MicroRNA-200c Contributes to Injury From Transient Focal Cerebral Ischemia by Targeting Reelin

Creed M. Stary, MD, PhD; Lijun Xu, MD; Xiaoyun Sun, MD; Yi-Bing Ouyang, PhD; Robin E. White, PhD; Jason Leong, MD; John Li; Xiaoxing Xiong, MD; Rona G. Giffard, MD, PhD

Background and Purpose—MicroRNA (miR)-200c rapidly increases in the brain after transient cerebral ischemia but its role in poststroke brain injury is unclear. Reelin, a regulator of neuronal migration and synaptogenesis, is a predicted target of miR-200c. We hypothesized that miR-200c contributes to injury from transient cerebral ischemia by targeting reelin.

Methods—Brain infarct volume, neurological score and levels of miR-200c, reelin mRNA, and reelin protein were assessed in mice subjected to 1 hour of middle cerebral artery occlusion with or without intracerebroventricular infusion of miR-200c antagonist, mimic, or mismatch control. Direct targeting of reelin by miR-200c was assessed in vitro by dual luciferase assay and immunoblot.

Results—Pretreatment with miR-200c antagonist decreased post–middle cerebral artery occlusion brain levels of miR-200c, resulting in a significant reduction in infarct volume and neurological deficit. Changes in brain levels of miR-200c inversely correlated with reelin protein expression. Direct targeting of the Reln 3′ untranslated region by miR-200c was verified with dual luciferase assay. Inhibition of miR-200c resulted in an increase in cell survival subsequent to in vitro oxidative injury. This effect was blocked by knockdown of reelin mRNA, whereas application of reelin protein afforded protection.

Conclusions—These findings suggest that the poststroke increase in miR-200c contributes to brain cell death by inhibiting reelin expression, and that reducing poststroke miR-200c is a potential target to mitigate stroke-induced brain injury. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.114.007041.)

Key Words: infarction, middle cerebral artery ▪ microRNAs ▪ reperfusion injury ▪ stroke

MicroRNAs (miRs) are endogenous, short (=20 nucleotides) single-stranded RNAs that regulate gene expression by inhibiting translation of specific target mRNAs. Members of the miR-200 family are upregulated in the brain after transient cerebral ischemia, but their role in stroke-related injury is not understood. Studies in tumor and endothelial cells demonstrate a proapoptotic role for miR-200c, suggesting that poststroke increases in miR-200c may contribute to neuronal cell death.

Computational analysis of potential neuronal targets of miR-200c identifies reelin, an extracellular matrix protein essential for proper neuronal migration in the developing brain and in maintaining synaptogenesis in adulthood. Reelin coordinates neuronal cell survival by inhibiting apoptosis and may play a protective role in the response to cerebral ischemia–reperfusion injury, as reelin-deficient mice are more susceptible to injury after transient cerebral ischemia. Therefore, in the present study, we sought to determine (1) whether miR-200c contributes to brain cell death subsequent to transient cerebral ischemia, (2) whether reducing miR-200c with antagonist pretreatment could decrease the severity of acute stroke injury, and (3) whether the effect of miR-200c on neuronal cell death is mediated by inhibition of reelin.

Methods

More details are provided in the online-only Data Supplement.

Animals and In Vivo Experimental Protocols

All experimental protocols using animals were approved by the Stanford University Animal Care and Use Committee, and in accordance with National Institutes of Health guidelines. Adult male CB57/B6 mice (aged, 8–10 weeks; Charles River) were randomly assigned by coin flip to either intracerebroventricular pretreatment with miR-200c antagonist, mimic, or mismatch-control and subjected to 1-hour middle cerebral artery occlusion (MCAO). Neurological score and infarct volume were assessed by a blinded observer after 24 hours of reperfusion. In a second set of experiments, animals were randomly divided and pretreated with either intracerebroventricular miR-200c antagonist or control infusion 24 hours before 1 hour MCAO, and then euthanized at 1, 3, and 24 hours of reperfusion for analysis of brain levels of miR-200c, reelin mRNA, and reelin protein.

