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In Vivo Inhibition of miR-155 Promotes Recovery after Experimental Mouse Stroke

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A multifunctional microRNA, miR-155, has been recently recognized as an important modulator of numerous biological processes. In our previous *in vitro* studies, miR-155 was identified as a potential regulator of the endothelial morphogenesis. The present study demonstrates that *in vivo* inhibition of miR-155 supports cerebral vasculature after experimental stroke. Intravenous injections of a specific miR-155 inhibitor were initiated at 48 h after mouse distal middle cerebral artery occlusion (dMCAO). Microvasculature in peri-infarct area, infarct size, and animal functional recovery were assessed at 1, 2, and 3 weeks after dMCAO. Using *in vivo* two-photon microscopy, we detected improved blood flow and microvascular integrity in the peri-infarct area of miR-155 inhibitor-injected mice. Electron microscopy revealed that, in contrast to the control group, these animals demonstrated well preserved capillary tight junctions (TJs). Western blot analysis data indicate that improved TJ integrity in the inhibitor-injected animals could be associated with stabilization of the TJ protein ZO-1 and mediated by the miR-155 target protein Rheb. MRI analysis showed significant (34%) reduction of infarct size in miR-155 inhibitor-injected animals at 21 d after dMCAO. Reduced brain injury was confirmed by electron microscopy demonstrating decreased neuronal damage in the peri-infarct area of stroke. Preservation of brain tissue was reflected in efficient functional recovery of inhibitor-injected animals. Based on our findings, we propose that *in vivo* miR-155 inhibition after ischemia supports brain microvasculature, reduces brain tissue damage, and improves the animal functional recovery.

Key words: **ischemia; microRNA; microvasculature; neuroprotection; regeneration; stroke**

Significance Statement

In the present study, we investigated an effect of the *in vivo* inhibition of a microRNA, miR-155, on brain recovery after experimental cerebral ischemia. To our knowledge, this is the first report describing the efficiency of intravenous anti-miRNA injections in a mouse model of ischemic stroke. The role of miRNAs in poststroke revascularization has been unexplored and *in vivo* regulation of miRNAs during the subacute phase of stroke has not yet been proposed. Our investigation introduces a new and unexplored approach to cerebral regeneration: regulation of poststroke angiogenesis and recovery through direct modulation of specific miRNA activity. We expect that our findings will lead to the development of novel strategies for regulating neurorestorative processes in the postischemic brain.

Introduction

Stroke-induced cerebral endothelial injury and inflammation impair endothelial function and thus increase cerebrovascular permeability and blood–brain barrier (BBB) leakage, leading to primary and secondary ischemic brain injury (del Zoppo et al., 2003; Brouns et al., 2009). Functional recovery is highly dependent on the effective restoration of blood supply to the damaged but viable peri-infarct area (Navaratna et al., 2009).

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Therefore, extensive investigation has been concentrated on poststroke vascularization with the aim of promoting the recovery process (Xiong et al., 2010; Chen et al., 2014).

Ischemic stroke induces significant changes in the gene profile of the neurovascular elements (Carmichael, 2003; Liu et al., 2007). microRNAs (miRNAs) are recently identified short mRNA-interfering molecules that regulate gene expression and thus significantly influence essential cellular processes (Kato et al., 2008; Fabian et al., 2010). miRNAs control posttranscriptional gene expression in many tissues, including the brain; are highly expressed in the vasculature; and serve as critical modulators for vascular cell functions (Suárez et al., 2007; Costa et al., 2013; Pecot et al., 2013). Considerable changes in miRNA profiles are associated with stroke at different perfusion times after experimental ischemia (Huang et al., 2010; Liu et al., 2010). Our previous studies demonstrated that miR-155 negatively regulates proangiogenic signaling pathways in mouse brain endothelial cells. Inhibition of miR-155 significantly enhanced, whereas overexpression significantly inhibited, *in vitro* endothelial morphogenesis (Roitbak et al., 2011). We therefore speculated that miR-155 could also coordinate vascular functions *in vivo*.

