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MiR-137–3p rescue motoneuron death by targeting calpain-2

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ABSTRACT

Brachial plexus root avulsion (BPRA) is a type of injury that leads to motor function loss as a result of motoneurons (MNs) degeneration. Here we identified that the reduced expression of rat miR-137–3p in the ventral horn of spinal cord was associated with MNs death. However, the pathophysiological role of miR-137–3p in root avulsion remains poorly understood. We demonstrated that the calcium-activated neutral protease-2 (calpain-2) was a direct target gene of miR-137–3p with miR-137–3p binding to the 3′-untranslated region of calpain-2. Silencing of calpain-2 suppressed the expression of neuronal nitric oxide synthase (nNOS), a primary source of nitric oxide (NO). After avulsion 2 weeks, up-regulation of miR-137–3p in the spinal cord reduced calpain-2 levels and nNOS expression inside spinal MNs, resulting in an amelioration of the MNs death. These events provide new insight into the mechanism by which upregulation of miR-137–3p can impair MN survival in the BPRA.

1. Introduction

Brachial plexus root avulsion is common in traffic accident, leading to limb muscle atrophy and chronic pain [1], which is predominantly ascribed by the deprivation of neurotrophic input from target muscles. As a consequence, that would cause important pathological changes which are responsible for motoneurons (MNs) death [2,3]. The current treatment of root avulsion show limited efficacy to be used routinely in repair so far [4]. Gene therapy is likely to be an approach to improve the outcome of root avulsion [5]. MicroRNA (miRNA), a novel group of small noncoding RNAs, serve as post-transcriptional regulators of downstream protein expression through inhibit mRNA transcription or degraded mRNA [6]. The characteristics of miRNAs include their small size and their capability of regulating multiple targeting genes [7]. Twenty-two oligonucleids of small size are able to cross the BBB with the aid of an appropriate vector such as nanoparticles [8] and one miRNA can regulate multiple protein functions, which produces important effects [9,10]. In our previous study, using microarray analysis, we have identified rat miR-137–3p gene involved in root avulsion. The reduced expression of miR-137–3p coincides with an increased MNs death [11]. Initial studies suggest that miR-137 is enriched in neurons [12] and play an important role in regulating ketamine induced hippocampal neurodegeneration through CDC42 [13]. However, the role of miR-137–3p in root avulsion has not been investigated.

In the present study, we measured miR-137–3p expression at 2 weeks after avulsion. Mechanistically, we examined an as yet undescribed miR-137–3p target, calcium-activated neutral protease-2 (calpain-2), a transcript predicted to be a miR-137–3p target by TargetScan, that is involved in cell death in the nervous system [14,15] like the apoptosis of adult motor neurons [16]. Once activated, calpains degrade cytoskeletal and membrane protein, which result in the breakdown of cellular architecture and finally apoptosis [17]. Besides, the role of neuronal nitric oxide synthase (nNOS) in MN death was recognized by Wu and our previous laboratory studies [18–20]. De novo expression of nNOS in injured MNs might be considered a signal of impending death of injured cells [18,21,22]. As the total number of MNs in the injured segment decreased gradually from 2 to 4 weeks following avulsion, focus of the present study was to evaluate the involvement of miR-137–3p and calpain-2 as well as their relationship with nNOS in 2 and 4 weeks of the injury.

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2. Material and methods

2.1. Animal surgery

Spinal root-avulsion of the right C7 was performed as described in our previous publication [23,24]. Briefly, Animals were anaesthetized with a mixture of ketamine (80 mg per kg of body weight) and xylazine (8 mg per kg), by intraperitoneal injection. Following the retraction of the paravertebral muscles, the right C6 laminectomy was performed under a surgical microscope, and then the dura mater was opened. After identifying the C7 segment of the spinal cord, the dorsal and ventral roots were selectively avulsed one by one. The proximal residual rootlets and the distal parts of the C7 avulsed roots were cut away to ensure that spinal MNs would not regrow axons into the C7 nerve in the avulsed rats. For sham operative control, only the right C7 was identified but not injured. All animal procedures were conducted with the approval of the Animal Research Ethics Board at Zhongshan School of Medicine at Sun Yat-sen University (protocol number: SCXK 2011–0029), and the animal procedures were performed in accordance with the Chinese National Institutes of Health Guide for Animal Care.

2.2. Lentiviral vector system construction

The chemical synthesis of miR-137–3p or control oligonucleotide were connected to the vectors GV271 by cutting the restriction sites of HpaI and XhoI. After identifying the bacterial clones, miR-137–3p DNA was extracted with a plasmid DNA extraction kit. Then miR-137–3p was transfected into 293T cells and GFP expression was observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Oligonucleotide sequences for the miR-137–3p gene were UUAUUGC UUAAGAAUACGCGUAG and the control sequences were TTCTCCGAA CGTGTCACGT.

