Original Contribution

Inhibition of miR-141-3p Ameliorates the Negative Effects of Poststroke Social Isolation in Aged Mice

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Background and Purpose—Social isolation increases mortality and impairs recovery after stroke in clinical populations. These detrimental effects have been recapitulated in animal models, although the exact mechanism mediating these effects remains unclear. Dysregulation of microRNAs (miRNAs) occurs in both strokes as well as after social isolation, which trigger changes in many downstream genes. We hypothesized that miRNA regulation is involved in the detrimental effects of poststroke social isolation in aged animals.

Methods—We pair-housed 18-month-old C57BL/6 male mice for 2 weeks before a 60-minute right middle cerebral artery occlusion or sham surgery and then randomly assigned mice to isolation or continued pair housing immediately after surgery. We euthanized mice either at 3, 7, or 15 days after surgery and isolated the perilesional frontal cortex for whole microRNAome analysis. In an additional cohort, we treated mice 1 day after stroke onset with an in vivo-ready antagomiR-141 for 3 days.

Results—Using whole microRNAome analysis of 752 miRNAs, we identified miR-141-3p as a unique miRNA that was significantly upregulated in isolated mice in a time-dependent manner up to 2 weeks after stroke. Posttreatment with an antagomiR-141-3p reduced the postisolation-induced increase in miR-141-3p to levels almost equal to those of pair-housed stroke controls. This treatment significantly reduced mortality (by 21%) and normalized infarct volume and neurological scores in poststroke-isolated mice. Quantitative PCR analysis revealed a significant upregulation of Tgfβr1 (transforming growth factor beta receptor 1, a direct target of miR-141-3p) and Igf-1 (insulin-like growth factor 1) mRNA after treatment with antagomiR. Treatment also increased the expression of other pleiotropic cytokines such as Il-6 (interleukin 6) and Tnf-α (tumor necrosis factor-α), an indirect or secondary target) in brain tissue.

Conclusions—miR-141-3p is increased with poststroke isolation. Inhibition of miR-141-3p improved mortality, neurological deficits, and decreased infarct volumes. Importantly, these therapeutic effects occurred in aged animals, the population most at risk for stroke and poststroke isolation.

Visual Overview—An online visual overview is available for this article. (***Stroke*. 2018;49:00-00. DOI: 10.1161/STROKEAHA.118.020627.)**

Key Words: cytokines ■ housing ■ microRNAs ■ neuroprotection ■ social isolation

Emerging evidence from experimental and clinical studies suggests that social isolation (SI) is not only a risk factor for a stroke but also contributes to increased stroke severity and delayed functional recovery.[1–3] Exacerbation of inflammation and a reduction in prosurvival growth factors mediate the detrimental effects of poststroke SI.[3–5] Social interaction can overcome these negative effects by promoting adaptive behaviors and favorable neuroendocrine responses to biological stressors.[6] SI is particularly relevant to the elderly as this population has a high risk of both strokes and isolation.[3,7] We have previously found that aged mice that were socially isolated after stroke do not recover completely and exhibit continued deficits in memory/motor function and elevation in inflammation even months after ischemic injury.[3] Given the complex pathophysiology and additional contribution of aging and SI, there is a critical need to concurrently target multiple effector pathways involved in stroke pathology.

MicroRNAs (miRNAs) are a class of small endogenously expressed noncoding RNAs that regulate gene transcription and translation to orchestrate mRNA and protein expression.[8] The role of miRNAs in stroke has been a subject of increasing interest because the first miRNA expression profiling study in cerebral ischemia was performed in 2008.[9]

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Poststroke recovery affects several miRNAs including miR-129, miR-141, miR-181a-d, and miR-200c, and treatment with mimics or antagomiRs of these miRNAs reduce injury and improve chronic behavioral recovery in young mice.[10] Similar studies are lacking in aged animals. miRNAs also mediate many aspects of social interaction. Social environments can directly influence miRNA expression, which then triggers expression of a plethora of downstream genes. For example, miR-124-5p is involved in social and behavioral deficits in frontotemporal dementia,[11] miR-200c in major depressive disorder,[12] and miR-181c-5p in the social withdrawal associated with autism.[13]

Poststroke inflammation plays a critical role in stroke injury and recovery, which is mainly initiated by rapid activation of microglia.[5,14,15] Advanced age and SI enhance microglia-mediated inflammation either by disturbing the homeostatic balance between proinflammatory and anti-inflammatory/reparative cytokine secretion and by reducing its scavenger functions (eg, phagocytosis), contributing to poststroke pathophysiology.[16] Interestingly, several miRNAs play critical regulatory roles in microglial activation and function.[17] This led us to hypothesize that targeted manipulation of miRNAs, which concurrently regulate multiple effector pathways, prevent the detrimental effect of poststroke SI on stroke recovery by altering the microglial response.