miR-155 has been implicated in regulating various physiological and pathological processes such as hematopoietic lineage differentiation, immunity, inflammation, cancer, and cardiovascular diseases (Faraoni et al., 2009; O'Connell et al., 2010). miR-155 is specifically expressed in hematopoietic cells and cells involved in vascular remodeling, including B cells, T cells, monocytes, and granulocytes, as well as endothelial cells and smooth muscle cells (Landgraf et al., 2007; Sun et al., 2012). miR-155 regulates the development of inflammatory T cells and promotes tissue inflammation. Specific inhibition of this miRNA is accompanied by reduced inflammation, which makes it a promising therapeutic target in proinflammatory conditions (Kurowska-Stolarska et al., 2011; Murugaiyan et al., 2011). Among other functions, miR-155 is significantly associated with endothelial and vascular function: its specific inhibition results in increased endothelial nitric oxide synthase (eNOS) expression and nitric oxide (NO) production and reduction of vascular inflammation, whereas overexpression alters endothelial cell morphology and impedes endothelial cell migration (Sun et al., 2012; Weber et al., 2014). miR-155$^{-/-}$ mice also exhibit accelerated wound healing processes, thus demonstrating an increased regeneration capacity (van Solingen et al., 2014). Interestingly, miR-155 is among the miRNAs significantly affected by cerebral ischemia (Liu et al., 2010).

Systemic administration of the miRNA inhibitors and mimics to regulate the activity of specific miRNAs had recently been identified as attractive target for therapeutic intervention (Linnstaedt et al., 2010; Zhang et al., 2013). *In vivo* regulation of miRNA activity is expected to provide a future therapeutic potential for the treatment of cancer and cardiovascular and diseases (Shi et al., 2010; Moon et al., 2013); delivery of synthetic miRNAs and miRNA inhibitors to tumor tissue is already used in animal studies (Takeshita et al., 2010; Wiggins et al., 2010).

A subacute phase of ischemia is associated with postischemic vascular remodeling (Ding et al., 2008; Beck et al., 2009) and our aim is to find a way to promote this natural regeneration process. The present investigation introduces a novel approach to cerebral regeneration: regulation of the vascular functions and overall recovery through direct modulation of specific miRNA activity.

Materials and Methods

Animal groups. The experimental procedures were performed in accordance with the University of New Mexico Office of Animal Care Compliance. All institutional and national guidelines for the care and use of laboratory animals were followed. Two-month-old C57BL/6 male mice (Jackson Laboratories) were used in our experiments. In total, 120 mice were subjected to the experimental cerebral ischemia; 59 were used for the *in vivo* evaluation of cerebral blood flow and capillary density and permeability and 22 were used for MRI-based evaluation of the brain injury and cerebral perfusion. An additional 20 mice were used for the pilot studies to assess the efficiency of miR-155 *in vivo* inhibition and for sham surgeries. A total of 50 mice were used for the concomitant behavioral studies. Experimental groups included intact mice, sham-operated mice, mice subjected to distal middle cerebral artery occlusion (dMCAO) and specific miR-155 inhibitor injections (Inhibitor group), and mice subjected to dMCAO and control (scrambled) miRNA inhibitor injections (Control group).

