Neuroscience Letters 622 (2016) 61–66

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research paper

MicroRNA-210 promotes sensory axon regeneration of adult mice in vivo and in vitro

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HIGHLIGHTS

- We show microRNA-210 promotes in vivo regeneration of adult mice sciatic nerve and in vitro axon regrowth from dissociated dorsal root ganglion (DRG) cultures.
- We reveal a new in vivo electroporation approach to transfect siRNAs to DRG neurons.
- We provide the first evidence miR-210-EFNA3 as a new signaling pathway can regulate sensory axon regeneration.

ARTICLE INFO

Article history:
Received 16 March 2016
Received in revised form 12 April 2016
Accepted 14 April 2016
Available online 19 April 2016

Keywords:
Axon regeneration
Peripheral nerve injury
MicroRNA-210
Electroporation
DRG

ABSTRACT

Axon regeneration as a critical step in nerve repairing and remodeling after peripheral nerve injury relies on regulation of gene expression. MicroRNAs are emerging to be important epigenetic regulators of gene expression to control axon regeneration. Here we used a novel in vivo electroporation approach to transfect microRNA-210 (miR-210) or siRNAs to adult mice dorsal root ganglion (DRG) neurons, measured the axon length 3 days after sciatic nerve crush or dissociated DRG cultures in vitro to detect the effect of miR-210 in sensory axon regeneration. Importantly, we found that miR-210 overexpression could promote sensory axon regeneration and inhibit apoptsosis by ephrin-A3 (EFNA3). In addition, inhibition of endogenous miR-210 in DRG neurons impaired axon regeneration in vitro and in vivo, the regulatory effect of miR-210 was mediated by increased expression of EFNA3 because downregulation of EFNA3 fully rescued axon regeneration. We thus demonstrate that miR-210 is a new physiological regulator of sensory axon regeneration, and EFNA3 may be the functional target of miR-210. We conclude that miR-210 may play an important role in sensory axon regeneration.

1. Introduction

Axons of mature neurons in the mammalian central nervous system (CNS) hardly regenerate after injuries due to diminished intrinsic ability to support axon growth and a hostile environment in the mature CNS, but they can robustly regenerate in the mammalian peripheral nervous system (PNS) [1]. In the axon regeneration process, regulation of gene expression determines the intrinsic ability of neurons to extend axons and has been a key approach for promoting axon regeneration after injuries [2].

MicroRNAs (miRNAs) are small non-protein-coding RNAs that function as negative gene expression regulators [3]. They have attracted immense attention because of their crucial role in human disease, and have been proposed as potential new therapeutic targets [4]. Recent studies demonstrated that miRNA inhibited mRNA transcription or degraded mRNA, subsequently regulated downstream protein expression [5]. MiRNAs are involved in many pathophysiological procedures including cell apoptosis and survival, cell proliferation, differentiation, migration and functioning [5].

MiR-210 was initially demonstrated to have functional link with hypoxia and it played a crucial role in the cellular response to hypoxia, regulating cell survival and angiogenesis [6,7]. In nerve regeneration areas, recent studies revealed that miR-210 could induce focal angiogenesis and neurogenesis in the adult mouse brain by upregulateing VEGF, and it could promote functional recovery of injured spinal cord through by enhancing angiogenesis via the inhibition of its target ephrin-A3 (EFNA3) [4,5]. In addition, one study showed that EphA4/ephrinA3 signaling was a key

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http://dx.doi.org/10.1016/j.neulet.2016.04.034
0304-3940/

mechanism for astrocytes to regulate synaptic function and plasticity, ephrin-A3 could regulate transporter currents in astrocytes and ephrin-A3 overexpression in astrocytes reduced glutamate transporter levels, produced focal dendritic swellings possibly caused by glutamate excitotoxicity [8]. But it is much less studied that the role of miR-210 in the regulation of neuronal morphogenesis in the PNS, including axon regeneration. In this study, we used a new in vivo electroporation method to transfect miR-210 mimics or miR-210 inhibitor (Anti-210) to DRG cells to investigate if miR-210 was necessary for axon regeneration [2]. We found that adult sensory neurons which overexpressed miR-210 leaded to enhanced intrinsic axon growth capacity and robust axon regeneration both in vivo and in vitro. Further, our study suggests EFNA3 may be a relevant target of miR-210, miR-210 can modulate it negatively. Lastly, we indicates that miR-210 may play an important role of sensory axon regeneration.

2. Materials and methods

2.1. Animals and reagents

All animals in experiments were approved by the Institutional Animal Care and Use Committee of China Medical University. 8–10 week-old CF-1 mice (weighing from 25 to 35 g) were purchased from the university animal facility. The plasmids pCMV–EGFP–N1 were from Clontech. The mouse miR-210 mimics, which double-stranded oligonucleotides designed to mimic the function of endogenous mature microRNA, or the miR-210 inhibitor, which is RNA oligonucleotides designed to inhibit the biogenesis of endogenous microRNA were from Dharmacon miRIDIAN microRNA reagents. The actin antibody was from Sigma-Aldrich (St. Louis, MO). The βIII tubulin (TuJ1) was from Covance. The antibodies against Ephrin-A3 and capsase-3, cleaved capsase-3 were from Cell Signaling Technology.