Materials and Methods

Data supporting the findings of this study are available from the corresponding or first author (raverma@uchc.edu) of the article on reasonable request.

Materials

We purchased miRNA primers, Invivofectamine 3.0, antagomiR-141-3p, mirVana miRNA Inhibitor negative Control (or antagomiR negative control), and other qPCR related supplies for in-house miRNA analysis from Ambion, Life Technologies (Camarillo, CA). Other laboratory chemicals and reagents were obtained from Sigma-Aldrich Chemical Co (St Louis, MO).

Experimental Animals

The Institutional Animal Care and Use Committee at the University of Connecticut approved all animal protocols, which we performed in accordance with National Institutes of Health guidelines. Aged C57BL/6 male mice (18–20 months old; 40±2 g; National Institute on Aging, Bethesda, MD) were acclimatized for at least 2 months in the animal care facility at ambient temperature and humidity with free access to food and water. Of those 144 mice that were pair-housed for 3 weeks (2 mice/cage) with a daily compatibility examination (eg, weight gain and absence of fight wounds), 10 pairs of mice were excluded because of incompatibility. Thus, 124 mice were randomly assigned to stroke or sham surgery. Immediately after surgery, we randomly assigned mice to 1 of 3 groups, as detailed previously[3]: stroke and sham pair-housed (ST-SH) consisting of 1 stroke (called as ST-PH) and 1 sham mouse (called as SH-PH), stroke isolated (ST-ISO), and sham isolated (SH-ISO). The assigned housing conditions were maintained until euthanize. If a mouse died in the pair-housed group, we excluded the partner from the study. A total of 92 surviving mice were used for the final analysis.

We conducted experiments in 4 separate cohorts: (1) subacute survival group for initial microRNAome (miRNAome) assay at day 15, (2) poststroke 3 and 7-day survival groups for temporal expression profiling of the miRNA, (3) in vivo treatment group for efficacy and target validation, and (4) in vivo treatment group for neuroprotection (infarct volume and Neurological deficit [ND] score) analyses. Table II in the online-only Data Supplement summarizes the number of mice used in each group of the 4 experimental cohorts.

Middle Cerebral Artery Occlusion

To induce focal transient cerebral ischemia, a midline ventral neck incision was made under isoflurane anesthesia, and a 60-minute unilateral right middle cerebral artery occlusion was performed by advancing a 6.0 silicone rubber-coated monofilament (Doccol Corporation, Sharon, MA) 10 to 11 mm from the internal carotid artery bifurcation via an external carotid artery stump.[5] We monitored rectal temperatures (Fine Science Tools, Foster City, CA) and maintained animals at ≈37°C with an automatic heating system. We used laser Doppler flowmetry (DRT 4, Moor Instruments, Devon, United Kingdom) to measure cerebral blood flow to confirm occlusion (ie, reduction to 15% of baseline cerebral blood flow) and reperfusion. All mice were fed wet mash for 1 week after surgery to ensure adequate nutrition for chronic end points. In sham mice, we performed an identical surgery but did not advance the suture into the internal carotid artery. The reperfusion period was 3, 7, or 15 days.

ND Score

The ND score is an assessment of neurological impairments during poststroke recovery. At several time points after stroke, investigators blinded to housing conditions recorded ND scores as follows[5]: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling.

Sample Preparation and RNA Isolation

We extracted total RNA from the perilesional ipsilateral cortex (Figure I in the online-only Data Supplement) of stroke mice using miRNeasy Mini kits (Qiagen, Germantown, MD) for miRNAome analysis or mirVana miRNA isolation kits (Thermo Fisher Scientific, Waltham, MA) for other analyses, according to the suppliers' protocols. We stored RNA at −80°C.