Received August 13, 2014; final revision received November 27, 2014; accepted December 15, 2014.
From the Department of Anesthesia, Stanford University School of Medicine, CA (C.M.S., L.X., X.S., Y.-B.O., J. Leong, J. Li, X.X., R.G.G.); and Department of Biology, Westfield State University, MA (R.E.W.).
The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.114.007041/-/DC1.
Correspondence to Rona G. Giffard, MD, PhD, Department of Anesthesia, Stanford University, 300 Pasteur Dr, Stanford, CA 94305-5117. E-mail rgiffard@stanford.edu
© 2015 American Heart Association, Inc.
Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.114.007041
Intracerebroventricular Pretreatment

Mice were anesthetized with 2% isoflurane by facemask and placed in a stereotactic frame. A 26-gauge brain infusion cannula was placed stereotaxically into the left lateral ventricle (bregma, −0.58 mm; dorsoventral, 2.1 mm; lateral, 1.2 mm) as previously described.10 miR-200c antagomir (3 pmol/g body weight in 2 μL), mimic or mismatch-control (Life Technologies) was mixed with cationic lipid DOTAP (4 μL; 6 μL total volume; Roche) and infused for 20 minutes.

Transient Focal Cerebral Ischemia (MCAO)

Mice (n=124 for all treatment groups and analyses) were anesthetized with 2% isoflurane and focal cerebral ischemia was produced by 1 hour of MCAO with a 6-0 monofilament followed by reperfusion as previously described.10,11 Sham-operated mice (n=12) underwent ligation of the external carotid artery but no suture insertion. Temperature and respiratory rate were monitored continuously and rectal temperature was maintained at 37±0.5°C with a heating pad. After the appropriate duration of reperfusion, mouse brains were rapidly removed after transcardial perfusion with ice cold PBS followed by 4% paraformaldehyde in PBS to assess infarct volume, or after perfusion only with PBS for reverse transcription quantitative polymerase chain reaction or protein analysis. Mice with no evidence of acute neurological deficit (control=3/24; antagomir=2/14; mimic=2/14), which died <24 hours after surgery (control=3/50; antagomir=4/48; mimic=1/17), or with evidence of significant bleeding (control=6/50; antagomir=7/48; mimic=2/17) were excluded from analysis. No significant difference (P<0.05) was observed between treatment groups in number of excluded animals.

Neurological Score and Measurement of Cerebral Infarction Area

Neurological performance was assessed and scored before euthanization as previously described10 from a score of 0 (no observable neurological deficit) to 4 (unable to walk spontaneously). Mice were then deeply anesthetized and transcardially perfused with saline followed by 4% paraformaldehyde, and brains were removed. After coronal sectioning of brains into 50-μm sections with a vibratome and staining with Cresyl Violet (EMD Chemicals), infarct volume was quantified by a blinded observer and corrected for edema using Image J software (v1.46, National Institutes of Health) as described previously.11

Dual Luciferase Target Validation and Reporter Assay

The luciferase reporter assay was performed as described previously.12 Briefly, mouse neuroblastoma (N2a) cells were cotransfected with 0.25-ng Firefly luciferase control reporter plasmid, 0.05-ng Renilla luciferase target reporter with Reln 3′ untranslated region (UTR), and 40 ng miRNA expression vector using Lipofectamine 2000 (Invitrogen). Luminescence was assessed using a Promega Dual-Luciferase assay kit (E1960) with automated microplate reader (Infinite M1000 Pro, Tecan). In an additional set of experiments, we tested a second potential target of miR-200c identified by computational predictive algorithms (Targetscan.org, release v6.2), Grp75 (Hsp90a/Hsp75/mortalin), a mitochondrial-localized member of the HSP70 family known to play a role in neuroprotection.13

