applied. All RT-PCR reactions were conducted on the ABI PRISM 7500 System (Applied Biosystems, Foster City, CA) with the following parameters: 95°C for 5 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 70°C for 10 s. GAPDH (for KLF7) and U6 (for miR-1b) were used as internal references. The primer sequences in this study were listed in Table I. The relative gene expression was analysed *via* the $2^{-\Delta\Delta Ct}$ method [21].

MTT assay

Neuron viability was detected *via* MTT assay. Firstly, neurons (1×10^4 cells/well) were seeded in a 384-well plate, and then 36 µl of MTT reagent (A13261, Thermo Fisher Scientific, USA) was added into each well. Subsequently, the neurons were incubated at 37° with 5% CO_2 overnight. And then 1.5 ml of dimethyl sulfoxide (DMSO) was employed to dissolve the formazan. A multifunction enzyme-linked analyser (Varioskan LUX, Thermo Fisher Scientific, USA) was used to detect the absorbance value OD at 490 nm for 4 hours.

Flow cytometry

Neuron apoptosis was measured by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (BMS500FI, Thermo Fisher Scientific, USA). After transfection, neurons were washed with cold PBS three times, followed by 48-hour incubation. Subsequently, the neurons were supplemented with 5 µl of Annexin V and 5 µl of propidium iodide (PI) together

Table I. Primer for qRT-PCR

Gene	Primers
MiR-1b	
Forward	5'-ATGTCGTATCCAGTGCAATTGC-3'
Reverse	5'-GTCGTATCCAGTGCGTGTCG-3'
KLF7	
Forward	5'-TTTCCTGGCAGTCATCTGCAC-3'
Reverse	5'-GGGTCTGTTTGTTTGTCAGTCTGTC-3'
GAPDH	
Forward	5'-GGAGAGTTTTTGGTTTTAGGGTTAG-3'
Reverse	5'-AAAACCTCCTATAATATCCCTCCTC-3'
U6	
Forward	5'-CCATCGGAAGCTCGTATACGAAATT-3'
Reverse	5'-GGCCTCTCGAACTTGCGTGTCAG-3'

without light for 15 minutes at room temperature. A flow cytometer (ACEA NovoCyte, ACEA Biosciences, Inc., USA) was applied to detect neuron apoptosis.

Western blot

The neurons were lysed using RIPA buffer (#9806, Cell Signaling Technology, Danvers, MA, USA). Protein concentration was determined with a Bicinchoninic protein assay (BCA) kit (7780S, Cell Signaling Technology, Danvers, MA, USA). All the protein samples were then electrophoresed *via* 10% SDS-PAGE (P0012A Beyotime, China). Later, the samples were transferred to polyvinylidene fluoride membranes (PVDF; Millipore, Bedford, MA) which were blocked by 5% skimmed milk at room temperature for 60 min. The membranes were then incubated with the following primary antibodies overnight at 4°C: anti-GAPDH antibody (rabbit, #5174, 1 : 1000, Cell Signaling Technology, USA), anti-NGF antibody (rabbit, ab221609, 1 : 1000, Abcam, UK), anti-C-caspase-3 antibody (rabbit, ab2302, 1 µg/ml, Abcam, UK), anti-CNTF antibody (rabbit, ab46172, 1 : 3000, Abcam, UK) and anti-BDNF antibody (rabbit, ab108319, 1 : 1000, Abcam, UK). The membranes were then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody goat anti-rabbit IgG H&L (HRP) (ab205718, 1 : 2000, Abcam, UK) for 1 h at room temperature and washed with tris-buffer saline tween (TBST) three times. GAPDH was employed as the normalization reference. An enhanced chemiluminescence (ECL) plus kit (K22030, Abbkine Scientific, China) was employed to measure the labelled proteins [36].

Prediction of target gene and dual-luciferase reporter assay

Target genes and potential binding sites of KLF7 and miR-1b were predicted by TargetScan V7.2 (http://www.targetscan.org/vert_72/) and then confirmed by dual-luciferase reporter assay.

PMIR-REPORT Luciferase vectors (AM5795; Thermo Fisher Scientific, USA) which contained KLF7 sequences (wild-type or mutated) were cloned into the pMirGLO reporter vector (Promega, Madison, WI, USA) to construct KLF7-WT and KLF7-MUT. Neurons (5×10^3 cells/well) were incubated in 96-well plates, and then luciferase reporter plasmids were co-transfected with miR-1b mimic (M), inhibitor (I) or their respective controls (mimic control – MC, inhibitor control – IC) into the neurons using Lipofectamine 2000 Transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). Luciferase detection was conducted in the dual-luciferase report assay system (E1910; Promega, USA) and the luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analysis

Values were represented as mean ± SD. Statistical analysis was performed *via* the SPSS software (version 22.0, SPSS Inc., Chicago, IL, USA). Multiple comparisons were performed using one-way ANOVA, followed by a post hoc test. *P*-values of less than 0.05 were considered statistically significant.

Results

Primary culture and characterization of rat neurons

After the neurons were successfully cultured, neuron morphological identification was performed using an optical microscope. On the third day, neuronal synapses were increased, and neural networks were gradually formed; on day 5, connections in neural networks were formed in synapses; and on day 7, the neurons were plump and grew into mature neurons (Fig. 1A). Microtubule-associated protein 2 (MAP-2) was a marker of neuronal differentiation, and its function was regulated by phosphorylation both during neuronal development and after differentiation during synaptic activity for maintenance of neuronal health [28]. The results of MAP-2 immunofluorescence staining and DAPI nuclear staining showed that neuron cell body and axon exhibited good morphology and the neurons were of high purity (Fig. 1B).

