Original Article

MicroRNA-21 promotes axonal regeneration of peripheral nerve cells through regulating EPHA4 expression in schwann cells

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Abstract: To investigate the molecular mechanism of microRNA-21 (miR-21) regulating EPHA4 in Schwann cells (SCs) in nerve injury and its promoting effect on axonal extension, providing a novel gene target for regeneration and repair after nerve injury. SC lines with miR-21 over-expression (MI-SC), inhibited miR-21 expression (IN-SC) and control miRNA expression (NC-SC) were constructed through the transfection of miR-21-mimic, miR-21-inhibitor and negative control miRNA (NC-miRNA) into RSC96 cells with liposome. Dorsal root ganglion neurons (DRGns) of newborn SD rats were co-cultured with 3 groups of SCs, respectively. Axonal growth of DRGns in different groups was compared. EPHA4 expression in SCs was detected by PCR and Western blot. After a 7-day co-culture of DRGns with SCs with different miR-21 expressions, axonal length in DRGns co-cultured with SCs with up-regulated miR-21 was significantly longer compared with other two groups; axonal length in DRGns co-cultured with SCs with down-regulated miR-21 was the shortest. PCR and Western blot showed that the up-regulation of miR-21 significantly decreased mRNA and protein expression of EPHA4. In SCs with down-regulated miR-21, mRNA and protein expression of EPHA4 were significantly higher compared with other two groups (P < 0.05). In vitro experiments demonstrate that miR-21 can promote axonal growth of DRGns through negatively regulating EPHA4, suggesting that miR-21 plays an important role in promoting axonal regeneration in injured peripheral nerves. The results may provide a potential therapeutic target for repair after peripheral nerve injury and boost the research on the repair therapy for nerve injury.

Keywords: MicroRNA-21, axonal regeneration, EPHA4, schwann cells, peripheral nerve

Introduction

The peripheral nervous system is different from the central nervous system, in which peripheral nerve injury can activate Schwann cells (SCs) [1, 2], and the functions of peripheral nervous system include the proliferation and chemotaxis of macrophages, the secretion of neurotrophic factors and extracellular matrix, and the formation of gap and tight junction with regenerated axons, which are of great importance in maintaining the survival of neurons after injury, inducing the regeneration of neuronal axons and promoting myelin formation. However, due to the special anatomical structures and functions of the peripheral nervous system and unclear repair mechanisms after nerve injury, functional recovery is poor after nerve injury in clinic [3]. To improve the prognosis of patients, it is necessary to explore the molecular mechanisms of repair and regeneration after peripheral nerve injury.

MicroRNAs (miRNAs), which extensively exist in eukaryotes, are a class of small non-coding RNAs, approximately 22-28 nucleotides in length. By completely or incompletely complementary binding with the 3'UTR (non-coding region) of mRNAs in a target gene, miRNAs cause degradation or translational suppression in the target gene, and thereby mediating the expression of the target gene and affecting the downstream signaling pathways [4]. In eukaryotes, miRNAs are involved in cell growth, proliferation, differentiation and apoptosis [5], as well as axonal regeneration [6]. It has been reported that in the peripheral nervous system, miRNA expressions in rat dorsal root ganglion (DRG)
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change at different degrees after the rat sciatic nerve was cut off, with increased microRNA-21 (miR-21) expression the most remarkable [7]. Moreover, our previous results have evidenced miR-21 expression in SCs [8]. However, the relationships among miR-21, SCs and axonal regeneration and their molecular mechanisms need further exploration.

Eph tyrosine kinase receptor A4 (EPHA4) has been proved to be a gene expressed in SCs, which is able to interfere with SC migration and inhibit axonal regeneration of nerve cells after peripheral nerve injury. When EPHA4 is inhibited, the functions of SCs and peripheral nerve regeneration are significantly improved [9, 10]. In this study, we aimed to explore the molecular mechanisms of neuronal repair after nerve injury. Furthermore, our study revealed that miR-21 might promote axonal regeneration of nerve cells through down-regulating the expression of EPHA4 gene in SCs.