Real-Time PCR of miRNA

We reverse transcribed 50 ng RNA in 50 µL reactions using the miRCURY LNA universal real-time miRNA complementary DNA system (Exiqon, Woburn, MA). We diluted the resulting complementary DNA (1:100) and assayed it in the miRNA ready-to-use PCR mouse and rat panel I and II (Exiqon) with the ExiLENT SYBR green master mix (Exiqon), according to the manufacturer's protocol. We performed the amplification in a LightCycler 480 PCR System (Roche, Basel, Switzerland) and analyzed the data using the quantification cycle (Cq) method (Light Cycler software, Roche). For in-house miRNA analysis, we used the TaqMan reverse transcription kit (Thermo Fisher Scientific) and the TaqMan universal PCR master mix (Thermo Fisher Scientific), according to the manufacturer's protocols.

miRNAome Data Analysis

We calculated the amplification efficiency using algorithms similar to LinReg software with Cq as the second derivative. We detected an average of 421 miRNAs per sample. To be included in the analysis, the assays were required to be detected with 5 Cqs less than the negative control and with Cq<37; for the Cq value of the global mean for each of the samples (Figure II in the online-only Data Supplement). We normalized all data to the average of assays detected in all samples, which NormFinder software found to be the best normalizer.

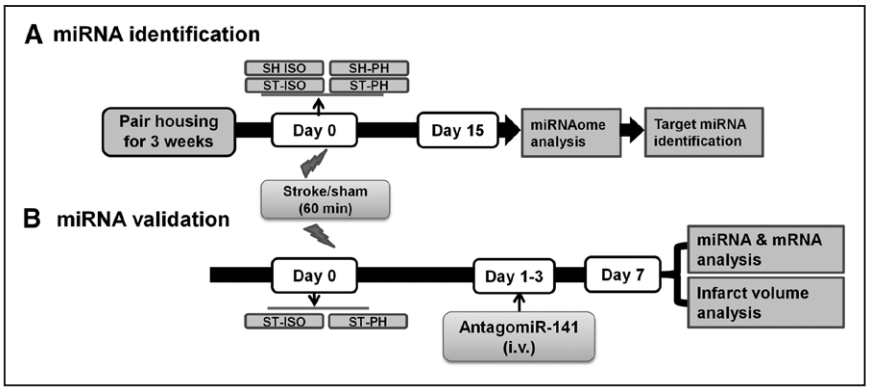


Figure 1. Schematic of research design for (**A**) miRNA identification and (**B**) miRNA validation. ST-ISO indicates poststroke social isolation; and ST-PH, poststroke pair-housed mice.

Cresyl Violet Staining for Infarct Volume and Tissue Atrophy Analysis

We measured tissue infarct volume after stroke as described previously.[5] Briefly, mice were euthanized 7 days after stroke surgery with an overdose of avertin (250 mg/kg by intraperitoneal injection). After blood collection by cardiac puncture, mice underwent transcardiac perfusion using cold phosphate-buffered saline followed by 4% paraformaldehyde. Brains were fixed overnight, placed in cryoprotectant (30% sucrose in phosphate-buffered saline) for 72 hours, and cut into 30 µm free-floating sections using a freezing microtome. Every eighth slice was mounted, stained with cresyl violet, and used for infarct volume calculations as described previously.[3]

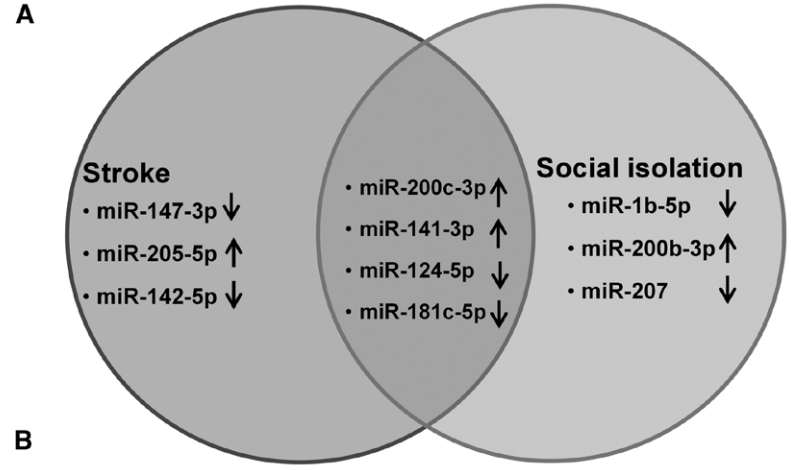
Statistical Analysis

We present data from individual experiments as mean±SD. We statistically evaluated the data by the Student *t* test (for comparison between 2 experimental groups) or by 1- or 2-way (housing condition and surgery as variables) ANOVA with a Bonferroni post hoc test to correct for multiple comparisons (GraphPad Prism Software Inc, San Diego, CA). As ND scores are ordinal in nature, we used the Mann–Whitney *U* test/Kruskal–Wallis test for statistical analysis for these experiments. A probability value of $P<0.05$ was considered statistically significant. An investigator blinded to the experimental groups performed the data analyses.