MiR-1b regulated neuron viability and regenerative ability

To discover the different expression of miR-1b in neurons, the neurons were transfected with miR-1b mimic (M) or inhibitor (I), and the corresponding control groups (MC and IC) were set up at the same time. As revealed in Figure 2A, the mRNA expression of miR-1b in the M group was higher in comparison with the MC group ($p < 0.001$), suggesting that overexpression of miR-1b was successfully transfected in neurons. In addition, the results of MTT assay demonstrated that neuron viability in the M group was lower than that in the MC group (Fig. 2B, $p < 0.001$). On the contrary, neuron viability in the I group was higher when compared with the IC group (Fig. 2B, $p < 0.01$), indicating

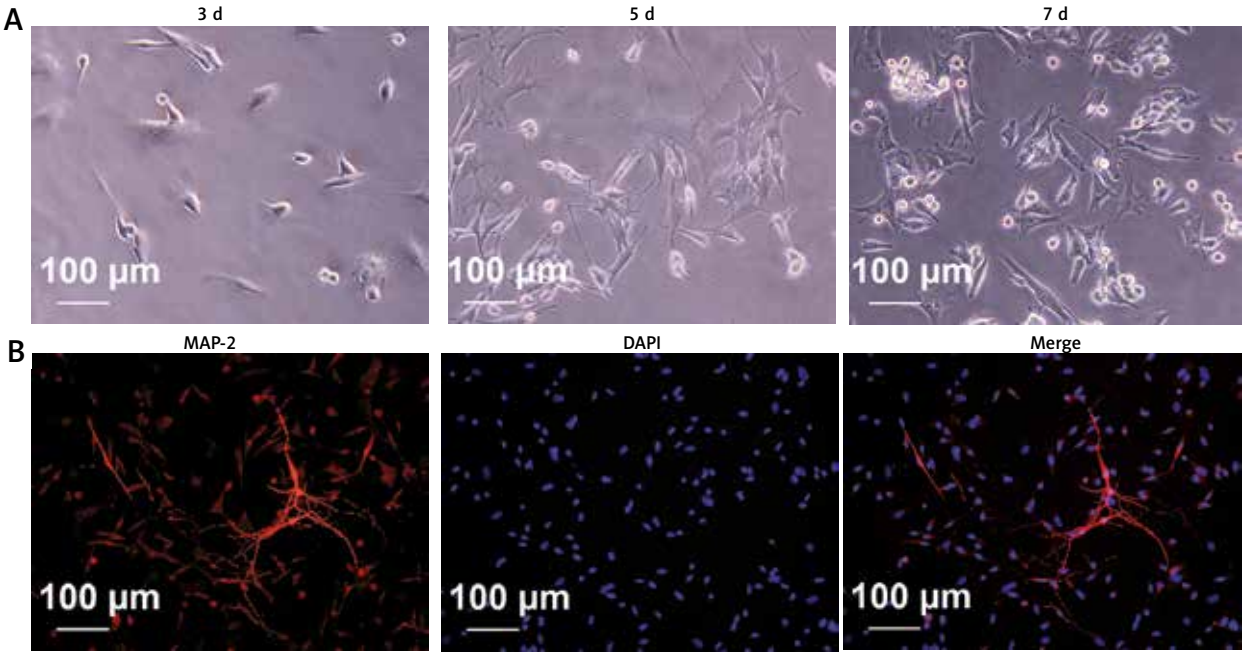

Fig. 1. Primary culture of rat neurons was successfully conducted. **A**) Rat neuron morphological identification was performed using a microscope. Magnification: 200×, scale bar = 100 μm. **B**) Neuronal identification was carried out by microtubule-associated protein 2 (MAP-2) immunofluorescence staining and DAPI nuclear staining. Magnification: 200×, scale bar = 100 μm. All experiments have been performed in triplicate and experimental data were expressed as mean ± standard deviation (SD).

that neuron viability was down-regulated *via* up-regulation of miR-1b, which was rescued by low expression of miR-1b.

Besides, neuron regenerative ability was investigated using a microscope and the Image J software after 48 hours of incubation. As shown in Figure 2C-E, the length of neurites per cell, the percentage of cells with neurites and the number of branches per cell in the M group were decreased in comparison with the MC group ($p < 0.05$). Interestingly, the above indicators in the I group were increased when compared with the IC group (Fig. 2C-E, $p < 0.05$). These data demonstrated that neuron regenerative ability was promoted by up-regulation of miR-1b, which was rescued by low expression of miR-1b.

MiR-1b regulated the neuron apoptosis and regeneration-related proteins expressions

After transfection, neuron apoptosis was detected through flow cytometry. It was clear that the apoptosis rate in the M group was significantly enhanced when compared with the MC group, while in the I group, it was lowered as compared with the IC group (Fig. 3A, $p < 0.001$).

Expressions of cell regeneration-related proteins in the transfected neurons were measured *via* Western blot. As shown in Figure 3B, the expressions of nerve growth factor (NGF), ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) in the M group were lower than those in the MC group, while these protein expressions in the I group were promoted when compared with the IC group ($p < 0.05$). Meanwhile, cleaved (C)-caspase-3 expression was up-regulated in the M group in comparison with the MC group, but it was down-regulated in the I group when compared with the IC group (Fig. 3B, $p < 0.001$), suggesting that up-regulation of miR-1b decreased the expressions of proteins related to cell regeneration but increased C-caspase-3 expression, which was rescued by low expression of miR-1b.