Materials and methods

Experimental materials

All the experimental procedures involving animals were conducted in accordance with guidelines for institutional animal care and approved ethically by the Experimental Animal Center of Sun Yat-sen University. Pregnant rats were monitored closely during predelivery period for any signs of discomfort. After delivery, the mother and newborn rats were housed in large cages with sawdust bedding, enough food and water. The DRGs were harvested from newborn rats (5-10 d) which were anaesthetized by an intraperitoneal injection of sodium phenobarbital and then received mercy killing. All efforts were made to minimize pain and discomfort and to reduce the number of animals used. SC line RSC96 was purchased from the Cell Bank of the Chinese Academy of Sciences. Apparatus included microscissors (Jinzong WaI030 made in Shanghai), two microforceps (Jinzong Wa30-40/Wa3050 made in Shanghai), an invert phase contrast microscope (Nikon eclipse TE2000-U), a digital camera (Nikon, Dxm1200F), a Laser Scanning Confocal Microscopy (Zeiss LSM710) and the Transwell system (Corning, 3460). Regents included PBS buffer, DMEM/F12, fetal bovine serum (Hyclone), Neurobasal culture medium (Gibco, 21103-049), B-27 additive agent (Gibco, 17504-044), L-glutamine (Gibco, 25030-081), papain, collagenase A (Gibco), matrigel (BD356234), NF200 mouse-anti-rat (Millipore Cat, No. mab5266), goat-anti-mouse IgG/Cy3 red fluorescent secondary antibody (Bioss, bs-0296G-Cy3, Lot: AE073003), and DAPI nuclear staining (Kaiji Biotech, Nanjing, China).

Transfection and culture of SCs

RSC96 cells in the logarithmic growth phase were seeded into a 6-well plate 24 hours prior to transfection. The cells were allowed to grow to 80% confluence, and then cell transfection was conducted in accordance with the operating instructions. miR-21-mimic, miR-21-inhibitor and NC-miRNA labeled by green fluorescent protein (GFP) were respectively transfected into RSC96 cells to construct SCs with miR-21 over-expression (Mi-SC), inhibited miR-21 expression (IN-SC) and control miRNA expression (NC-SC). Observation and photographing were carried out under an inverted phase-contrast microscope, and transfection efficiency was detected using Image J software. The cells were further cultured in an incubator at 37°C, 5% CO₂ and saturated humidity for following experiments.

RSC96 cells were cultured in DMEM/F12 culture medium containing 10% fetal bovine serum in an incubator at 37°C, 5% CO₂ and saturated humidity. They were digested by 0.25% trypsin containing EDTA for further cell pas-
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sage. After suspended, the cells were transferred to a centrifuge tube and digestion was terminated by DMEM/F12 culture medium containing 10% fetal bovine serum. Subsequently, the cells were transferred to a new culture bottle.

RNA extraction

The cells in each group were transferred to centrifuge tubes, and culture medium was removed after centrifugation. Then, 1 mL Trizol was added, followed by well-mixing on ice and transferring into new EP tubes. The tubes were subject to room temperature for 5 min, then also room temperature for 5 min after addition of 0.2 mL chloroform and well-shaking for 15 s, and finally centrifugation at 12,000 r/min under 2°C~8°C for 15 min. Subsequently, the supernatant was carefully transferred to new EP tubes, which were added with 0.5 mL isopropanol, followed by reaction under room temperature for 10 min and centrifugation at 7,500 r/min under 2°C~8°C for 5 min. After the supernatant was removed, the solution stood still until sediment turned transparent, and then 1 mL 75% ethanol solution was added, followed by reaction under room temperature for 10 min and centrifugation at 7,500 r/min under 4°C for 5 min. Next, 75% ethanol was removed; the sediment was dried on ice for 5 min; and 30 ul DEPC was added for full dissolution of RNA. The value of A260/A280 and RNA concentration were measured by spectrophotometer.

Real-time PCR for detection of miR-21 and EPHA4 expression

The 7500 Fast Real-Time PCR System was applied under reaction conditions as follows: 40 cycles of 95°C for 30 s, 95°C for 5 s, and 60°C for 34 s. U6 and GAPDH were used, respectively, as the internal reference of miR-21 and EPHA4. The data were obtained from 3 independent samples in 3 independent experiments, and relative quantitative analysis was performed through comparing CT values. The reverse primers of U6, miR-21, GAPDH and EPHA4 and PCR primers are shown in Table 1.

Western blot for detection of EPHA4 expression

Cells were collected and protein samples were extracted using a total protein extraction kit.

Figure 1. Extraction of DRG. A. After the skin was cut open, soft tissue around the spine was stripped to expose the spine completely; B. After the spine was cut open and the whole spinal cord was picked out, circular and semitransparent DRGs were visible in bilateral intervertebral foramen. Bar = 0.5 mm.