dMCAO procedure. dMCAO was used as an experimental model of cortical ischemia (Kuraoka et al., 2009; Pena-Philippides et al., 2014). This model produces an infarct in the frontal and parietal cortex, which over time progresses into adjacent temporal, frontal, and cingulate cortex. In addition to its high reproducibility and survival rate, the advantage of this model is that it produces a smaller infarct comparable to human stroke (Carmichael, 2005). In addition, dMCAO induces mostly cortical ischemia and thus represents a convenient model for *in vivo* imaging, which is limited by its depth penetration capacities. dMCAO was performed on 2-month-old male C57BL/6 mice (Jones et al., 2008; Kuraoka et al., 2009). The mice were anesthetized using isoflurane gas (induction dosage 2–3%; maintenance dosage 1.5–2%) and a mixture of O_2:N_2O gases in the ratio 2:1 delivered during the surgery. The middle cerebral artery (MCA) was exposed via a transtemporal approach (Jones et al., 2008; Kuraoka et al., 2009). A small burr hole (located 1 mm rostral to the fusion of zygoma and squamosal bone and 3 mm ventral to the parietal bone) was made on the left side of the skull surface and the MCA was coagulated with low-heat electrocautery (Bovie Medical). In sham-operated animals, the MCA was exposed but not coagulated.

miRNA inhibitor injections. Our study used a novel, second-generation-specific inhibitor for miR-155, which has been recently developed by Exiqon. Specific miRNA inhibitors are designed using novel locked nucleic acid (LNA) technology, which greatly increases the affinity of the inhibitors for their target miRNAs and improves their resistance to enzymatic degradation. These features are especially important for the *in vivo* studies because the oligonucleotides have high knock-down efficiency at low concentrations, which reduces the risk of negative and off-target side effects. Injections of a specific anti-miR-155 miRCURY LNA inhibitor or control inhibitor (scrambled oligonucleotide) from Exiqon were initiated 48 h after dMCAO and performed for 3 consecutive days. Oligonucleotides were introduced via mouse lateral tail vein; the dose was 10 mg/kg in saline, total injected volume was 100 μl.

In vivo *two-photon laser scanning microscopy*. *In vivo* two-photon laser scanning microscopy (2PLSM) was performed 7, 14, and 21 d after dMCAO as described previously (Kleinfeld et al., 1998; Bragin et al., 2011). Imaging was performed at the stereotactic coordinates 2 mm caudal and 2.5 mm lateral to bregma through the optical windows (1 mm^2) over both lesioned and nonlesioned hemispheres. According to 2PLSM measurements and MRI performed 24 h after dMCAO, this region is characterized by reduced blood flow and is localized within ~400 μm from the infarct border. Based on our own measurements and on the existing literature, this region was defined as a peri-infarct area of stroke (Li et al., 2008; Murphy et al., 2009; Ueno et al., 2012; López-Valdés et al., 2014). An Olympus BX51WI upright microscope and water-immersion LUMPlan FL/IR 20×/0.50 W objective were used for the imaging. Excitation (740 nm) was provided by a Prairie View Ultima multiphoton laser scan unit powered by a Millennia Prime 10 W diode laser source pumping a Tsunami Ti:sapphire laser (Spectra-Physics) tuned up to 810 nm center wavelength. Images (512 × 512 pixels, 0.15 μm/pixel in the *x*- and *y*-axes) or line scans were acquired using Prairie View software. Cranial windows (1 mm in diameter) for the imaging were made at the stereo-

tactic coordinates bregma −2 mm and L 2.5 mm over both lesioned and nonlesioned hemispheres.

Microvascular red blood cell (RBC) flow velocity was measured by the line scan mode of 2PLSM. Blood plasma was labeled by the injection of ~100 μl of fluorescein-labeled dextran (70 kDa; Sigma-Aldrich) at an initial concentration of 150 μM. Fluorescence was band-pass filtered at 500–550 nm. A total of ~100 microvessels (2–50 μm diameter) per hemisphere in an imaging volume of 500 × 500 × 300 μm = 0.075 mm^3 were scanned. A line scan through a microvessel led to a sequence of alternating bright and dark diagonal bands in a space–time image corresponding to labeled fluorescent plasma and unlabeled dark RBCs and slope of the stripes inversely reflects RBC velocity. The width, sharpness, and angle of the dark bands representing the RBC motion, as well as the distance between them, were evaluated to calculate the RBC flow velocity in offline analyses using Rincon version 7.7 software.