KLF7 was the target of miR-1b

The prediction software TargetScan V7.2 showed that KLF7 was a target of miR-1b, with the target site located in the 3'-UTR (Fig. 4A). With the aim to further validate that miR-1b directly binds to KLF7, the

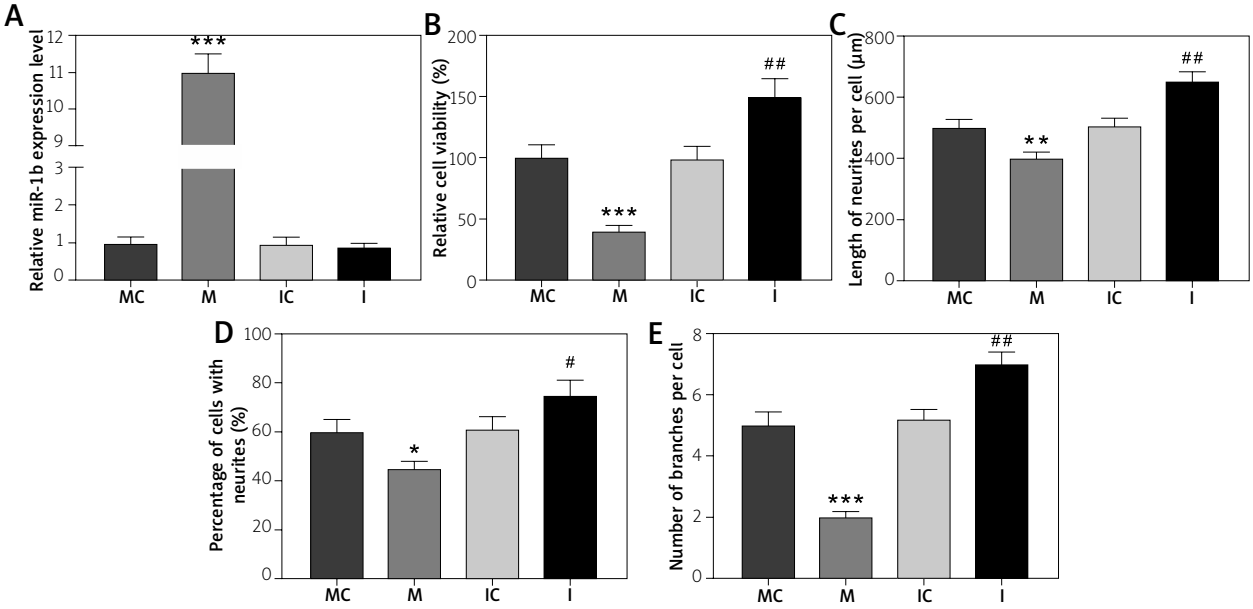

Fig. 2. MiR-1b regulated neuron viability and regenerative ability. **A**) The transfection rate of miR-1b in neurons was measured by quantitative real-time polymerase chain reaction (qRT-PCR). U6 was used as the internal reference. **B**) After transfection, neuron viability was detected through MTT assay. **C-E**) The length of neurites per cell, the percentage of cells with neurites and the number of branches per cell after transfection were investigated using a microscope and the Image J software. All experiments have been performed in triplicate and experimental data were expressed as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs. MC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. IC. MC – mimic control, IC – inhibitor control, M – miR-1b mimic, I – miR-1b inhibitor.

dual luciferase reporter assay was performed. It was discovered that the luciferase activity was reduced in the KLF7-WT-mimic group in comparison with the MC group, while it was enhanced in the KLF7-WT-inhibitor group when compared with the IC group (Fig. 4B, C, $p < 0.01$), indicating that KLF7 was targeted by miR-1b. Moreover, the results of qRT-PCR showed that miR-1b mimic mitigated the up-regulation of the KLF7 level after transfection of overexpressed KLF7 plasmid, and miR-1b inhibitor rescued the down-regulation of KLF7 level after knockdown of KLF7, which further proved that KLF7 was the target gene of miR-1b (Fig. 4D-G, $p < 0.001$).

Effects of miR-1b on neuron viability, regeneration and apoptosis through targeting KLF7

The outcomes of MTT assay revealed that the viability of neurons transfected with overexpressed KLF7 plasmid was promoted compared with the negative control (NC) group (Fig. 5A, $p < 0.01$). Besides, co-transfection of overexpressed KLF7 plasmid and miR-1b mimic weakened neuron viability compared with the KLF7 group (Fig. 5A, $p < 0.01$). Conversely, the viability of neurons transfected with specific siRNA against KLF7 or co-transfected with miR-1b inhibitor and specific siRNA against KLF7 displayed an opposite result (Fig. 5B, $p < 0.05$). These findings demonstrated that overexpressed KLF7 partially rescued the effects of up-regulation of miR-1b on neuron viability.

Through microscope observation and Image J software analysis, it was found that the length of neurites per cell, the percentage of cells with neurites and the number of branches per cell after transfection of overexpressed KLF7 plasmid were evidently promoted in comparison with the NC group (Fig. 5C-E, $p < 0.05$). Meanwhile, these regeneration indicators were up-regulated *via* co-treatment with overexpressed KLF7 plasmid and miR-1b mimic compared with the M group (Fig. 5C-E, $p < 0.05$). By contrast, transfection of specific siRNA against KLF7 or co-transfection of miR-1b inhibitor and specific siRNA against KLF7 into neurons was counter-productive (Fig. 5F-H, $p < 0.05$). These data proved that overexpressed KLF7 partially rescued the effects of up-regulation of miR-1b on neuron regeneration.