Cell Culture Transfection and Injury

N2a cells were grown in high-glucose DMEM (Invitrogen, Carlsbad) supplemented with 8% fetal bovine serum (Hyclone) and antibiotics (50 U/mL penicillin+50 μg/mL streptomycin; Invitrogen) in a humidified atmosphere containing 5% CO2 at 37°C. Cells were transfected with 20 pmol miR-200c mimic, inhibitor, or mismatch-control (Thermo Scientific), with and without 20 pmol Reln small interfering RNA (Life Technologies) using Lipofectamine 2000 (Invitrogen). Cells were either harvested for analysis of miR-200c, reelin mRNA or reelin protein or subjected to injury 24 hours after transfection. In an additional set of experiments, cells were incubated with 0, 50, 100, or 500 ng/μL recombinant reelin protein 1 hour before, and during, injury. Injury was induced by exposure to 500 μmol/L H2O2 in serum-free DMEM for either 18 or 24 hours14 and cell death was quantified by measuring lactate dehydrogenase–released dead cells as previously described.15

Reverse Transcription Quantitative Polymerase Chain Reaction

Total RNA was isolated with TRIzol (Invitrogen). Reverse transcription was performed as previously described16 using the TaqMan MicroRNA Reverse Transcription Kit for miR-200c and total RNA (Applied Biosystems). Predesigned primer/probes for polymerase chain reaction were obtained from Life Technologies for mouse Reln and GAPDH mRNA, miRNA-200c, and U6 small nuclear RNA (U6). Quantitative polymerase chain reaction reactions were conducted as previously described16 using the TaqMan Assay Kit (Applied Biosystems). Measurements for reelin mRNA were normalized to within-sample GAPDH, whereas miR-200c was normalized to U6 (ΔΔCT). Comparisons were calculated as the inverse log of the ΔΔCT from controls.17

Reelin Protein Analysis

Immunoblotting was performed as previously described.18 Briefly, 50 μg of protein/sample was separated on a 4% to 10% Bis–Tris mini-gel (Life Technologies), and electrotransferred to immobilized polyvinylidene fluoride membrane (Millipore Corp). Membranes were blocked and incubated with primary antibody against full-length reelin (1:500, Abcam, No. ab78540) and β-actin (1:3000, LiCOR Bioscience, No. 926–42210), washed and incubated with 1:15 000 the appropriate conjugated secondary antibody (LiCOR Bioscience, No. 926–32212, No. 926–68021). Immunoreactive bands were visualized using the LiCOR Odyssey infrared imaging system. Densitometric analysis was performed using Image J software (v1.46, National Institutes of Health). Reelin band intensity was normalized to β-actin.

Results

Reduction of miR-200c Protects the Brain From Transient Focal Cerebral Ischemia

Decreasing miR-200c has been shown to improve cell survival after in vitro ischemia in neuronal SH-SY5Y cells.18 We investigated the effect of miR-200c inhibition in transient focal cerebral ischemia. Pretreatment with intracerebroventricular infusion of miR-200c antagomir resulted in significant knockdown of miR-200c to ≈13% of control values, whereas pretreatment with miR-200c mimic increased miR-200c ≈40-fold (Figure 1A). After miR-200c antagomir pretreatment and MCAO infarct volume was significantly decreased, ≈30% relative to control animals (Figure 1B and 1C). Gross motor function, estimated by neurological score 24 hours after reperfusion, was significantly improved with antagomir pretreatment (Figure 1D). In contrast, animals pretreated with miR-200c mimic were not significantly different from mismatch-control in either infarct volume or neurological score.
Brain Levels of Reelin Decrease After MCAO