Results

miRNAome Analysis Identifies miRNA Leads Involved in Poststroke SI

To examine the contribution of miRNAs to the detrimental effects of SI after stroke, we performed whole miRNAome analysis in aged mice subjected to ischemic stroke. After 15 days of reperfusion, we isolated RNA from perilesional ipsilateral brain tissue (collected as a 2 mm coronal section, from bregma 0.00–2.00 mm) for whole miRNAome analysis. Figure 1 shows a schematic of our approach. Using sham pair-housed mice as controls, we found several differentially expressed miRNAs across conditions (>2 fold up or downregulation, $P<0.05$). Comparative analysis for the



Name of miRNA	P value	Fold change
miR-200c-3p	0.02132	49 fold upregulation
miR-141-3p	0.0249	9.6 fold upregulation
miR-124-5p	0.0058	6.4 fold downregulation
miR-181c-5p	0.032	2.6 fold downregulation

Figure 2. Lead miRNAs after poststroke social isolation (**A**) Venn diagram illustrating differentially regulated miRNAs in the brains of aged mice during the stroke, social isolation (housing condition) and their overlap. **B**, List of differentially regulated brain miRNAs, their *P* values, and their fold changes in aged male mice subjected to poststroke pair-housed mice (ST-PH) compared with poststroke social isolation (ST-ISO).

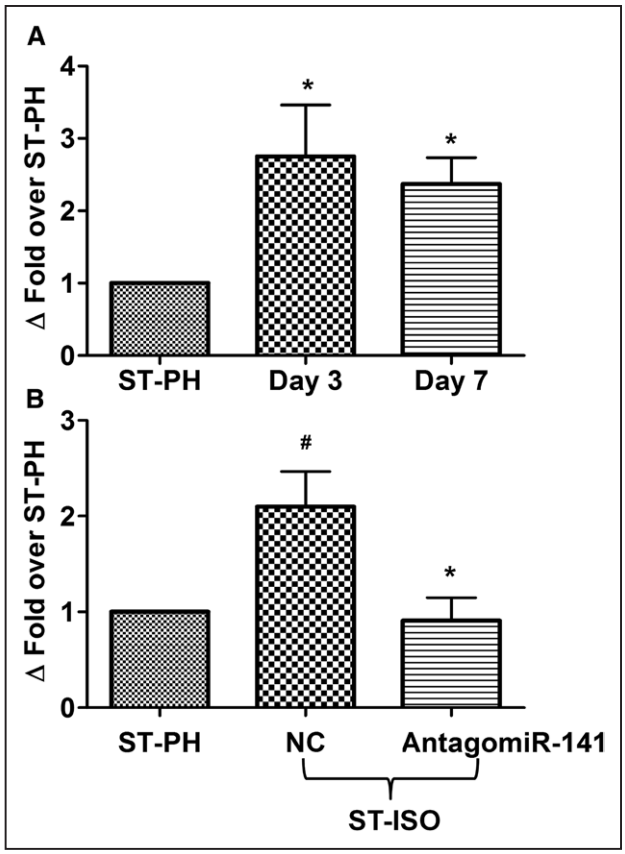


Figure 3. **A**, Temporal profile of miR-141-3p expression in mice subjected to poststroke social isolation, as compared with their respective pair-housed controls. Data are expressed as mean±SD. (n=4 per group/time point; **P*<0.05; ST-PH vs ST-ISO, 1-way ANOVA). **B**, AntagomiR-141-3p reduces miR-141-3p expression levels in socially isolated stroke mice. miR-141-3p levels were higher in the brain after middle cerebral artery occlusion (MCAo); antagomiR-141-3p reduced miR-141-3p levels measured at 7 d after stroke. Data are expressed as mean±SD and values are presented as fold change in gene expression of poststroke socially isolated (NC [antagomiR negative control] and antagomiR-141) mice at day 7 against pair-housed stroke mice (#*P*<0.05 ST-PH vs ST-ISO NC; **P*<0.05 ST-ISO NC vs ST-ISO antagomiR; n=8, n=6, and n=7 for ST-PH/SH-PH, ST-ISO NC, and ST-ISO antagomiR-141, respectively). ST-ISO indicates poststroke social isolation; and ST-PH, poststroke pair-housed mice.