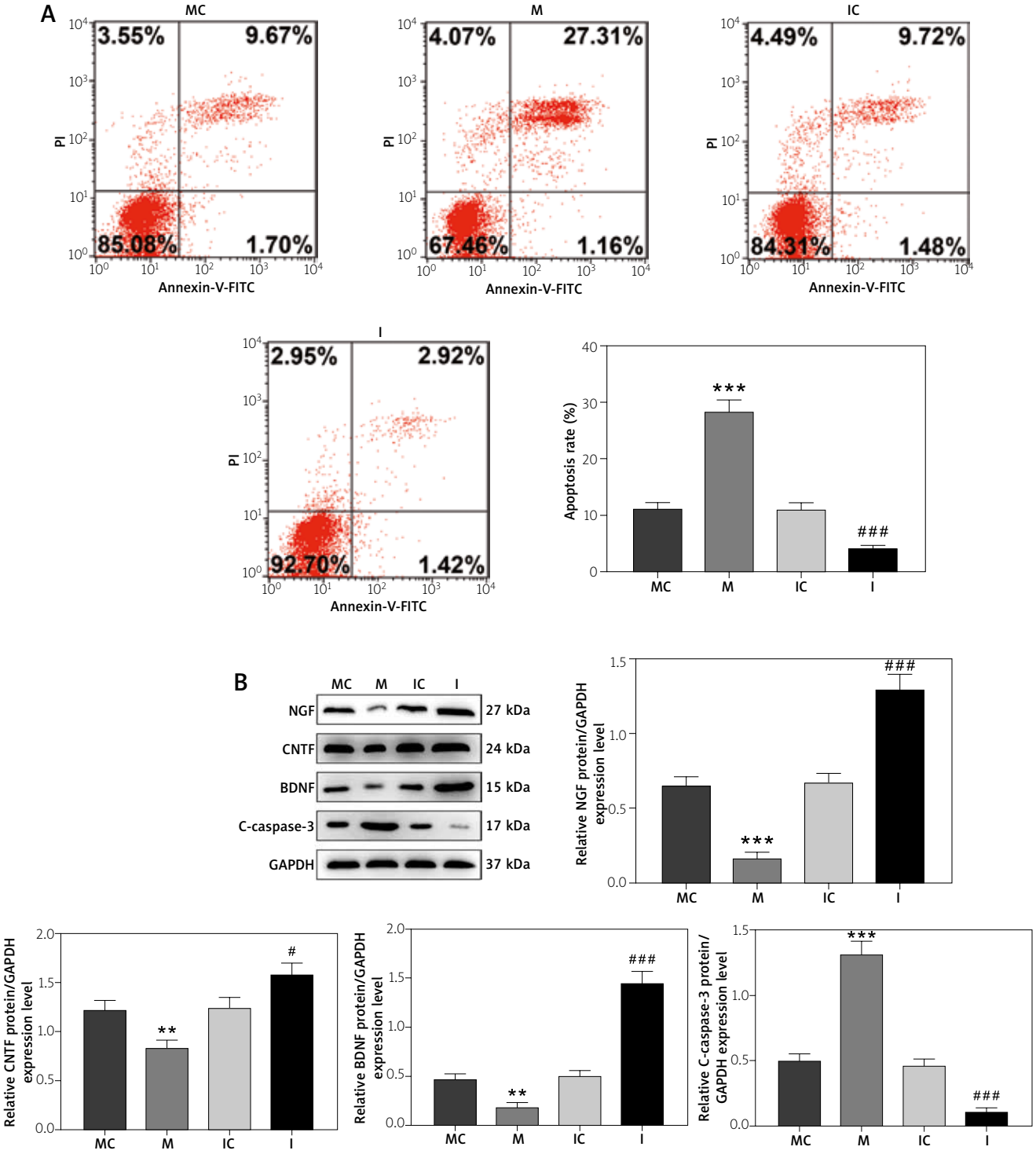

Fig. 3. MiR-1b regulated neuron apoptosis and the expressions of regeneration-related proteins. **A**) Neuron apoptosis after transfection was measured by flow cytometry. **B**) Expressions of nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF) and C-caspase-3 after transfection were detected through Western blot. GAPDH was used as the internal reference. All experiments have been performed in triplicate and experimental data were expressed as mean ± standard deviation (SD). $^{**}p < 0.01$, $^{***}p < 0.001$, vs. MC; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, vs. IC), MC – miR-1b mimic control, IC – miR-1b inhibitor control, M – miR-1b mimic, I – miR-1b inhibitor.