Brain levels of miR-200c after MCAO significantly increased ≈17-fold at 1 hour of reperfusion (Figure 2A), before returning to baseline levels by 24 hours of reperfusion. Conversely, by 3 hours of reperfusion brain levels of reelin protein were significantly decreased (Figure 2A and 2B). These findings agree with previous data showing an inverse correlation between reelin and miR-200c expression in developing submandibular cells. To further define a functional relationship between miR-200c and reelin in the development of injury after transient cerebral ischemia, we assessed post-MCAO brain levels of reelin mRNA and uncleaved reelin protein in animals subjected to miR-200c knockdown before MCAO. We observed a significant decrease in miR-200c with antagomir relative to mismatch-control–pretreated animals at baseline and all reperfusion time-points (Figure 3A). An effect of miR-200c knockdown on reelin expression was also observed: both pre-MCAO levels and levels at the 3 hours reperfusion time-point in miR-200c antagomir-pretreated animals demonstrated significantly increased reelin protein (Figure 3C and 3D). However, miR-200c suppression by antagomir did not alter reelin mRNA levels (Figure 3B), suggesting that inhibition of reelin expression by miR-200c occurred via translational silencing rather than mRNA degradation.

Validation of the 3′UTR of Reln as a Direct Target of miR-200c

To date, only predictive and correlative evidence has been presented suggesting reelin mRNA is a direct target of miR-200c (mature miR-200c sequence listed in Figure IA in the online-only Data Supplement). The 3′ UTR of Reln contains 2

Figure 1. A, Brain microRNA (miR)-200c levels after intracerebroventricular (ICV) pretreatment decrease with miR-200c antagomir (Antag) and increase with mimic, relative to mismatch-control (cont). B, Representative cresyl violet–stained brain sections from mice subjected to ICV pretreatment, 1 hour middle cerebral artery occlusion (MCAO), and 24 hours of reperfusion. Regions of infarct are lighter in color. C, Effect of altering miR-200c levels on injury; quantification of infarct volume averaged for 4 levels demonstrated significant protection with miR-200c antagomir pretreatment compared with either control or mimic. D, Neurological score in post-MCAO mice: miR-200c antagomir pretreatment resulted in significantly improved neurological outcome (lower score). *P<0.05 compared with control (n=11–23 animals per group).

Figure 2. A, Time course of brain levels of microRNA (miR)-200c (), reelin mRNA (), and uncleaved reelin protein () in mice subjected to 1 hour middle cerebral artery occlusion (MCAO). B, Examples of immunoblots for reelin and actin after MCAO at the 3 hours reperfusion time-point. Although the levels of miR-200c increased by 1 hour of reperfusion (A), levels of uncleaved reelin protein (A and B) decreased by 3 hours, suggesting translational inhibition. *P<0.05 compared with sham control (n=8 animals per group).

Figure 3. In mice pretreated with intracerebroventricular infusion of microRNA (miR)-200c antagomir, after middle cerebral artery occlusion (MCAO) miR-200c expression in the brain significantly decreased relative to control (A). Although reelin mRNA remained unchanged (B), uncleaved reelin protein increased (C and D), suggesting silencing by miR-200c rather than degradation. *P<0.05 compared with pre-MCAO control (n=8–11 animals per group).
potential binding sites for miR-200c (Targetscan.org, release v6.2; Figure 4A). To validate direct targeting of the Reln 3′UTR by miR-200c, we used the dual luciferase gene reporter assay in N2a cells. The full-length mouse Reln 3′UTR (1119 nt) was cloned (forward primer: GGACTTGGCCAGAACAAGAC; reverse primer: CTAGTCAGAGGCTACAGGGC) and inserted into the Renilla luciferase reporter vector phRL-TK (Promega). We generated a mutant of mouse miR-200c with 3 base substitutions within the seed region (nt 47–53 of the mouse sequence, AAUACUG to AAAAAU). Both wild-type and mutant inserts were confirmed by DNA sequencing (Stanford Protein and Nucleic Acid Facility). Luciferase activity with the 3′UTR of Reln present was significantly decreased by exposure to miR-200c compared with the miR-200c seed mutant control (Figure 4B). We further assessed the effect of miR-200c levels on reelin protein expression in N2a cells by immunoblot after changing levels with miR-200c mimic, inhibitor, or mismatch-control. Transfection with miR-200c mimic significantly decreased, and inhibitor significantly increased, reelin protein expression (Figure 4C), indicating that the luciferase data are also reflected in changes in protein levels in this cell line.