Figure 2. Co-culture system. Transwell culture system, mixture of DRGns and matrigel was seeded to the lower chamber, and SCs were seeded to the upper chamber.
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The concentration of the total protein was measured using a microplate reader with the wavelength of A562 nm by BCA method. The protein samples were separated by 10% SDS-PAGE, and then transferred to the PVDF membrane using an electroporator at a constant current of 200 mA for 90 min, which was subject to 5% non-fat milk powder for sealing at room temperature for 1 hour, and TBST for membrane washing for 3 times with 5 min for each time. Subsequently, the first antibodies of EPHA4 and GAPDH were added, followed by shaking incubation at 4°C overnight, and membrane washing in TBST for 3 times with 5 min each time. Next, the secondary antibodies labeled by HRP were added, which was followed by incubation at room temperature for 1 hour, and membrane washing in TBST for 3 times with 5 min each time. Development was conducted by an ECL chemiluminescence kit.

Luciferase reporter assay

Report gene vectors for wild-type EPHA4 (EPHA4 3’UTR-WT) and mutant-type EPHA4 (EPHA4 3’UTR-MUT) were constructed. Report gene vectors and miR-21 mimics were co-transfected into 293 T human embryonic kidney cells, respectively, using liposome, which were tested by the dual luciferase reporter assay kit. Two groups were assigned and luciferase activity was compared: EPHA4 3’UTR-WT group: co-transfection of miR-21 mimics and EPHA4 3’UTR-WT was compared with co-transfection of NC mimics and EPHA4 3’UTR-WT; EPHA4 3’UTR-MUT group: co-transfection of miR-21 mimics and EPHA4 3’UTR-MUT was compared with co-transfection of NC mimics and EPHA4 3’UTR-MUT.

Extraction of dorsal root ganglion neurons (DRGns)

After anesthesia of newborn SD rats (1-5 d) by an intraperitoneal injection of sodium phenobarbital and mercy killing, the skin was cut open from the dorsum to expose the spine under an anatomical microscope (Figure 1A). The spine was then cut open. The whole spinal cord was picked out, and circular and semi-transparent DRGs were extracted one by one in bilateral intervertebral foramen (Figure 1B). The extracted DRGs were subsequently placed into DMEM/F12 culture medium at 4°C. After rinsing, DRGs were sheared into a paste, and 2 mg/mL papain was added, which was followed by digestion in a constant-temperature incubation.
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tor at 37°C for 20 min, shaking once every 5 min. After centrifugation, the supernatant was removed, and 4 mg/mL collagenase I was added, which was followed by digestion in a constant-temperature incubator at 37°C for 20 min, shaking once every 5 min. DMEM/F12 + 10% FBS culture medium were used to terminate digestion. After centrifugation, the supernatant was removed. DMEM/F12 + 10% FBS culture medium was added to re-suspend cells, and the single cell suspension was obtained by gently blowing using a blue pipette tip. The single cell suspension (10^5/mL) was mixed with already frozen matrigel at a ratio of 1:1, which was subject to the lower chamber of the Transwell system in a constant-temperature incubator at 37°C for 30 min for gelling. Next, NB culture medium was added. Thereafter, the whole medium was changed every 24 hours, and 5 μmol/L cytosine arabinoside (Ara-c) was added 48 hours later for purified culture.

Co-culture of DRGns with SCs

After purified culture of DRGns in the lower chamber of the Transwell system for 7 days (Figure 2), the density of SCs was adjusted to 10^5 cells/mL. Then, SCs were seeded into the upper chamber of the culture plate. The culture medium in both upper and lower chambers was changed to low serum medium (neurobasal + B27 + 0.5 mM Gln + 5% FBS). Seven days later, axonal length was observed under an inverted phase-contrast microscope.

Immunocytochemistry

The culture medium was sucked out, followed by washing with PBS for 3 times. After soaked up, 4% paraformaldehyde was used for fixation for 30 min, which was followed by washing with PBS for 3 times, and reaction at room temperature changed with 0.1% Triton for 10 min. After washing with PBS for 3 times, 2% bovine serum albumin (BSA) was used for sealing at 37°C for 1 h; then, after complete washing with PBS, the first antibody (NF200 mouse-anti-rat, Millipore Cat, No. mab526; 1:200) was added and maintained overnight at 4°C; subsequently, PBS was used for washing for 3 times, and the secondary antibodies [goat-anti-mouse IgG (H + L) red fluorescent secondary antibody; ABGAB Cat (No. 24-00-0296); 1:200]; were added at 37°C for 2 h. Next, PBS was used for washing for 3 times with 3 min each time, and DAPI staining solution was added at room temperature for 5 min, followed by washing with PBS for 3 times. Under an invert phase contrast microscope, cell purity was measured, and axonal growth was observed. Axonal length in each group was calculated under a laser scanning confocal microscope (Zeiss LSM710).