2.3. Intraspinal microinjection

The injection protocol was similar to previous studies [25,26]. Lentiviral vectors encoding miR-137–3p or lentiviral control vectors (Gikai, shanghai, China) were injected into the gray matter of the spinal cord at C7 in adult rats. Injections were placed at 0.5 mm lateral to the midline at a depth of 1.3 mm to target motor neurons in the ventral horn. Injections were made using a heat-pulled glass capillary needle (tip diameter 60 um) to limit damage to the spinal cord. For injection site, a volume of up to 2 ul of lentivirus (10^5 TU) was slowly infused over 5 min. Intraspinal injection of miR-137–3p or miR-NC was verified by immunofluorescence staining, PCR and western blot.

2.4. Cell culture

Differentiated PC12 cells, a rat neuronal cell line derived from pheochromocytoma cells, were purchased from the Cell Resource Center, Shanghai Institute for Biological Sciences, China Scientific Academy (Shanghai, China). This cell line has already been well differentiated and is genetically stable. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose; Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin A and 100 g/l streptomycin; Gibco-BRL) at 37 ˚C in a humidified 5% CO2 incubator. The infection experiment was performed following 3–4 passages when cells were in a stable state without add NGF to differentiate PC12 cells.

2.5. Cell infection with lentiviral virus

PC12 cells were plated in a 60-cm dish with complete medium and infected by lentiviral mU6-MCS-Ubi-GFP. Appropriate amount of virus [miR-137–3p or negative control, at multiplicity of infection of 100 (MOI = 100)] were diluted into fresh complete media (contain 5 ug/mL of polybrene) on the next day. Then the old medium was changed with the medium containing virus, and cells were incubated overnight. The following day, the medium containing virus was removed and replaced with fresh and complete culture medium. PC12 cells were harvested 96 h after transduction and the total RNA or protein was isolated. Meanwhile, half of miR-137-3p- or miR-NC-PC12 cells were passaged in the 25-cm^2 flask.

2.6. RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from cultured cells using Trizol Isolation Reagent (TAKARA, China) according to the manufacturer's instructions. 500 ng RNA samples were converted to cDNA by using the PrimeScript™ RT reagent Kit (TAKARA, China). The expression levels of miR-137–3p and mRNA level was detected TaKaRa SYBR® Premix Ex Taq™ II (TAKARA, China) and U6 was used as an internal control. The miRNA and U6 primers were purchased from Ribobio (Guangzhou, China). The calpain-2 and nNOS primers were purchased form TAKARA (China).

calpain-2 primers
Forward:5′-gAAATCgAggCCAACATTgAAgA-3′
Reverse: 5′-CTCCAgCCAgCTgAgCAAAC-3′
nNOS primers
Forward:5′-CCTATgCCAAgACCCTgTgTgA-3′
Reverse: 5′-CATTgCCAAAggTgCTggTg-3′

qRT-PCR was performed on conditions: 95 ˚C for 30 s, 40 cycles of 95 ˚C for 5 s, 60 ˚C for 30 s using the iCycler iQ5 detection system (Bio-Rad, Cambridge, MA, USA). Relative expression was determined by calculating the mean difference between cycle thresholds of the target gene from the U6 normalized control for each sample [Δ cycle threshold (ΔCT)] within each sample group and expressed as -ΔCT for relative change in expression. Fold change in target gene expression was expressed as fold-change (2-ΔΔCT) [27].

2.7. Immunoblotting

Proteins were separated in SDS-polyacrylamide gels, electrophoretically (Bio-Rad, Cambridge, MA, USA) transferred to polyvinylidenedifluoride membranes(Millipore, Darmstadt, Germany) then detected with antibodies. The sources of antibodies were as follows: mouse anti-calpain-2 primary antibody (1:1000; Dallas, Texas, Santacruz, USA), rabbit anti-CaMKII primary antibody (1:500; Dallas, Texas, Santacruz, USA), goat anti-p-nNOS at Ser847 primary antibody (1:400; Dallas, Texas, Santacruz, USA), mouse anti-GAPDH primary antibody (1:2000; ABclonal, Maryland, USA) was used as an internal control. All results were reproduced in three independent experiments, and the representative immunoblots were shown.

2.8. Immunofluorescence

At 2w and 4w post-lesion period, animals were administered a lethal dose of chloral hydrate and transcardially perfused with normal saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). The cervical spinal cord and the brachial plexus were carefully dissected under a microscope. The C7 spinal segments, which were defined as the region between the uppermost and the lowermost roots of the C7 nerve of the contralateral spinal cord. After postfixation by immersion in 4% PFA followed by overnight immersion in 30% (v/v) sucrose in PB solution at 4 ˚C, the transverse sections of the C7 spinal cord (35μm), and collected in 0.01M PBS. Every third section of the spinal cord was used for the investigation of the choline acetyltransferase (ChAT), calpain-2 and nNOS immunofluorescence reaction (n = 6) under a fluorescence microscope.

PC12 cells were seeded onto poly-lysine-coated 4-well plates at a