We tested a second potential target of miR-200c predicted by homology, the mitochondrial chaperone Grp75 (Hsp9a). The 3′UTR of Hsp9a shares the same seed sequence homology with miR-200c as the Reln 3′UTR (Figure 1B in the online-only Data Supplement). The Hsp9a 3′UTR was cloned (primers listed in Figure IC in the online-only Data Supplement) and assessed with dual luciferase assay. In N2a cells, dual luciferase assay with the Hsp9a 3′UTR and miR-200c yielded no change in luciferase activity (Figure 4D), indicating that the luciferase data are also reflected in changes in protein levels in this cell line.

**Figure 4.** Reln 3′-untranslated region (UTR) is a direct target of microRNA (miR)-200c. Predicted binding for miR-200c:Reln 3′UTR (A) was assessed by dual luciferase activity assay in N2a cells cotransfected with Renilla (Ren) Reln 3′UTR target reporter, Firefly (Ff) control reporter, plus either wild-type miR-200c or seed mutant (SM). A reduction in luciferase activity with wild-type but not SM control indicated that miR-200c recognizes the Ren 3′UTRs (B). Uncleaved reelin protein expression (C) decreases in N2a cells treated with mimic, and increases in cells treated with inhibitor, relative to mismatch-control–treated cells. All cell culture experiments were performed in triplicate, *P<0.05 compared with control (n=4–6 wells per condition).

miR-200c Contributes to Neuronal Cell Death by Inhibiting Reelin Expression

Because miRs can theoretically target a large number of different genes, we investigated whether reduction of reelin expression by miR-200c might be a mechanism contributing to cell death. We compared cells with increased or decreased levels of miR-200c using mimic and inhibitor. Cultures were also treated with small interfering RNA to Reln or control to reduce reelin mRNA expression. After altering miR-200c levels using mimic and inhibitor (2513±113- and 0.68±0.06-fold change, respectively, relative to control), N2a cells were subjected to 500 μmol/L H2O2 exposure in serum-free medium for 18 hours and assayed for cell death. Inhibition of miR-200c significantly decreased, whereas miR-200c mimic increased cell death from this injury paradigm (Figure 5A). Relin mRNA was effectively decreased (to 0.22±0.05) with small interfering RNA, and this abolished the protective effect of miR-200c inhibition (Figure 5A), confirming a role for reelin in the mechanism of miR-200c–mediated cell death. Finally, exogenous application of recombinant reelin at 50, 100, or 500 ng/μL provided significant protection (Figure 5B) from 24 hours of H2O2/serum deprivation.

**Discussion**

In the present study, we investigated the role of miR-200c in the evolution of injury after transient focal cerebral ischemia by altering brain levels of miR-200c before transient focal cerebral ischemia. Using antagonir pretreatment, we have demonstrated for the first time that preventing the early increase in miR-200c in the brain subsequent to transient focal cerebral ischemia is protective. We confirmed the protective effect of miR-200c knockdown in an in vitro model using oxidative stress.

We most consistently observed a protective effect with miR-200c inhibition, although increasing levels with miR-200c mimic did not result in further injury in vivo. This may be because of the high endogenous poststroke levels of miR-200c, whereby any further increase with mimic did not result in additional translational suppression. Previous studies investigating the role of miR-200c in neuronal injury have reported varying outcomes. Lee et al. demonstrated that inhibition of miR-200c decreased cell death after in vitro ischemia in neuronal SH-SY5Y cells. This finding agrees with several previous studies in other cells implicating miR-200c as contributing to cell death by targeting and silencing antiapoptotic genes. However, increased survival subsequent to in vitro ischemia in the absence of reperfusion was also reported with overexpression of miR-200c. These seemingly conflicting results may be because of different ischemic injury protocols; previous findings indicate that brain levels of miR-200c increase substantially 3 hours after a transient period of ischemia, but not after a 3-hours period of fixed ischemia, suggesting that reperfusion may be an important component in miR-200c induction and function. This agrees with previous findings in endothelial cells where oxidative stress induced increased expression of miR-200c, causing apoptosis and growth arrest.