effect of surgery (stroke or sham) and housing condition (pair-housed or isolated) by a 2-way analysis determined the number of miRNAs affected by surgery, housing, or both conditions ($P<0.05$). Further validation by RT-qPCR brought our list down to 4-lead miRNAs that were modulated by poststroke SI. Direct comparison between ST-PH (as control group) and ST-ISO confirmed our miRNAome finding (Figure 2A and 2B).

miR-141-3p Expression Progressively Increases After Stroke

Among the 4 selected miRNAs, we were interested in miRNAs that showed a persistent increase or decrease in expression at different time points of ischemic/reperfusion injury. We performed a similar experiment as above, using cohorts that underwent either 3 or 7 days of reperfusion before RNA isolation. Among the 4 leads, miR-141-3p showed a progressive increase in expression (Figure 3A).

miR-141-3p Targets Pathways Involving Cytokines and MAPK Pathway Genes in Socially Isolated Mice After Stroke

To identify the pathways that our lead miRNAs target, we used DIANA-mirPath versus software and the microT-CDS database.[18] This approach identifies mRNA targets of input miRNAs within a KEGG pathway. This analysis predicted a total of 22 pathways significantly affected by the combined effect of the at least 3 out 4-lead miRNAs (Figure IIIA in the online-only Data Supplement). On performing a similar analysis for miR-141-3p, we identified 4 major biological functional categories (Figure IIIB in the online-only Data Supplement). Among these 4, 2 pathways viz gap junction and axon guidance pathways involve several cytokines and MAPK signaling pathway genes, which play a role in microglia-mediated inflammation and its resolution. This finding suggested that miR-141-3p is a strong candidate as a regulator of several common genes seen with poststroke SI.

Inhibiting miR 141-3p Improves Stroke Outcomes in Socially Isolated Mice

We next examined the effects of inhibiting miR-141-3p on stroke outcomes in socially isolated mice. We treated mice with an antagomiR-141-3p (7 mg/kg, intravenously) for 3 days starting 24 hours after middle cerebral artery occlusion. We first validated the successful delivery and efficacy of the treatment by measuring its target gene expression. Indeed, after the treatment protocol, miR-141-3p levels reduced to almost 50% of the control levels, nearly equivalent to the pair-housed surgery controls (Figure 3B). After validating the in vivo efficacy of the antagomiR-141-3p, we assessed its effects on infarct damage and ND score. At 3 days posttreatment with antagomiR, the SI-induced increase in infarct volume and ND score were abolished (Figure 4A through 4C). Furthermore, antagomiR-141-3p treatment reduced mortality by 20% in poststroke-isolated mice at 7 days after stroke (Figure 4D).

miR-141-3p Inhibition Increases the Expression of Neuromodulator Cytokines

We previously found that poststroke SI increases M1-type proinflammatory gene expression and reduces M2-type anti-inflammatory gene expression.[5] Therefore, we hypothesized that antagomiR-141-3p treatment would increase several neuroprotective and anti-inflammatory gene targets of miR-141-3p. Indeed, mRNA levels of several neuroprotective and anti-inflammatory genes such as Tgfβr1 (transforming growth factor beta receptor 1), a receptor for neuroprotective cytokine *Tgfβ1* and Igf-1 (insulin-like growth factor 1) were significantly upregulated in brain tissue from socially isolated stroke mice after miR-141-3p treatment (Figure 5). Besides the above, mRNA levels of Il-6 (interleukin 6), the most implicated cytokine in poststroke SI, and Tnf-α (tumor necrosis factor-α) were also increased by antagomiR treatment. Together, these data indicate that neuroprotective effect of antagomiR-141-3p might be mediated by increased expression of several neuromodulatory genes, including both direct (Tgfβr1; Figure IV in

