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MicroRNA-146a promotes oligodendrogenesis in stroke

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Abstract

Stroke induces new myelinating oligodendrocytes that are involved in ischemic brain repair. Molecular mechanisms that regulate oligodendrogenesis have not been fully investigated. MicroRNAs (miRNAs) are small non-coding RNA molecules that post-transcriptionally regulate gene expression. MiR-146a has been reported to regulate immune response, but the role of miR-146a in oligodendrocyte progenitor cells (OPCs) remains unknown. Adult Wistar rats were subjected to the right middle cerebral artery occlusion (MCAo). In situ hybridization analysis with LNA probes against miR-146a revealed that stroke considerably increased miR-146a density in the corpus callosum and subventricular zone (SVZ) of the lateral ventricle of the ischemic hemisphere. In vitro, over-expression of miR-146a in neural progenitor cells (NPCs) significantly increased their differentiation into O4⁺ OPCs. Over-expression of miR-146a in primary OPCs increased their expression of myelin proteins, whereas attenuation of endogenous miR-146a suppressed generation of myelin proteins. MiR-146a also inversely regulated its target gene-IRAK1 expression in OPCs. Attenuation of IRAK1 in OPCs substantially increased myelin proteins and decreased OPC apoptosis. Collectively, our data suggest that miR-146a may mediate stroke-induced oligodendrogenesis.

Keywords

miR-146a; oligodendrocyte progenitor cells; differentiation; IRAK1; survival; stroke

Introduction

Stroke induces long term neurological deficits. Mature oligodendrocytes are vulnerable to stroke and damaged oligodendrocytes no longer produce myelin proteins [1–3]. Loss of myelinating oligodendrocytes exacerbates neurological deficits [1–3]. New myelinating oligodendrocytes are solely derived from differentiated oligodendrocyte progenitor cells (OPCs)[1–3]. At present, molecular mechanisms controlling oligodendrocyte differentiation

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are poorly understood. Therefore, identifying molecular mechanisms underlying the regulation of oligodendrogenesis in adult brain could lead to the development of therapies for neurological diseases including stroke by amplifying the generation of endogenous oligodendrocytes.

MicroRNAs (miRNAs) are small, single-stranded RNA molecules, approximately 20 nucleotides long. They suppress protein translation by binding to the 3'-untranslated region (UTR) of target mRNAs in a complementary dependent manner [4–6]. Recent reports indicate that many miRNAs are critically important to central nervous system (CNS) development and physiology, with roles described in neuronal and glial differentiation, synaptic plasticity, and homeostasis [7–9]. Stroke alters the miRNA profiles of peripheral blood and brain tissues [10–14]. However, the functions of these altered miRNA are largely unknown. Using miRNA array, several studies have identified miRNAs related to oligodendrocyte maturation [15–17]. Multiple miRNAs including miR-219, miR-138, miR17-92 and miR-338 have been found to modulate the proliferation or differentiation of OPCs into mature oligodendrocytes [15,18–22]. However, the functional roles of miRNAs in stroke-induced oligodendrogenesis have not been reported.

The function of miR-146a has been extensively studied in the immune response and is regarded as an inflammatory miRNA [23]. Our previous study showed that miR-146a mediated the neuroprotective effect of Velcade in combination with tPA for the treatment of stroke, primarily targeting endothelial cells after stroke [24]. The miR-146aG allele has been associated with the pathogenesis of ischemic stroke in humans [25]. In addition, miR-146a was substantially increased in NPCs after stroke, suggesting a role for miR-146a in ischemic brain repair processes [14]. The present study describes the pivotal role of miR-146a in promotion OPC differentiation into myelinating oligodendrocytes.

Materials and Methods

All experimental procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital (IACUC).

Model of Middle Cerebral Artery Occlusion (MCAo)

Male Wistar rats (3–4 months, Jackson Laboratory, Maine, USA) were employed in this study. The right middle cerebral artery (MCA) was occluded by placement of an embolus at the origin of the right MCA, as previously described [26]. All rats were sacrificed 7 days after MCAo.