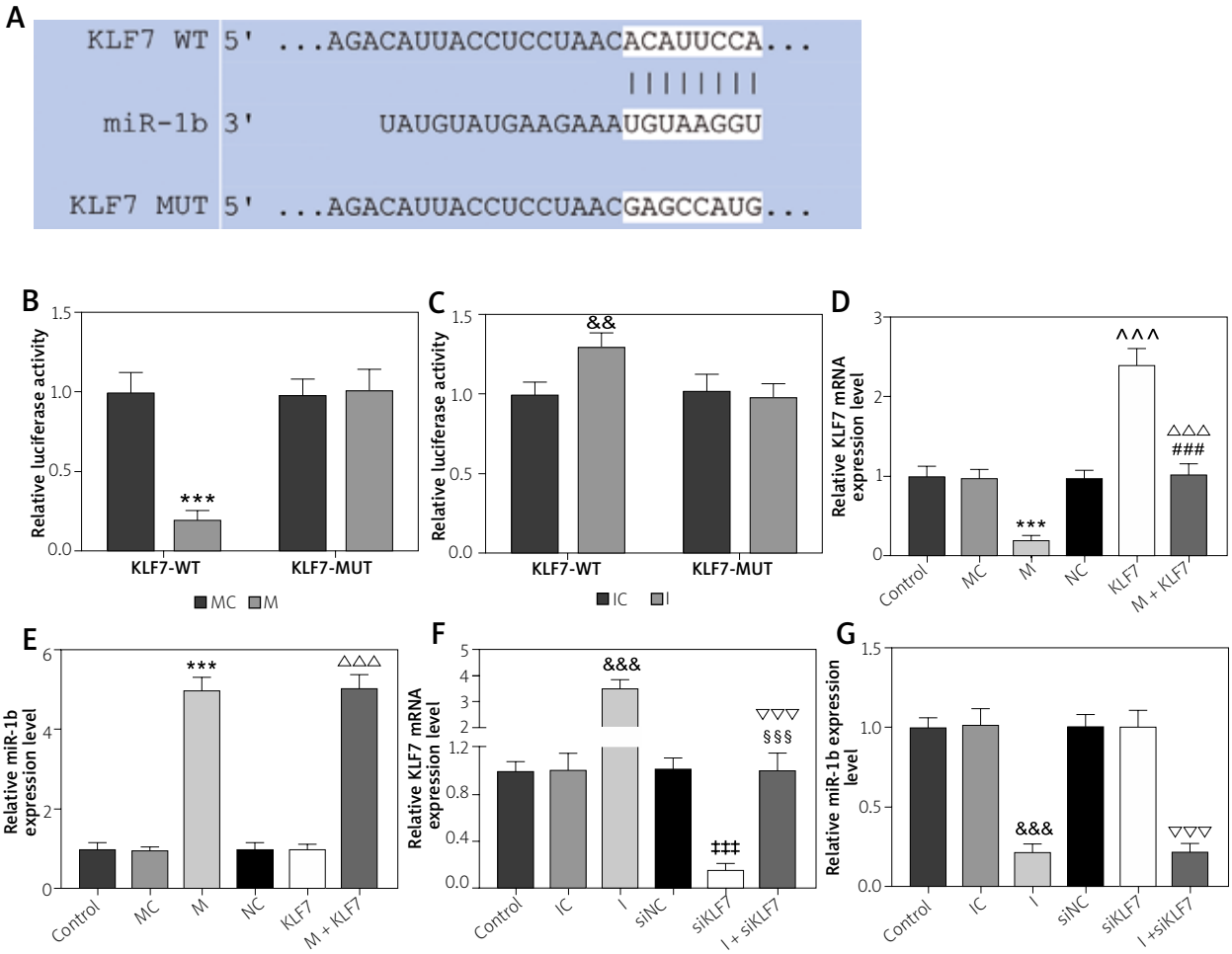

Fig. 4. KLF7 was the target of miR-1b. **A)** TargetScan V7.2 was used to predict the binding sites of miR-1b and KLF7. **B, C)** Wild-type or mutant KLF7 (KLF7-WT, KLF7-MUT) was separately co-transfected with miR-1b mimic and miR-1b inhibitor into the neuron, and luciferase activity was measured by dual-luciferase reporter assay. **D-G)** The mRNA expressions of KLF7 and miR-1b in neurons after transfection were detected by qRT-PCR. GAPDH (for KLF7) and U6 (for miR-1b) were used as internal references. All experiments have been performed in triplicate and experimental data were expressed as mean ± standard deviation (SD). $^{***}p < 0.001$, vs. MC; $^{\#\#\#}p < 0.001$, vs. M; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. IC; $^{\wedge\wedge\wedge}p < 0.001$, vs. NC; $^{\Delta\Delta\Delta}p < 0.001$, vs. KLF7; $^{\ddagger\ddagger\ddagger}p < 0.001$, vs. siNC; $^{\nabla\nabla\nabla}p < 0.001$, vs. siKLF7; $^{\S\S\S}p < 0.001$, vs. I. KLF7 – Krüppel-like factor 7, M – miR-1b mimic, I – miR-1b inhibitor, MC – miR-1b mimic control, IC – miR-1b inhibitor control, NC – negative control, siKLF7 – small interfering RNA for KLF7 (siKLF7), siNC – negative control for small interfering RNA for KLF7.

In addition, the results of flow cytometry demonstrated that neuron apoptosis was significantly reduced after transfection of overexpressed KLF7 plasmid compared with the NC group (Fig. 6A, $p < 0.001$). Co-transfection of overexpressed KLF7 plasmid and miR-1b mimic mitigated neuron apoptosis in comparison with the M group. However, the apoptosis rates of neurons transfected with specific siRNA against KLF7 or co-transfected with miR-1b inhibitor and specific siRNA against KLF7 exhibited an opposite result (Fig. 6B, $p < 0.001$). The results herein were sufficient to support that miR-1b regulated neuron viability, regeneration and apoptosis through targeting KLF7.

Effects of miR-1b on the expressions of C-caspase-3 and neuron regeneration-related proteins through targeting KLF7

The results of Western blot proved that the expressions of NGF, CNTF and BDNF were evidently increased yet C-caspase-3 expression was