Evaluation of microvessel density. The 3D anatomy of the vasculature in the region of interest was reconstructed from *z*-series of 2D (planar) scans (500 × 500 μm) obtained at consecutive depths in the cortex with 10 μm steps from brain surface to 300 μm in depth. In offline analyses using ImageJ, the microvessel diameters were measured to differentiate capillaries from other microvessels and density was quantified assuming that one capillary is a microvessel from bifurcation to bifurcation. The capillaries were identified by diameters (3–8 μm) (Schiszler et al., 2000), mean of ~5 μm (Motti et al., 1986; Seylaz et al., 1999), tortuosity, and single-file RBCs (Hudetz et al., 1995; Hauck et al., 2004).

Microvascular BBB permeability. BBB permeability was evaluated by measuring perivascular tissue fluorescence in planar images of the cortex taken at 50 μm depth 30 min after fluorescein dextran injection, as described previously (Bragin et al., 2011; Bragin et al., 2013). In offline analyses using ImageJ, the fluorescence of five randomly chosen regions of interest over the vessel areas and five to 10 areas over perivascular brain parenchyma in both lesioned and nonlesioned/intact hemispheres were measured. The obtained measurements in the interstitial space were normalized to the maximal fluorescence intensity in the blood vessels and expressed as a percentage fluorescence intensity (modified from Egawa et al., 2013). Thirty minutes after fluorescein-dextran injection in a healthy brain, bright vessels filled with fluorescein-dextran are seen clearly over the dark background of unstained tissue, reflecting intact BBB. Increased perivascular tissue fluorescence due to fluorescein leakage from microvessels into the brain parenchyma indicates impaired BBB.

Electron microscopy. For electron microscopy (EM), mice were perfused with 0.5% glutaraldehyde, 2.5% paraformaldehyde in 0.1 M Sorensen's buffer. Cortical tissue (1×1 mm) was dissected from the areas imaged during 2PLSM analysis (identified by the cranial window openings) from the hemisphere both ipsilateral and contralateral to ischemic damage. Samples were fixed in 2% osmium, dehydrated, embedded in Araldite, thin-sectioned to 80–90 nm, and stained with 4% uranyl acetate in methanol followed by lead citrate as described previously (Lippman et al., 2008). Images were acquired using a Hitachi H7500 transmission electron microscope equipped with an AMT X60 camera. Three to four animals per experimental group (Control and Inhibitor) were used for analysis; high-magnification (800–15,000×) images were taken both in the lesioned and intact hemispheres. The following analysis methods were used for the ultastructural evaluation of capillaries and neurons.

To evaluate the vascular damage and TJ integrity 7 d after dMCAO, 205 capillaries (<8 μm in diameter) from the lesioned and unlesioned hemispheres were imaged and analyzed. On average, 85–87 capillaries/group were evaluated in the lesioned hemispheres and 30–40 in the intact hemispheres. Endothelial cell integrity, intracellular (inside the endothelial cells and pericytes) and perivascular vacuoles, perivascular astrocytic processes, and basal lamina were evaluated to assess microvascular damage. The major ultrastructural criterion for TJ integrity was an existence of the intermediate dark (electron-dense) layer that is formed by multilayered membrane units of the TJs (Castejón, 2012). Capillaries with all intact TJs were considered normal/intact, whereas capillaries with at least one partially or fully opened/disrupted TJ unit were considered partially opened or opened, respectively. In addition, 186 capillaries from the lesioned and unlesioned hemispheres of both groups of animals were imaged and analyzed for assessment of microvasculature 21 d after dMCAO.

To evaluate neuronal damage 21 d after dMCAO, 430 neurons from the lesioned and unlesioned hemispheres were imaged and analyzed. On average, 172 neurons per group were evaluated in the lesioned hemispheres of the Control and Inhibitor groups and 43 neurons/group in the nonlesioned hemispheres of both groups. Major criteria for neuronal damage evaluation were as follows: shape and integrity of the nuclei, presence of intracellular vacuoles, mitochondrial swelling, ER dilation and damage, chromatin accumulation, and overall cytoplasm appearance.