Neural Progenitor Cell Cultures

Neural progenitor cells (NPCs) were dissociated from the subventricular zone (SVZ) of the lateral ventricle of adult mice, as previously described [14,27–30]. The cells were plated at a density of 2×10^4 cells/ml in growth medium. Growth medium contains DMEM/F-12 medium (Life Technologies, NY, USA), 20 ng/mL of epidermal growth factor (EGF, R&D System, MN, USA), and basic fibroblast growth factor (bFGF, R&D System). DMEM/F-12 medium contains L-glutamine (2 mmol/L, Life Technologies), glucose (0.6%, Sigma

Aldrich, MO, USA), putrescine (9.6 mg/ml, Sigma Aldrich), insulin (0.025 mg/mL, Sigma Aldrich), progesterone (6.3 ng/ml, Sigma Aldrich), apo-transferrin (0.1 mg/mL, Sigma Aldrich), and sodium selenite (5.2 ng/mL, Sigma Aldrich). The generated neurospheres (primary spheres) were passaged by mechanical dissociation and reseeded as single cells at a density of 20 cells/ μ l. SVZ cells from ischemic brain were extracted 7d after MCAo, a peak time of increase of neurogenesis [31][32]. SVZ cells used in all experiments were from passages 2–5.

Primary Oligodendrocyte Precursor Cell Culture

For in vitro experiments, primary OPCs were isolated, as previously described [33,34], with the procedure modified to accommodate embryonic day 18 rat embryos. In brief, pregnant Wistar rats were decapitated under deep anesthesia and a c-section was performed. The scalp and meninges were removed and cortices were dissected out. Cortices were then rinsed twice in ice cold Hank's buffered salt solution and incubated at 37°C for 15 min with 0.01% trypsin and DNase (Life Technologies). The tissue was then triturated and filtered through a 40 μ m sterile cell strainer to remove insoluble debris. Cells were plated in poly-D-lysine (Sigma Aldrich) coated culture flasks in DMEM with 20% FBS until the cells were confluent (10 days), in which time a bed of astrocytes grew with a layer of OPCs on top. The flasks were then shaken at 200 rpm for 1h to dislodge dead cells and microglia; the media were then changed, and the flasks shaken overnight at 200 rpm to dislodge OPCs. The OPCs were collected and plated onto poly-D,L-ornithine (Sigma Aldrich) coated culture dishes with serum free DMEM supplemented with human Apo-transferrin (Sigma Aldrich), BSA, L-glutamine, sodium selenite, D-biotin, penicillin–streptomycin, hydrocortisone, insulin. Proliferation medium also contains OPC mitogens such as PDGF-AA and NT-3 (both from PeproTech, NJ, USA); differentiation medium includes triiodothyronine (T3; Sigma Aldrich) and ciliary neurotrophic factor (CNTF, 10 ng/ml; PeproTech) without OPC mitogens.

Laser Capture Microdissection (LCM)

Briefly, frozen brain coronal sections stored at –80°C were immediately immersed in acetone for 2 min fixation and air-dried for 30 secs, previously described [14,29]. After a brief rinse with 0.1% diethylpyrocarbonate treated phosphate-buffered saline (PBS), sections were stained with propidium iodide dye (1:5000 dilution, Sigma Aldrich) for 5 mins, and rinsed with PBS twice. All reaction steps were performed in RNase-free solutions. Sections were then air-dried under laminar flow for 10 min and immediately used for LCM. Dense SVZ cells on sections stained by propidium iodide were readily distinct from the ependymal cells that have cilia along the lateral wall of the lateral ventricle and from the adjacent striatal cells [14,29,35]. Propidium iodide-positive cells within the SVZ and corpus callosum were selected and cut with a Leica AS LMD System (Leica Microsystems Inc, IL, USA) and collected into the cap of tube. Approximately 1,000 cells were isolated in the SVZ or corpus callosum from each animal. Extra care was taken to minimize the contamination of materials from other cell types while laser dissecting neural or oligodendrocyte progenitor cells from the SVZ and corpus callosum, respectively.