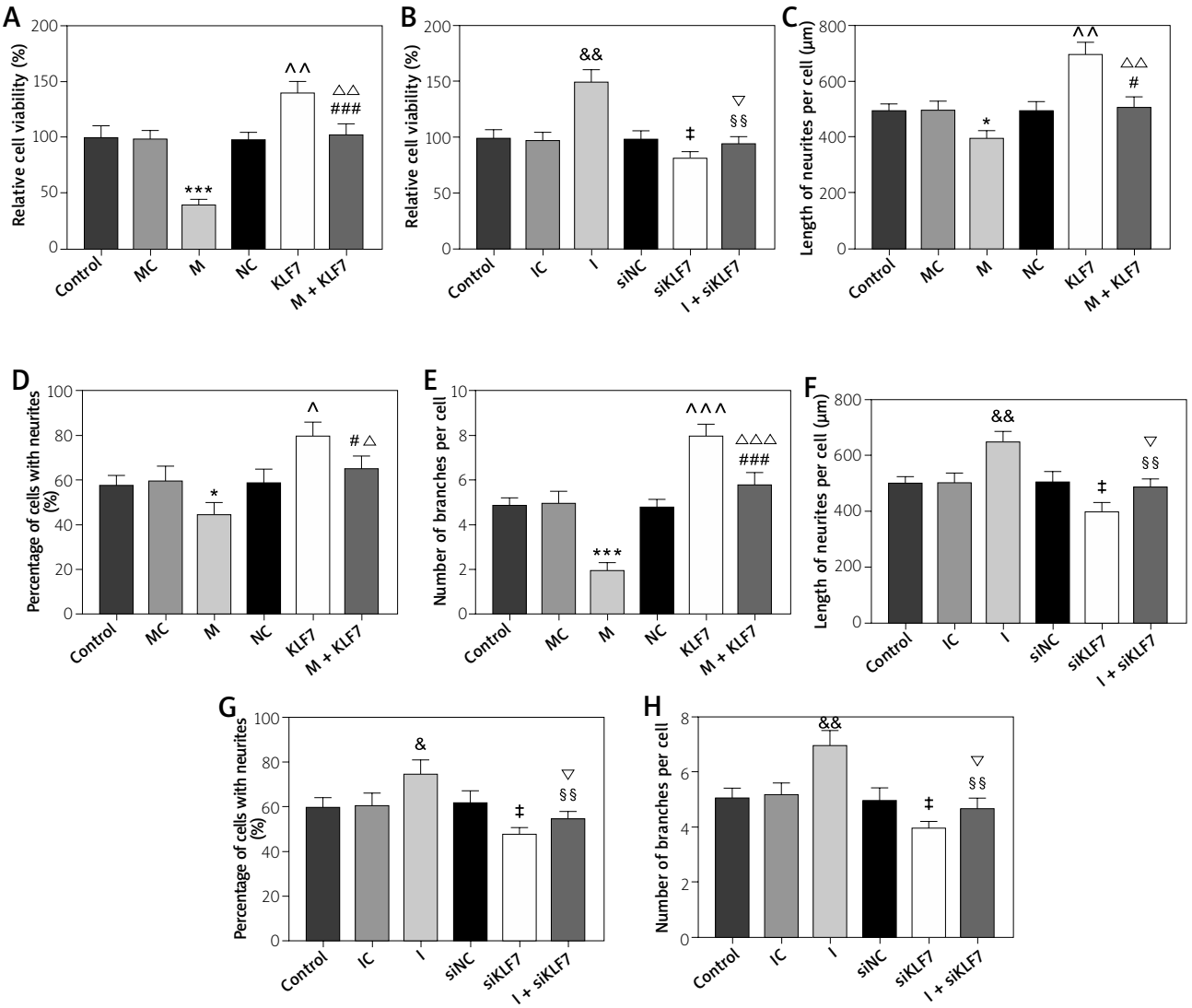

Fig. 5. Effects of miR-1b on neuron viability and regeneration through targeting KLF7. **A, B)** Inhibitory effect of overexpressed miR-1b on neuron viability *via* targeting KLF7 was detected by MTT assay, and low expressions of miR-1b and KLF7 performed the opposite function. **C-E)** Suppressive role of overexpressed miR-1b in the length of neurites per cell, the percentage of cells with neurites and the number of branches per cell *via* targeting KLF7 was investigated using a microscope and ImageJ. **F-H)** Promoting role of down-regulation of miR-1b on the length of neurites per cell, the percentage of cells with neurites and the number of branches per cell via targeting KLF7 was determined using a microscope and ImageJ. All experiments have been performed in triplicate and experimental data were expressed as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{***}p < 0.001$, vs. MC; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$, vs. NC; $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$, vs. KLF7; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, vs. M; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, vs. IC; $^{\ddagger}p < 0.05$, vs. siNC; $^{\nabla}p < 0.05$, vs. siKLF7; $^{\S\S}p < 0.01$, vs. I.

decreased in the neurons that were transfected with overexpressed KLF7 plasmid compared with the NC group (Fig. 7A, $p < 0.01$). In addition, co-transfection of overexpressed KLF7 plasmid and miR-1b mimic facilitated the expressions of NGF, CNTF and BDNF while mitigating C-caspase-3 expression compared with the M group (Fig. 7A, $p < 0.05$). On the contrary, the expressions of NGF, CNTF, BDNF and C-caspase-3 in neurons that were transfected with specific siRNA against KLF7 or co-transfected with miR-1b inhibitor and specific siRNA against KLF7 showed an opposite result (Fig. 7B, $p < 0.01$). These data revealed that