A second major finding of this study is that reelin is a direct target of miR-200c, and that reduction of reelin by miR-200c...
with antagonim pretreatment contributes at least in part to stroke-related injury. In the present study, we observed only a modest and transient increase in reelin expression in vivo after miR-200c suppression with antagonist, which may be the result of cell-type or regional heterogeneity in reelin expression, and additional cellular factors regulating reelin expression.\(^2\) The half-life of antagonim to miR-200c is not known, so the decrease in reelin expression at 24 hours may also be because of loss of antagonim. However, our observations in cell culture demonstrated a greater alteration in reelin expression with changing levels of miR-200c, supporting our findings using the dual luciferase assay that reelin is a direct target of miR-200c. Because additional targets of miR200c may be relevant for stroke, future studies will need to knockdown-specific targets to assess their relevance for protection.

Although previous studies have established the role of reelin in neurodevelopment,\(^2\) little is known about the functional role reelin plays in neuroprotection. Reelin-deficient mice provide a loss-of-function model but display early neurologic dysfunction and greater susceptibility to excitotoxicity,\(^2\) and it is currently unknown whether they develop compensatory changes in cell survival signaling which could complicate interpretation of results using these mice. Therefore, we performed in vitro reelin loss-of-function experiments using small interfering RNA to demonstrate that the protective effect of miR-200c inhibition is lost when reelin expression is concurrently downregulated. Moreover, our findings demonstrate for the first time that exogenous application of recombinant reelin provides significant protection from oxidative cell injury model.

regulation of neuronal cell survival occurs, at least in part, by altered reelin expression; and (5) treatment with reelin protein promotes neuronal cell survival. These results suggest that inhibiting brain levels of miR-200c and upregulating reelin expression in the acutely injured brain may have clinical use to both minimize the evolution of injury and enhance recovery.

Acknowledgments

We would like to thank William Magruder for assistance in article preparation, and Jeong-mi Moon, MD, for assistance with the N2a cell injury model.

Sources of Funding

This work was supported by the American Heart Association 14FTF-19970029 and National Institutes of Health T32-GM089626 awarded to Dr Stary and National Institutes of Health R01-NS084396 and R01-NS80177 to Dr Giffard.

Disclosures

None.

References


15. Xu L, Kounenis IL, Tilly JL, Giffard RG. Overexpression of bel-2L protects astrocytes from glucose deprivation and is associated with higher glutathione, ferritin, and iron levels. *Anesthesiology.* 1999;91:1036–1046.


MicroRNA-200c Contributes to Injury From Transient Focal Cerebral Ischemia by Targeting Reelin
Creed M. Stary, Lijun Xu, Xiaoyun Sun, Yi-Bing Ouyang, Robin E. White, Jason Leong, John Li, Xiaoxing Xiong and Rona G. Giffard

Stroke. published online January 20, 2015; Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2015/01/20/STROKEAHA.114.007041

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2015/01/20/STROKEAHA.114.007041.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at: http://stroke.ahajournals.org/subscriptions/
miR-200c Contributes to Injury From Transient Focal Cerebral Ischemia by Targeting Reelin

Creed M. Stary, M.D., Ph.D., Lijun Xu, M.D., Xiaoyun Sun, M.D., Yi-Bing Ouyang, Ph.D., Robin E. White, Ph.D., Jason Leong, John Li, Xiaoxing Xiong, M.D., and Rona G. Giffard, M.D., Ph.D.
**Supplemental Figure I.**

**A,** Mature miR-200c sequence.

B, *Hsp9a* 3'UTR has one potential binding site for miR-200c. C, Primers for *Hsp9a* 3'UTR. D, Binding for miR-200c:*Hsp9a* 3'UTR was assessed by dual luciferase activity assay in N2a cells co-transfected with Renilla (Ren) *Hsp9a* 3'UTR; Firefly (Ff), plus either wild type miR-200c or seed mutant (SM) control. No reduction in luciferase activity was observed with application of wild type miR-200c, indicating that miR-200c does not target *Hsp9a* 3'UTR.