MiRNA in Situ Hybridization

In situ hybridization was performed, as previously [14,36]. Briefly, rats subjected to 7 day MCAo or sham surgery were sacrificed under anesthesia by intracardiac TBS-paraformaldehyde perfusion. Coronal brain sections (20 μ m thick) from each rat were post-fixed and acetylated by incubating in acetic anhydride/triethanolamine solution followed by washes in 1 $\times$ PBS. The sections were incubated in hybridization solution (50% formamide, 5 $\times$ SSC, 200 μ g/mL yeast tRNA, 500 μ g/mL salmon sperm DNA, 0.4 g Roche blocking reagent, and 5 $\times$ Denhardt's solution) at room temperature for 2h. The sections were incubated overnight in hybridization solution containing 3 pmol of digoxin (DIG)-labeled locked nucleic acid (LNA) miRCURY probes (Exiqon Inc, Denmark) at below -20° predicted melting temperature (T_m) value of the miR-146a. The sections were washed at 55°C for 30m in 1 $\times$ SSC and for 10 min in 0.1 M Tris-HCl buffer (pH 7.5) and incubated in the blocking solution (10% fetal calf serum in 0.1 M Tris-HCl buffer) for 1 h at room temperature followed by labeling with anti-DIG-FAB peroxidase (POD, Roche Applied Science, IN, USA) for 1h at room temperature. The signals were amplified using the Individual Indirect Tyramide signal amplification Kit (TSA, PerkinElmer Life Science, MA, USA), according to the protocol. Alkaline phosphatase was used for the detection of the miRNA signals.

For semiquantitative measurements of miR-146a density, one coronal section/rat (N=5 rats) was employed. The corpus callosum area was digitized with a 40 $\times$ objective (BX20 Olympus Optical, PA, USA) using a 3-CCD color video camera (DXC-970 MD; Sony) interfaced with a MCID image analysis system. The entire corpus callosum area and areas with miR-146a signals in the corpus callosum were measured, as described previously [14]. Data are presented as a percentage of miR-146a signals within the corpus callosum.

MiRNA Fluorescence in Situ Hybridization Combined with Immunofluorescence

FISH combined with immunohistochemical staining were performed, as previously described [36]. Briefly, the sections were hybridized with miR-146 LNA probe, and the probe was detected with peroxidase-conjugated anti-FAM (Roche) followed by incubation tyramine-signal-amplification (TSA)-Cy3 substrate for 10 min at room temperature. Primary antibody was added and incubated at 4 degrees overnight and detected with FITC-conjugated secondary antibody (Jackson ImmunoResearch). Slides were mounted with mounting medium with DAPI (Invitrogen).

MiRNA and SiRNA Transfection

To efficiently introduce the miRNA or siRNA into OPCs, the N-TER nanoparticle transfection system (Sigma Aldrich) was employed. Briefly, N-TER Peptide was diluted into water in a sterile tube and incubated in a sonicating water bath at maximum output and continuous power for 3–5 minutes. Then 5mM miR-146a mimics (mature sequence: UGAGAACUGAAUCCAUGGGUU, Dharmacon, CO, USA) or miRNA mimic control was diluted with N-TER Buffer in a sterile tube.

To knock down the endogenous interleukin-1 receptor-associated kinase 1 (IRAK1) expression, we introduced a siRNA against IRAK1 (Dharmacon, 5mM) and a scrambled

siRNA as a negative control, which was mixed with N-TER buffer as the miRNA mimics. The nanoparticle formation solutions were prepared by combining the appropriate diluted miRNA solutions with diluted N-TER peptide solutions, and the tubes containing the nanoparticle formation solutions (combined miRNA or siRNA and N-TER peptide solutions) were incubated at room temperature for 20 minutes to allow the nanoparticles to form. A solution of nanoparticle formation solutions was mixed in 1400 μ L of growth medium. This solution was added to the cells and slightly agitated to mix. After 24 h at 37°C, the solution was removed from the cells and replaced with 37°C growth medium or differentiation medium.

MiRNA or siRNA were delivered into SVZ NPCs using nucleofector electroporation. SiRNAs or miRNAs oligonucleotides (200 pmol, Dharmacon) were mixed with 100 μ L of nucleofector solution and were introduced into cultured NPCs using a Nucleofector™ kit (Lonza, NJ, USA), as previously described [35].

Neurosphere Assay

A neurosphere assay was employed to investigate the effect of miRNAs on SVZ NPCs, as previously described [14,29,35]. The SVZ NPCs were transfected with miRNA mimics or inhibitors of miR-146a and incubated in growth medium in the presence of EGF and bFGF for 24 h to allow the cells to recover. To analyze cell proliferation, bromodeoxyuridine (BrdU, 30 μ g/ml, Sigma Aldrich), the thymidine analog that is incorporated into the DNA of dividing cells during S-phase, was added 18 h before the termination of incubation. BrdU positive cells were measured (see below for quantification).