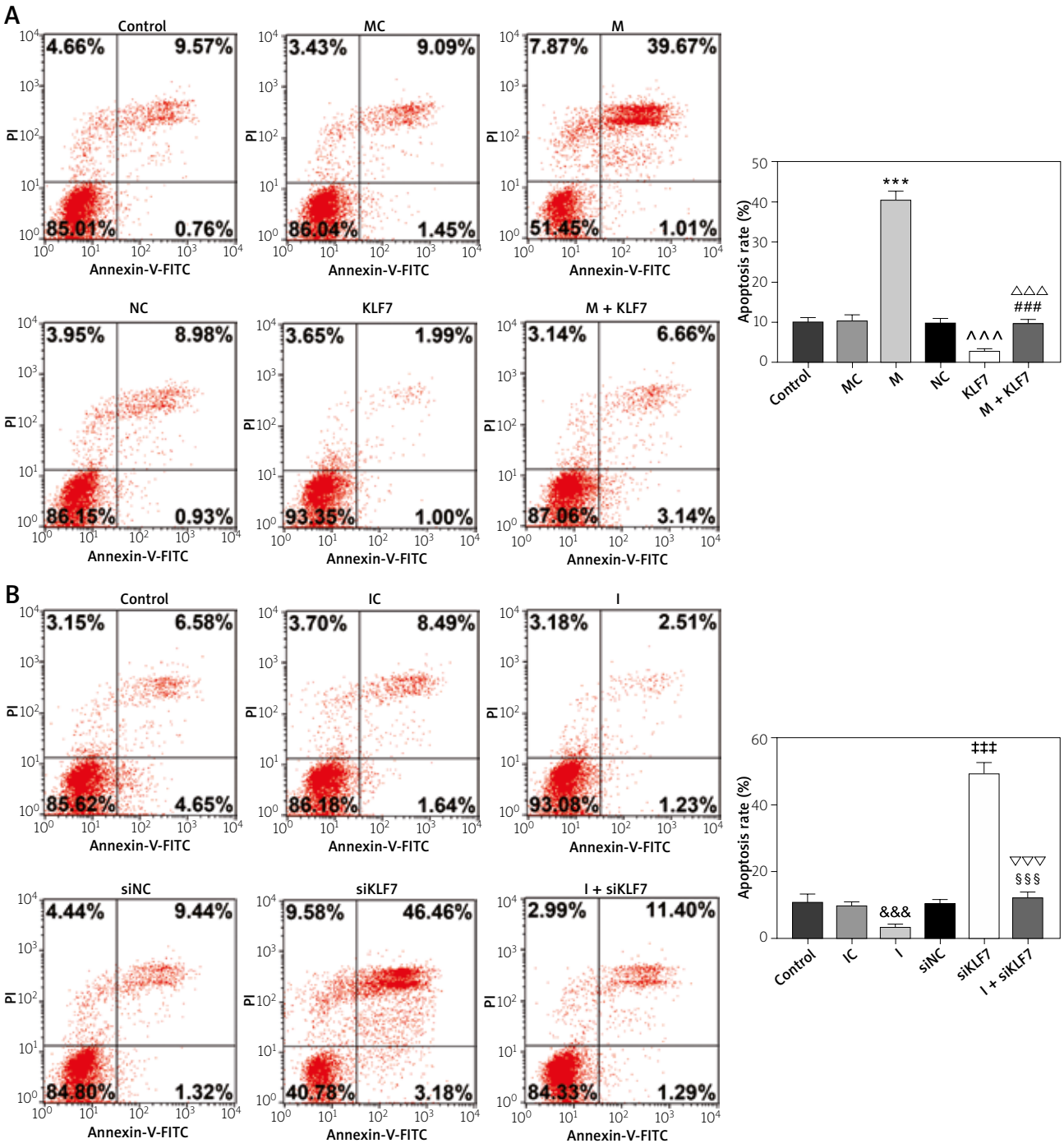

Fig. 6. Effects of miR-1b on neuron apoptosis through targeting KLF7. **A)** Positive effect of overexpressed miR-1b on neuron apoptosis *via* targeting KLF7 was measured through flow cytometry. **B)** Repressive effect of low expression of miR-1b on neuron apoptosis *via* regulating KLF7 was identified by flow cytometry. All experiments have been performed in triplicate and experimental data were expressed as mean ± standard deviation (SD). $^{***}p < 0.001$, vs. MC; $^{\wedge\wedge\wedge}p < 0.001$, vs. NC; $^{\Delta\Delta\Delta}p < 0.001$, vs. KLF7; $^{\#\#\#}p < 0.001$, vs. M; $^{\&\&\&}p < 0.001$, vs. IC; $^{\ddagger\ddagger\ddagger}p < 0.001$, vs. siNC; $^{\nabla\nabla\nabla}p < 0.001$, vs. siKLF7; $^{\S\S\S}p < 0.001$, vs. I.