Assessment of axonal growth in DRGns

Analysis of axonal growth was carried out by two experimenters independently. Axons were
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Real-time PCR results showed that as compared to the NC-SC group, EPHA4 mRNA expression in the MI-SC group decreased significantly (P < 0.05), while increased significantly in the IN-SC group (P < 0.05), as seen in Figure 5.

**Effect of miR-21 on EPHA4 protein expression**

Western blot results were analyzed by Image J software, demonstrating that EPHA4 protein expression in the IN-SC group was obviously higher than that in the NC-SC group, while EPHA4 protein expression in the MI-SC group was evidently lower than that in the NC-SC group (P < 0.05) (Figure 6).

**Target gene of miR-21 verified by luciferase reporter system**

It was predicted by bioinformatics methods that the 3'-UTR of EPHA4 contains binding sites of miR-21 (Figure 7), suggesting that EPHA4 may be one of the target genes of miR-21. Dual-luciferase reporter system showed that in the EPHA4 3'-UTR (WT) group carrying wild-type reporter gene vectors, luciferase activity in the cells transfected with miR-21 mimics was down-regulated significantly compared to the cells transfected with NC mimics (P < 0.05); in the EPHA4 3'-UTR (MUT) group carrying mutant-type reporter gene vectors, luciferase activity in the cells transfected with miR-21 mimics and NC mimics presented no obvious changes (Figure 8).

**Purity detection after purified culture of DRGns**

After a 7-day culture, DRGns were observed under an inverted phase-contrast microscope (Figure 9A). Immunofluorescence staining was conducted, with red fluorescence indicating DRGns and their synapses (Figure 9B), and...
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After image fusion, the proportion of the number of cells with red fluorescence to the number of cells with blue fluorescence was calculated (Figure 9D), suggesting that the purity of DRGns was higher than 90%, which met the requirements of following experiments.

Figure 9. Purified culture of DRGns and purity detection. (A) After culture for 7 days, the morphology of DRGns was observed under an inverted phase-contrast microscope; (B) After purification, DRGns presented red with NF200 staining; (C) Nuclei presented blue with DAPI staining; (D) A fused image of (B and C) Bar = 200 μm.

Figure 10. EPHA4 protein expression level after 7 day co-culture. Relative protein expression of EPHA4 in MI group, NC group and IN group detected by Western blot after co-culture for 7 days. Values are expressed as Mean ± SD, significance is referred to the NC group. $t$ test, N = 3. *$P < 0.05$ vs. NC-SC group.

Comparison of axonal length among DRGns co-cultured with 3 groups of SCs

After co-culture for 7 days, EPHA4 protein expression in each group was detected, showing that EPHA4 protein expression in the MI-SC group was the lowest (Figure 10). Then, immunofluorescence staining was conducted and photographed under the laser scanning confocal microscopy, and axonal length in each group was calculated and compared using Image J software. Comparison in the length of the longest axon in a single cell in each group (Figure 11) revealed that the length in the MI group was far longer than that in the NC group (344.7 ± 26.5 μm vs. 177.8 ± 7.9 μm; $P < 0.05$); while the length in the IN group was shorter than that in the NC group (56.5 ± 4.9 μm vs. 177.8 ± 7.9 μm; $P < 0.05$). Additionally, further comparison in the total axonal length of a single cell in each group showed that the length in the MI group was obviously longer than that in the NC group (875.8 ± 84.4 μm vs. 338.6 ± 32.5 μm; $P < 0.05$), and the length in the IN group was shorter than that in the NC group (89.8 ± 13.1 μm vs. 338.6 ± 32.5 μm; $P < 0.05$). Integrated data from the 3 groups suggested that over-expression of miR-21 promoted axonal elongation of DRGns and their regeneration.

Discussion

Peripheral nerve injury is usually followed by Wallerian degeneration, whose products combining with macrophages can stimulate SCs to secrete nutrition-support materials [11, 12], by which SCs play a crucial role in peripheral nerve repair [1, 2]. Therefore, the study on peripheral nerve repair and regeneration is mainly focused on the exploration of SCs and their secretory substances.