To examine the SVZ cell differentiation, neurospheres were plated directly onto laminin coated glass cover slips in DMEM-F-12 medium containing 2% fetal bovine serum (FBS), which is referred to as differentiation medium. Every 4 days, one-half of the medium was replaced with fresh medium. Incubation was terminated 10 days after plating, and immunostaining for neuronal and astrocyte markers was performed for evaluation of cell differentiation.

Assay of Caspase-3 and Caspase-7 Activity

OPCs transfected with miR-146a mimics or inhibitors after 48 hours were seeded into 96-well plates at a density of 3×10^4 cells per well in 6 replicates, and cultured for 24 hours in growth medium. Cells transfected with cel-miR-67 mimics or inhibitors were used as a negative control, respectively. The combined activity of caspase-3 and caspase-7 (caspase-3/7) was evaluated using the Caspase-Glo 3/7 Assay Kit (Promega, WI, USA) according to the manufacturer's instructions. Briefly, the Caspase-Glo 3/7 reagent was added to the cells and incubated at room temperature for 1 hour. Results were detected by a plate reader (Perkin Elmer, MA, USA) at 482 nm wavelength.

MiR-146a Target PCR Array

The Rat miR-146a Targets RT² Profiler PCR Array (Qiagen, CA, USA) profiles the expression of 84 rno-miR-146a-5p target genes. The protocol was conducted according to the vendor's manual. Briefly, for reverse transcription, total RNA was incubated with Buffer

GE for 5 min at 42°C to eliminate the genomic DNA, and thereafter was mixed with reverse transcriptase mix at 42°C for 15 min, and the reaction was stopped by incubating at 95°C for 5 min. For miR-146a Targets PCR Array, a volume of 25 µl PCR components including RT products and 2×SYBR Green Master mix was prepared. The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression of mRNAs was normalized against the expression of housekeeping genes from the array as an endogenous normalization control. The n-fold change in target mRNAs expression was determined according to the method of 2^{-CT} .

Quantification of Mature miRNAs by Real-time qRT-PCR

Total RNAs (10 ng) were reverse transcribed using a TaqMan® MicroRNA Reverse Transcription (RT) kit (Applied Biosystems, NY, USA). Each RT reaction contained 1× stem-loop RT specific primer, 1× reaction buffer, 0.25 mM each of dNTPs, 3.33 U/µl Multiscribe RT enzyme and 0.25 U/µl RNase inhibitor. The 15-µl reactions were incubated for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C and then held at 4°C. The PCR reaction was performed using a standard TaqMan® PCR kit protocol (Applied Biosystems). Briefly, following the RT step, 1.33 µl of the RT reaction were combined with 1 µl of a TaqMan MicroRNA Assay (20×; forward primer, reverse primer and probe) and 17.67 µl of TaqMan® Universal PCR Master Mix, No AmpErase® UNG in a 20 µl final volume. The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression of miRNAs was normalized against the expression of U6 snRNA as an endogenous normalization control. All assays were performed in triplicate and were calculated on the basis of the 2^{-CT} method. The n-fold change in miRNAs expression was determined according to the method of 2^{-CT} [37].

Immunocytochemistry and Quantification

Immunofluorescent staining was performed on cultured cells. The following primary antibodies were used in the present study: mouse anti-BrdU (1:1,00; Boehringer Mannheim, IN, USA), rat anti-myelin basic protein (MBP, 1:50, Abcam, MA, USA), markers for oligodendrocytes, rabbit anti-chondroitin sulfate proteoglycan (NG2, 1:800, Chemicon, MA, USA), a marker of OPCs, glial fibrillary acidic protein (GFAP), a marker of astrocytes, and chicken anti-neurofilament-H (NF-H, 1:10,000, ABR Affinity Bioreagents, CO, USA). Cultured cells were fixed in 4% paraformaldehyde for 20 min at room temperature. Nonspecific binding sites were blocked with phosphate-buffered saline with 1% bovine serum albumin goat serum for 1 h at room temperature. The cells were then incubated with the primary antibodies listed above and with CY3-conjugated secondary antibodies. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (1:10,000, Vector Laboratories, CA, USA).