MRI. T2-weighted MRI. Infarct size was evaluated using MRI at 24 h and 7, 14, and 21 d after dMCAO using a 4.7 T Biospec dedicated research MR scanner (Bruker) equipped with a single tuned surface coil for mouse brain (RAPID Biomedical). The mice were anesthetized using isoflurane gas (induction dosage 2–3%; maintenance dosage 1.5–2%) and a mixture of O_2:N_2O gases in the ratio 2:1 delivered during the measurements. Real-time monitoring of physiological parameters (heart rate and respiratory rate) was performed during the entire duration of the study. A tri-pilot scan using gradient echo sequence was used to acquire initial localizer images. T2-weighted MRI was performed with a fast spin-echo sequence (RARE), TR/TE = 5000/56 ms, FOV = 4 cm × 4 cm, slice thickness = 1 mm, interslice distance = 1 mm, number of slices = 12, matrix = 256 × 256, number of average = 3.

Infarct volume was calculated based on the widely used method proposed previously (Swanson et al., 1990) using ImageJ analysis software. The optical density threshold was determined for the gray matter in the unlesioned control hemisphere in each slice and used for the recognition of normal gray matter in the lesioned hemisphere. The areas of noninfarcted (normal) brain tissue were measured in nonlesioned intact (*I*) and lesioned (*L*) hemispheres in each T2-weighted MRI slice. The measured areas were summarized and the respective V_I and V_L volumes were calculated by multiplying each sum by the distance between slices (1 mm). The infarction volume was expressed as a percentage of the volume of the control hemisphere, using the following formula: $\%I = 100 \times (V_I - V_L)/V_I$.

Quantitative measurements of cerebral blood flow using MRI. In the same MRI sessions, tissue perfusion was measured using noninvasive arterial spin labeling method (ASL) (Kim, 1995). The sequence Flow-sensitive Alternating Inversion Recovery Rapid Acquisition with Relaxation Enhancement (FAIR-RARE) was used to implement ASL with parameters: TE/TR = 46/16000 ms, FOV = 2.56 cm × 2.56 cm, slice thickness = 1 mm, number of slices = 1, matrix = 128×128. A perfusion map was calculated using the ASL Perfusion Processing macro in ParaVision 5.1 (Bruker). Perfusion associated with specific areas of the brain was determined using interpolation method and corresponding T2-weighted MRI scans. Cortical areas of interest from the *L* and *I* hemispheres were selected from the corresponding T2-weighted MRI scans and interpolated to perfusion maps. Because the exact borders of the peri-infarct region are difficult to define, the measurements were performed in the whole sensorimotor cortex area adjacent to the dMCAO-induced cortical lesion and the analogous area of the intact hemisphere. Similar to infarct size measurements, brain tissue perfusion in the hemisphere ipsilateral to the lesion was expressed as a percentage of that in the intact hemisphere.

PCR. Three and 7 d after completion of miR-155 or control inhibitor injections (which started 48 h after dMCAO and lasted for 3 consecutive days) in dMCAO-subjected mice, 3 brains per experimental group (Inhibitor and Control) were used to generate separate sample triplicates for the analyses. Brain cortices were dissected and stored in RNAlater solution (Ambion). Total RNA was isolated using mirVana miRNA (Ambion) isolation kit from the hemispheres ipsilateral to dMCAO injury. qRT-PCR was used to evaluate miR-155 expression in the samples using a TaqMan MicroRNA reverse transcription kit and miR-155 specific TaqMan microRNA assay (Ambion). PCR array experiments were performed in collaboration with the University of New Mexico CUGR (Keck-UNM Genomic Resource) Affymetrix facility. Expression of miR-155 target genes was assessed using miR-155 Targets PCR Array (Qiagen). Mouse Cytokines and Chemokines PCR Array (Qiagen) was used