In present studies, the tyrosine kinase receptor family (ephs) and their ligands (ephrins) have been identified to play a critical role in cell
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migration, axonal growth and the formation of neuronal network. Also, they are all found to be expressed in the whole nervous system, with a positive or negative effect based on different microenvironments [13-15]. For instance, the expression of EPHA7 increases significantly after spinal cord injury, while the down-regulation of EPHA7 expression can evidently promote the motor recovery of rat hind limbs [16]. After transecting the spinal cord, EPHB3 expression is up-regulated in astrocytes of adult rats in vivo, and is involved in scar formation [17]. Moreover, in an in vitro experiment of retinal ganglion in adult rats, EPHB3 supports axonal growth [18]. EPHA4 involved in this study, a member of the tyrosine kinase receptor family, is up-regulated in astrocytes after spinal cord injury and inhibits the formation of nerve growth cone in broken ends [19]. Furthermore, artificially down-regulated EPHA4 expression can promote neural axonal regeneration and functional recovery [9]. Similarly, after peripheral nerve injury, increased EPHA4 expression can be detected in SCs, and inhibiting the function of this gene can obviously promote peripheral nerve repair and regeneration [9]. These previous studies have revealed that after peripheral nerve injury, EPHA4 is an important factor in promoting scar formation and inhibiting nerve repair and regeneration, and effectively inhibition of the expression of EPHA4 gene is the key for further progress in the field of peripheral nerve repair and regeneration.

It is worth noting that miR-21 is another important factor in the field of peripheral nerve repair and regeneration. After sciatic nerve transection, miR-21 level in DRGs presents the most obvious increase [7]. Previous research on miR-21 has evidenced that miR-21 is involved in the regulation of various tumor suppressor genes, and the negative regulation to

Figure 11. Comparison of axonal length. A-C. photographs of 3 groups (MI, NC, IN) after co-culture for 7 days under a confocal microscopy; D. Comparison of the longest axonal length in a single cell in each group; E. Comparison of total axonal length of a single cell in each group. Values are expressed as Mean ± SD, significance is referred to the NC group. t test, N = 3. *P < 0.05 vs. NC-SC group. Bar = 50 μm.
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tumor suppressor genes is closely related to the occurrence and development of tumors [20]. In addition, research on cardiovascular diseases has revealed that miR-21 is also involved in the occurrence and development of the diseases through the negative regulation of target genes [21]. However, in the process of repair and regeneration after nerve injury, the regulatory mechanism of miR-21, the regulatory relationship between miR-21 and EPHA4, and the effect of the interaction on axonal growth are the focuses of this study.

In the present study, comparison of PCR and Western blot results among the MI group, NC group and IN group revealed that when miR-21 expression in the MI group was up-regulated, the mRNA and protein expressions of EPHA4 were obviously lower than those in the other 2 groups, while the mRNA and protein expressions of EPHA4 in the IN group were evidently higher than those in the NC group and the MI group, suggesting a significant negative regulation between miR-21 and EPHA4. In order to further verify the interaction, we employed bioinformatics method (Targetscan) to predict the binding sites between miR-21 and 2.236-2.243 bp (AUAAAGCUA) in the 3'UTR (non-coding region) of EPHA4, which was also confirmed by dual-luciferase reporter system. On this basis, Transwell culture system (Corning company) was applied for the co-culture of DRGns with 3 groups of SCs, respectively. The bore diameter of polycarbonate membrane was 0.4 μm, so as to facilitate the exchange of nutrient substances and avoid cell migration. Moreover, different from other mixed culture systems, the cell components in each chamber in this culture system are single, and have no effect on the observation of a single cell. Seven days later, the axonal length of neurons in each group was significantly different, the axonal length of DRGns: the MI group > the NC group > the IN group, suggesting that miR-21 in SCs might influence the axonal growth of peripheral neurons through negatively regulating EPHA4.

In summary, this study directly demonstrated the relationships among SCs, miR-21 and EPHA4. It is clear that miRNA-21 and EPHA4 play important roles in the complex network of axonal regeneration. Therefore, local implantation of SCs with high expression of miR-21 might provide a new approach for the treatment of repair and regeneration after peripheral nerve injury. Even so, direct mechanisms between EPHA4 and neurons are still unclear, and further researches are needed. Moreover, this study is based on in vitro experiments, so the promoting effect of SCs with high expression of miR-21 on nerve regeneration and functional recovery requires further verification by in vivo experiments.

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Disclosure of conflict of interest

None.

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