For all measurements, we counted cells from three wells/group (n = 3 individual cultured cells). Ten fields of the view per well were randomly imaged under a 20× objective and measured using MCID system. All analysis was conducted with the examiner blinded to the identity of the samples being studied.

SDS-PAGE and Western Blot

Cells were lysed in RIPA buffer, and lysate was sonicated and then centrifuged for 10 min at 12,000 rpm to remove cell debris. Protein concentrations were determined using a BCA assay (Thermo Scientific, MA, USA). Equal amounts of proteins were then separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membrane was probed with an appropriate primary antibody and a secondary antibody conjugated to horseradish peroxidase. The following antibodies were utilized: β -actin (1:10,000 dilution, Abcam), Cleaved Caspase-3 (1:500 dilution; Cell signaling, MA, USA), 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase, 1:1000 dilution, Chemicon), interleukin-1 receptor-associated kinase 1 (IRAK1, 1:1000 dilution; Santa Cruz), MBP (1:1000 dilution, Abcam), myelin proteolipid protein (mPLP, 1:1000 dilution, Abcam), NF-KB p65 (1:1000 dilution, Abcam), NG2 (1:1000 dilution, Santa Cruz), platelet-derived growth factor receptor α (PDGFR- α ; 1:1000 dilution; Santa Cruz), TNF receptor associated factor (TRAF6, 1:1000 dilution; Santa Cruz). Proteins were visualized by enhanced chemiluminescence (Thermo Fisher Scientific).

Statistical Analysis

The data are presented as mean $\pm$ SE. Independent sample t-test was used for two-group comparisons from the non-MCAo and MCAo samples. One-way analysis of variance followed by Student-Newman-Keuls test was performed for multiple sample analysis. A value of $p < 0.05$ was taken as significant.

Results

Stroke upregulates miR-146a expression

Using miRNA microarray, we previously demonstrated that stroke robustly increased miR-146a expression in cultured SVZ neural progenitor cells [14]. To confirm this finding in vivo, we measured miR-146a levels in cells captured by LCM in the SVZ and the corpus callosum by means of quantitative real-time RT-PCR, and found that stroke significantly and considerably upregulated miR-146a in these cells (Fig. 1A–F). In addition, in situ hybridization was performed on brain coronal sections with DIG-labeled LNA probes that target the mature form of miR-146a. We found that stroke significantly and substantially increased miR-146a signals in the ipsilateral SVZ and corpus callosum compared to that in homologous regions of the contralateral hemisphere (Fig. 1G–L). These data are consistent and extend our published microarray data showing that stroke upregulates miR-146a expression in the SVZ [14], which consist of a heterogeneous cell population [38]. FISH analysis of cultured NPCs revealed that cells with miR-146a positive particles in the cytoplasm were nestin (Fig. 2A, B), Tuj1 (Fig. 2C), PDGFR alpha (Fig. 2D), and GFAP positive (Fig. 2E), suggesting that all NPCs express miR-146a.

MiR-146a promotes oligodendrocyte differentiation

To examine the effect of miR-146a on oligodendrocyte differentiation, primary OPCs isolated from rat brain at E18 were transfected with miR-146a mimics. We previously demonstrated that more than 90% of these cells are O4 expressing OPCs [34]. Transfection

of OPCs with miR-146a mimics considerably elevated miR-146a levels compared to OPCs transfected with mimic control, cel-miR-67 (Fig. 3A). Immunocytochemistry analysis revealed that elevation of miR-146a in OPCs resulted in a significant increase in the number of MBP positive oligodendrocytes (Fig. 3B, C). Moreover, Western blot analysis showed that miR-146a mimics robustly increased myelin proteins, CNPase, MBP, and PLP, while OPC marker proteins, NG2 and PDGFR- α were remarkably reduced (Fig. 3E). In contrast, attenuation of endogenous miR-146a expression in OPCs by miR-146a hairpin inhibitors blocked OPCs from differentiating into mature oligodendrocytes, as assayed by immunocytochemistry and Western blot analysis (Fig. 3C–E).