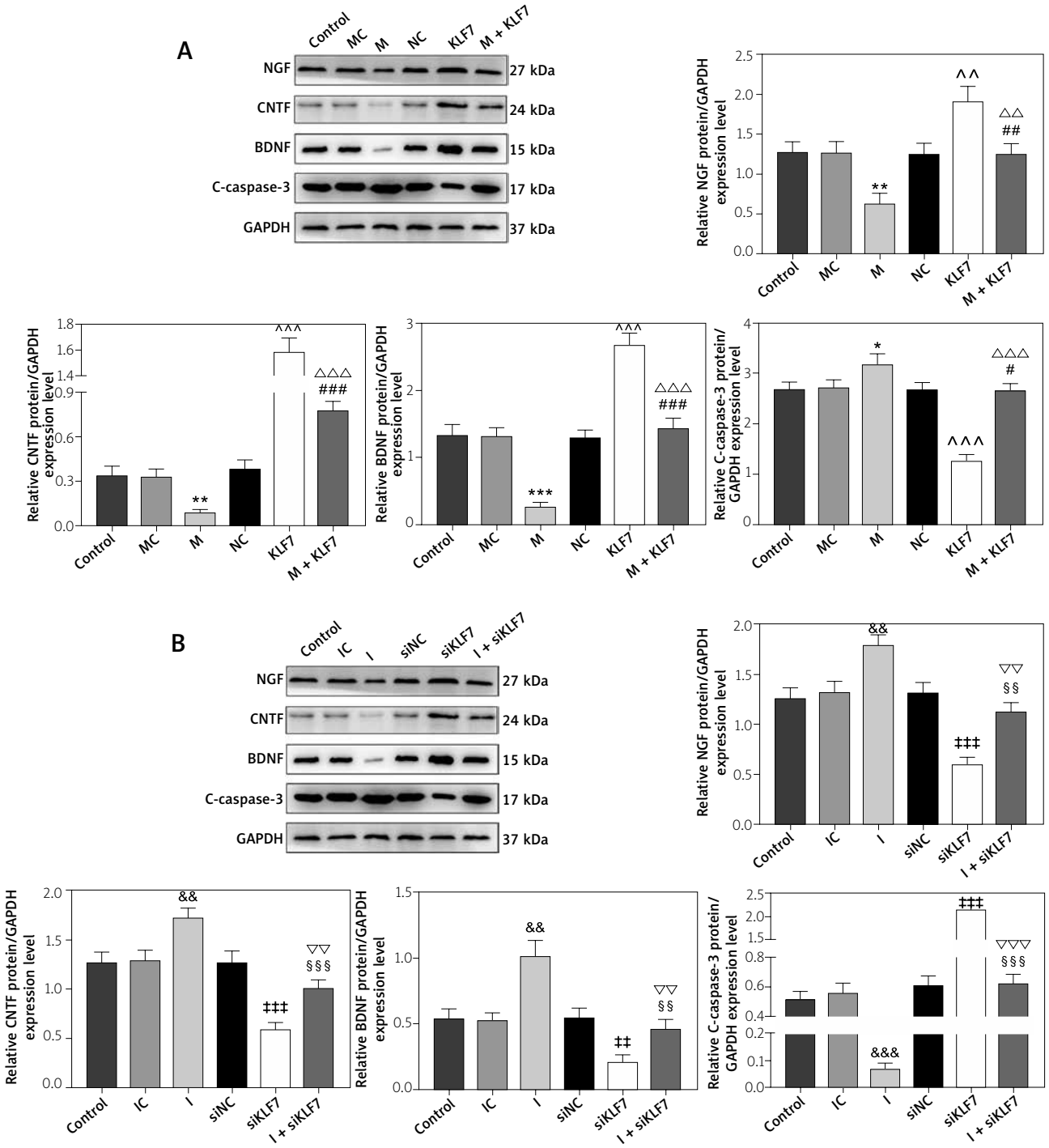

Fig. 7. Effects of miR-1b on the expressions of C-caspase-3 and neuron regeneration-related proteins through targeting KLF7. **A)** Effect of overexpressed miR-1b on inhibiting the expressions of nerve growth factor (NGF), ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) and promoting cleaved (C)-caspase-3 expression *via* targeting KLF7 was detected by Western blot. GAPDH was used as the internal reference. **B)** Effect of low expression of miR-1b on promoting the expressions of NGF, CNTF and BDNF but suppressing C-caspase-3 expression *via* targeting KLF7 was investigated by Western blot. GAPDH was used as the internal reference. All experiments have been performed in triplicate and experimental data were expressed as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs. MC; $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$, vs. NC; $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$, vs. KLF7; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. M; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, vs. IC; $^{\ddagger\ddagger}p < 0.01$, $^{\ddagger\ddagger\ddagger}p < 0.001$, vs. siNC; $^{\nabla\nabla}p < 0.01$, $^{\nabla\nabla\nabla}p < 0.001$, vs. siKLF7; $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$, vs. I.

overexpressed KLF7 partially rescued the effects of up-regulation of miR-1b on the expressions of C-caspase-3 and neuron regeneration-related proteins.

Discussion

Recovery from central nervous system (CNS) injury is limited by the inability of axons to regenerate and rebuild effective communication [33]. Besides, axon growth can be promoted *via* targeting the intrinsic growth capacity of neurons through forced expression of regeneration-associated transcription factors [2]. In addition, increasing evidence has shown that a great number of miRNAs are involved in neuron generation *via* regulating their target genes, which may be transcription factors [10,22].

For figuring out the regulatory role of miRNAs in neurons of the brain, it is essential to conduct neuron-specific analysis of miRNAs and their target genes. It has been reported that miR-1 was down-regulated following sciatic nerve injury that increased the expression and secretion of BDNF, which benefits nerve growth and regeneration [34]. Neumann *et al*. discovered that miR-1 expression in the sciatic nerve was time-dependently decreased and the protein expressions of the miR-1 targets BDNF and Connexin 43 were upregulated in the sciatic nerve and dorsal root ganglion [27]. MiR-1b is a member of the miR-1 family with a highly homologous sequence to miR-1 [20]. In our study, miR-1b mimic and inhibitor were transfected into primarily cultured neurons, and consistent with previous findings, we discovered that a low expression of miR-1b rescued the reduction of neuron viability and regenerative ability caused by up-regulation of miR-1b [20].

Nerve growth factor is a well-studied neurotrophic factor and one of the first identified growth factors [11]. Ciliary neurotrophic factor is a potent inducer of axonal regeneration [12]. Moreover, BDNF is a member of the neurotrophin family of secreted proteins [29] and Caspase-3 is a major mediator of activated apoptosis [35]. According the results of Western blot in our study, the expressions of NGF, CNTF and BDNF were down-regulated but cleaved (C)-caspase-3 expression was up-regulated in neurons by overexpression of miR-1b, which further proved that overexpression of miR-1b inhibited neuron regeneration.

The Krüppel-like transcription factors (KLFs) are important regulators of cell proliferation and differentiation as well as embryonic development [14]. More recently, KLFs have proved as important regulators of the neuron-intrinsic capacity for regeneration, with various members displaying either anti- or pro-regenerative roles [32]. For example, knocking out KLF4 enhanced axon growth and regeneration in developing retinal ganglion cells, and KLF4 acted independently on cell survival to suppress axon and dendrite initiation and elongation in hippocampal neurons *in vitro* [25]. The inhibition of miR-124 expression promoted motor neuron regeneration through targeting KLF6 and STAT3 [26]. KLF7, a member of the KLFs family, has been described as a regulator of axon outgrowth [31]. Moreover, Kajimura *et al*. demonstrated that loss of KLF7 primarily affects the activity of genes involved in olfactory sensory neuron differentiation, axonal growth, cytoskeletal dynamics, cell adhesion and synaptogenesis [13]. In central nervous system and peripheral nerve injury, KLF7 plays a positive role through promoting growth, axonal regeneration, and sprouting in injured nerves [3,16]. Importantly, KLF7 has been reported to be the target gene of miR-146b, and inhibition of miR-146b promotes sciatic nerve regeneration *via* regulating KLF7 [17], suggesting that KLF7 may be beneficial to neuron regeneration. However, whether KLF7 was the target of miR-1b and the interaction of KLF7 and miR-1b in neurons remain elusive.

In our study, we further identified that KLF7 was the target gene of miR-1b. Our experimental results uncovered that overexpressed KLF7 rescued the effects of up-regulating miR-1b on neuron viability, regeneration and apoptosis, and also partially rescued the effect of up-regulating miR-1b on the expressions of NGF, CNTF, BDNF and C-caspase-3. However, the mechanism by which KLF7 played its role in regulating neuron apoptosis and regeneration-related proteins was not well illuminated, which was a limitation in our study. It was reported that KLF7 may directly regulate TrkA, which is a crucial nerve growth factor receptor during embryogenesis and in adulthood [15]. It is also known that TrkA and NGF were both target genes of KLF7 [4]. These discoveries suggested that KLF7 might participate in neuron regeneration through directly regulating the transcription progress of regeneration-related genes. However, the effects of miR-1b through regulating KLF7 in neuron death-associated diseases still needed further investigation.

Taken together, the investigations of our study demonstrated that up-regulation of miR-1b could inhibit rat neuron proliferation and regeneration yet promote apoptosis *via* targeting KLF7. In any event, the involvement of the miRNA system in gene expression regulation for neuron regeneration may be an additional mechanism to fully understand the mechanisms underlying nerve regeneration.

Disclosure

The authors report no conflict of interest.

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