2.2. Primary culture of adult mouse DRG neurons

For in vitro experiments, the adult mouse sensory neurons were cultured using the same protocol as our previous study [9]. All DRGs were digested with collagenase A (1 mg/ml) for 90 min, followed by trypsin-EDTA (0.05%) for 15–20 min at 37.0 °C. Digested DRGs were then washed 3 times with culture medium (MEM with L-glutamine, 1 × penicillin/streptomycin and 5% fetal calf serum). The cells were dissociated with a 1 ml pipette tip in culture medium. To transfect RNA oligos and/or DNA plasmids into DRGs, the dissociated neurons were centrifuged to remove the supernatant and resuspended in 80 μl–100 μl of Amaxa 300 electroporation buffer (for mouse neuron) with the EGFP (10 μg per transfection) plasmid and/or RNA oligos (siRNA and microRNA mimics or inhibitor, 0.2 nmol per transfection). Suspended cells were then transferred to a 2.0-mm cuvette and electroporated with the Amaxa Nucleofector apparatus. After electroporation, cells were immediately mixed to the desired volume of prewarmed culture medium and plated on culture dishes coated with poly-D-lysine (100 μg/ml) and laminin (10 μg/ml). After 4 h when neurons were fully attached to the coverslips, the medium was changed to remove the remnant electroporation buffer. For in vitro axon growth assay, the transfected neurons were cultured for 3 days. After 3 days culture, the adult DRG neurons were fixed with 4% PFA and then stained with anti-β III tubulin antibody.

2.3. In vivo electroporation of adult DRG neurons and sciatic nerve crush

The mice were anaesthetized with intra-peritoneal injection of a mixture of katamine (100 mg/kg) and xylazine (10 mg/kg). The in vivo electroporation of adult DRG neurons were performed according to our previous published papers [10]. Briefly, the L4 and L5 DRGs on one side of the mouse were surgically exposed. Solution of siRNAs and/or EGFP plasmid (1 μl) were injected into the DRGs using pulled-glass capillary pipette connected to a Picospritzer II (Parker Ins.; pressure, 30 psi; duration: 8 ms). The electroporation was then performed using a custom-made tweezer-like electrode and a BTX ECM830 Electro Square Porator (5 times, 15 ms pulses at 35 V with 950 ms interval). After electroporation, the wound was sutured and the mice were allowed to recover. 2 days after electroporation, the sciatic nerve on the side of the electroporated DRGs was exposed and crushed (3 times,10s per crush by using forceps), the crush site was labeled with nylon epinural suture. 3 days later, the mice were terminally anesthetized and perfused with ice-cold 4% paraformaldehyde (PFA). The whole sciatic nerve was dissected out and further fixed in 4% PFA overnight at 4 °C.

2.4. Measurement of axon growth and TUNEL-Positive cell counts

For measurement of axon growth in vitro, the fixed neurons were washed with PBS and blocked in blocking buffer (2% BSA, 0.1% Triton X −100, 0.1% sodium azide in PBS) for 1 h.

The neurons were then immunostained with the antibody against neuron specific tubulin (Tuj1). To examine apoptosis, the fixed cells were immunostained with the antibody against terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). Only neurons both with axons longer than twice the diameter of cell bodies and with successful transfection of EGFP and/or RNA oligos (siRNA and microRNA mimics or inhibitor) were selected and the longest axon of each neuron was traced manually. 50–100 neurons per condition were measured, and at least 3 independent experiments were performed to calculate the mean value of axon length. TUNEL-positive cells double-labeled with DAPI (Vector laboratories) were counted randomly.

To quantify axon regeneration in vivo, the fluorescence images of the whole mount nerves were first obtained. All identifiable EGFP −labeled axons in the sciatic nerve were manually traced from the crush site to the distal axonal tips to measure the length of the regenerated axons. At least 15 axons of nerves were measured and data from 6 mice for each experimental condition were used to calculate the mean axon length.

2.5. Quantification of mature microRNA

The microRNA-210 level of in vitro cultured DRG neurons or in vivo DRG tissues were tested with qRT-PCR 3 days after miR-210 mimics or inhibitor transfection. The experiment was performed according to the method described by a previous study [11]. In brief, total RNA was isolated with the TRizol reagent (AidLab, China), reverse transcription and real-time PCR were performed according to the manufacturer's protocol of the Hairpin-it microRNA and U6 snRNA Normalization RT-PCR Quantitation Kit (GenePharma, China) by a fast real-time PCR system (7900HT, ABI). The sequences of the microRNA-210 primers used were forward, 5′-CGAATGATTTCGCTTACCC-3′; and reverse, 5′-CTGAAGTTGGGCCGAGAG-3′. Each assay was carried out in triplicate for each sample tested. Relative expression was calculated using the comparative Ctmethod ($2^{-\Delta\Delta Ct}$) [8]. Different samples were normalized to miR-210 expression.

2.6. Western blot analysis

DRG tissues or dissociated DRG neurons 3 days after miR-210 mimics or inhibitor transfection were collected and lysed by using the RIPA buffer. The extracted proteins were separated by 4–12% gradient SDS/PAGE gel electrophoresis, and transferred onto PVDF