In addition, we analyzed the effect of miR-146a on OPC proliferation and survival under normoxia conditions. Transfection of OPCs with miR-146a mimics significantly decreased the number of BrdU positive cells compared to OPCs transfected with mimic control (Fig. 3F, G), suggesting that miR-146a inhibits OPC proliferation. Caspase-3 and -7 are key factors in the apoptosis signaling. Using a Caspase-3/7 luciferase assay, we found that overexpression of miR-146a significantly decreased the Caspase-3/7 luciferase activity, but inhibition of miR-146a induced the Caspase-3/7 activity (Fig. 3H), suggesting that miR-146a protects oligodendrocytes from apoptosis.

To examine the effect of miR-146a on NPCs, primary NPCs were isolated from the SVZ of the lateral ventricle in the adult rats. Transfection of NPCs with miR-146a mimics considerably increased Tuj1 positive neuroblasts (Fig. 4A, B) and O4 positive OPCs (Fig. 4C, D), but did not significantly alter GFAP positive astrocytes ($32 \pm 4\%$ in miR-146a mimic groups vs $27 \pm 4\%$ in mimic control group, $p=0.14$). In addition, miR-146a mimics substantially reduced proliferating NPCs, assayed by BrdU positive cells, compared to mimic controls (Fig. 4E, F).

Collectively, these data indicate that elevation of miR-146a in OPCs promotes their differentiation, while in NPCs, miR-146a enhances differentiation of NPCs into neuronal and oligodendrocyte lineage cells.

Interleukin-1 receptor-associated kinase1 (IRAK1) mediates miR-146a-promoted OPC differentiation

Using a miR-146a target PCR array, we screened 88 genes including experimentally verified and bioinformatically predicted target genes in OPCs transfected with miR-146a mimic and control miRNAs. Compared to the control miRNAs, miR-146a mimics substantially reduced the transcripts of IRAK1, IRAK2, TRAF6 and Numb (Fig. 5A), which are well validated genes targeted by miR-146a [39–41]. In addition, miR-146a downregulated target genes such as a disintegrin and metalloproteinase with thrombospondin motifs 3 (Adams3), breast cancer 1, 2 (Brca1, 2), Fas-associated via death domain (Fadd), Notch 2, Sortilin 1 (Sort1) (Fig. 5A), which are known to regulate biological function of OPCs [42–46].

The toll-like receptor (TLR) signaling in adult neural stem cells regulates adult neurogenesis, and IRAK1 is an adaptor protein of TLRs [47–50]. However, it has not been studied whether IRAK1 in NPCs is involved in miR-146a-mediated NPC and OPC differentiation. We thus, first examined whether elevation of miR-146a in NPCs alters

IRAK1 proteins. NPCs harvested from non-ischemic and ischemic SVZ were transfected with miR-146a mimics. Western blot analysis showed that miR-146a mimics substantially reduced IRAK1 proteins in a dose-dependent manner (Fig. 5B). In parallel, miR-146a also decreased TRAF6 protein levels, another gene targeted by miR-146a (Fig. 5B). Reduction of IRAK1 and TRAF6 by miR-146a were associated with a decrease of NF- κ B p65 proteins (Fig. 5B), that are known to be regulated by IRAK1 [39].

We then examined whether IRAK1 mediates miR-146a-promoted OPC differentiation. Western blot analysis revealed that transfection of OPCs with miR-146a mimics reduced IRAK1 protein levels by approximately 30% of mimic control (Fig. 5C). In contrast, attenuation of endogenous miR-146a in OPCs by siRNA against miR-146a substantially elevated IRAK1 protein levels (Fig. 5C). These data suggest that miR-146a targets IRAK1. More importantly, attenuation of endogenous IRAK1 increased myelin protein levels of MBP, CNPase, and PLP, and decreased protein levels of NG2 and PDGFR α (Fig. 5D). In addition, knockdown of IRAK1 significantly decreased the Caspase-3/7 activity (Fig. 5E) and Caspase-3 protein levels (Fig. 5D), suggesting IRAK1 also regulates oligodendrocyte survival. Thus, these data suggest that IRAK1 mediates miR-146a-promotion of oligodendrocyte differentiation.

Discussion

The present study for the first time reveals a new role for miR-146a in oligodendrogenesis in response to cerebral ischemia. The elevation of miR-146a levels in NPCs increases oligodendrocyte lineage cells, while miR-146a in OPCs promote their differentiation by repressing the levels of IRAK1. OPCs are the only cell population to generate mature oligodendrocytes and they originate from NPCs in the adult rodent brain [51,52]. Thus, miR-146a has potential therapeutic effect for amplifying oligodendrogenesis in the ischemic brain and in other demyelination disorders. [3,53].

The present paper provides evidence that stroke upregulates miR-146a not only in the SVZ but also in the corpus callosum during brain repair. Using primary culture NPCs in vitro, we demonstrated that overexpression of miR-146a increased the number of Tuj1 positive cells, suggesting miR-146a can promote the neurogenesis. More importantly, in cultured NPCs, miR-146a mimics significantly increased the number of O4 positive cells, while in OPCs miR-146a increased myelin proteins and decreased proteins produced by OPCs. Inhibition of endogenous miR-146a has the inverse impact on OPCs. The effect of miR-146a on enhanced differentiation of neuronal and oligodendrocyte lineage cells appears specific because miR-146a did not alter GFAP positive astrocytes. Collectively, these data indicate that miR-146a promotes NPCs to differentiate into neuronal and oligodendrocyte lineage cells and facilitates OPCs to differentiate into myelinating oligodendrocytes. The present data are consistent with studies by us and others showing that miR-146a mediates thymosin β 4-enhanced OPC differentiation [54] and that miR-146a levels are increased in OPCs during the transition from A2B5⁺ OPCs to premyelinating GalC⁺ cells [3].

Multiple miRNAs have been associated with oligodendrocyte maturation during embryonic development [15,18,20,21]. Whether these miRNAs play important roles in adult

oligodendrocytes are poorly understood. Recently, we demonstrated serum response factor (SRF) expression in OPCs and OLs, and that SRF levels are mediated by miR-200c and miR-9, which regulate OPC differentiation after stroke [34]. In addition, the miR17-92 cluster that has been reported to regulate the proliferation of oligodendrocytes, was found to be significantly upregulated in NPCs after stroke [35]. It is plausible that the upregulation of miR17-92 is also involved in the oligodendrogenesis induced by stroke and that additional miRNAs may also contribute to oligodendrogenesis after stroke.

TLR2/4 is expressed in oligodendrocytes [49,55] and IRAK1 is the key adaptor molecule of the toll-like receptor (TLR2/4)-IRAK1 mediated NF- κ B activation pathway for the activation of cells in the adaptive immune system [56]. TLR2/4 or IRAK1-deficient mice are resistant to experimental autoimmune encephalomyelitis, exhibiting little or no CNS inflammation [55,57,58], suggesting the importance of TLR signaling in regulating oligodendrocyte pathogenesis and demyelination. The present study showed that gain-of-function of miR-146a decreased the expression of IRAK1 in NPCs and OPCs, and loss-of-function of miR-146a increased the expression of IRAK1. Moreover, knockdown of endogenous IRAK1 significantly increased myelin protein levels and decreased the OPC apoptotic key gene-cleaved Caspase-3. Levels of IRAK1 were inversely correlated with the function of miR-146a in OPCs, suggesting that IRAK1 signaling mediates miR-146a-induced OPC differentiation and survival. Apart from IRAK/TRAF genes, we also found that multiple pro-inflammatory genes, including ADAMTS3, a secretory factor, and apoptosis associated gene-FADD, were substantially downregulated in OPCs. These genes are putative miR-146a targets, suggesting that they may also be involved in the effect of miR-146a on OPC differentiation. Further studies on the functions of these target genes would help to clarify the pathophysiological mechanisms of oligodendrocyte impairment after stroke.

In summary, the present study demonstrates that miR-146a promotes OPC differentiation and increases neuronal differentiation of NPCs. Mature oligodendrocytes myelinate axons [1]. Thus, miR-146a could potentially be used as a target for development of novel therapies for brain injuries, such as stroke and traumatic brain injury, and other neurodegenerative diseases.

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Figure 1.

Quantification of miR-146a in corpus callosum oligodendrocytes and SVZ neural progenitor cells after stroke. Panels A, B and D, E show the corpus callosum oligodendrocytes and SVZ neural progenitor cells captured before and after laser capture microdissection, respectively. Panels C and F show qRT-PCR data of miR-146a in captured oligodendrocytes (C) and neural progenitor cells (B). The fold change of miRNAs was normalized against the expression of U6 snRNA as an endogenous normalization control. In situ hybridization shows miR-146a signals in non-ischemic (G, outlines) and ischemic (H, outlines) corpus

callosum. Panels J and K show the miR-146a signal in non-ischemic (J) and ischemic (K) SVZ neural progenitor cells. Panels I and L show quantitative data of miR-146a in the corpus callosum and SVZ, respectively. *Arrowheads show the dissected OPCs in corpus callosum (B) and NPCs in SVZ (E) by LCM.* CC = corpus callosum; LV = lateral ventricle; Str = striatum; SVZ = subventricular zone. Scale bar=40µm.

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Figure 2.

FISH in combination with immunofluorescent staining of cultured NPCs shows the distribution of miR-146a (A) and the co-localization of miR-146a (green) with nestin positive neural progenitor cells (2B, red), Tuj1 positive neuroblasts (2C, red), PDGFRα positive OPCs (2D, red), and GFAP positive astrocytes (2E, red). Scale bar=40μm.

Figure 3.

The effects of miR-146a on the differentiation and survival of oligodendrocyte progenitor cells (OPCs). Panels A and B demonstrate the introduction of miR-146a mimics (A) or inhibitors (B) significantly increased or decreased the expression of miR-146a in OPCs, respectively. Panel C shows representative immunostaining images of MBP positive cells after miR-146a mimic transfection. Panel D shows quantitative data of the number of MBP positive cells in OPCs after treatment with miR-146a mimics or inhibitors. OPCs transfected with cel-miR-67 mimics or inhibitors was employed as a negative control (D, control).

Western blots (E) show that delivery of miR-146a mimics increased protein levels of MBP, proteolipid protein (PLP), and 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), markers of mature oligodendrocytes as well as considerably decreased oligodendrocyte progenitor cell protein levels, PDGFR α and NG2, nevertheless inhibition of miR-146a using inhibitor against miR-146a. *Panel F shows representative BrdU positive cells in OPCs after transfection of miR-146a mimic. Panel G shows quantitative data of the number of BrdU positive cells in OPCs after treatment with miR-146a mimics.* Panel H shows that delivery of miR-146a mimics dramatically decreased the Caspase-3/7 activity tested by a luciferase reporter in OPCs, but miR-146a inhibitor inversely increased the Caspase-3/7 activity. * $p < 0.05$, $N = 3$ /group. Scale bar = 20 μ m.

Figure 4.

The effects of miR-146a mimics on the differentiation and proliferation of ischemic neural progenitor cells. Panels A, C and E show representative immunostaining images of Tuj1 (A), O4 (B) and BrdU (C) positive cells, respectively, in neural progenitor cells after treatment with miR-146a mimics or cel-miR-67 (control). Panels B, D and F show quantitative data of Tuj1 (B), O4 (D) and BrdU (F), positive cells, respectively, after treatment with miR-146a mimics or cel-miR-67 (control). *p<0.05. Scale bar=5μm.

Figure 5.

miR-146a regulates oligodendrocyte differentiation by targeting IRAK1. Using a miR-146a target PCR array, panel A showed that the transcripts of IRAK1, IRAK2, TRAF6 and Numb were decreased in OPCs after overexpression of miR-146a by introducing exogenous miR-146a mimics, compared with those transfected with miRNA mimic control. Western blot demonstrated that overexpression of miR-146a decreased its target genes-IRAK1 and another target gene TRAF6 as well as NF- κ B p65 subunit in neural progenitor cells isolated from both contralateral and ischemic SVZ cells with a dose-dependent manner (B). Panel C

shows that nanoparticle-delivered mature miR-146a resulted in a substantial decrease of IRAK1 protein levels compared with the miRNA mimics control as well as attenuation of endogenous miR-146a increased IRAK1 levels in primary cultured OPCs. Panel D shows that protein levels of IRAK1 were decreased in OPCs transfected with its siRNA and knockdown of endogenous IRAK1 increased protein levels of MBP, CNPase, PLP and cleaved Caspase-3 whereas decreased protein levels of NG2 and PDGFR α . Panel E shows that knockdown of IRAK1 significantly increased the Caspase-3/7 activity in OPCs.