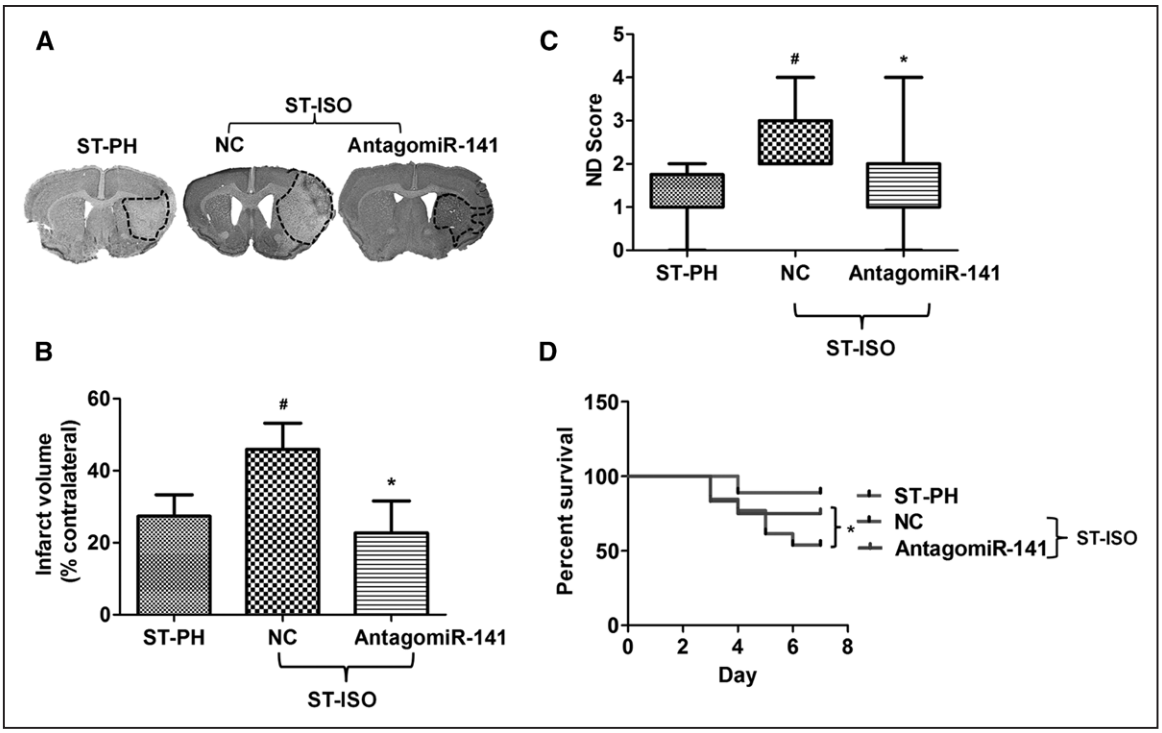


Figure 4. AntagomiR-141-3p improves stroke recovery in socially isolated mice. **A**, Representative coronal section of ST-PH, ST-ISO NC, and ST-ISO 141 anta-treated brains stained with cresyl violet. **B**, AntagomiR treatment reduced infarct volume in poststroke socially isolated mice to levels comparable to pair-housed stroke controls (#*P*<0.05 ST-PH vs ST-ISO NC; **P*<0.05 ST-ISO NC vs ST-ISO antagomiR-141). **C**, Similarly, antagomiR treatment reduced the neurological deficit (ND) score to levels comparable to pair-housed stroke controls at day 7 of stroke (#*P*<0.05 ST-PH vs ST-ISO NC; **P*<0.05 ST-ISO NC vs antagomiR-141; Kruskal–Wallis test, n=10, n=6, and n=7 for ST-PH/SH-PH, ST-ISO NC, and ST-ISO antagomiR-141, respectively). **D**, Kaplan–Meier survival curve shows that antagomiR-141 treatment reduced poststroke mortality by 21% compared with the negative control (ST-ISO NC; **P*<0.05) in socially isolated mice at day 7 after stroke. ST-ISO indicates poststroke social isolation; and ST-PH, poststroke pair-housed mice.

the online-only Data Supplement) and indirect or secondary miRNA targets (Il-6, Tnf-α, and Igf-1) of miR-141-3p.

Discussion

SI is associated with increased mortality and morbidity in patients with the established vascular disease, including stroke. Experimentally, poststroke SI increases ischemic damage and infarct volume in both young and aged mice.[3,5,19] Given that the deleterious effects of SI are mediated by poststroke inflammation and that miRNAs are upstream of select inflammatory genes, we sought to examine the contribution of miRNAs to poststroke SI. We identified several miRNAs, including some previously linked to recovery after stroke or social interaction (ie, miR-181c-5p, miR-200c-3p, miR-141-3p, and miR-124-5p), that were modulated in poststroke, socially isolated, aged mice. Among these, miR-141-3p expression increased consistently after stroke over a period of 2 weeks. An in vivo-ready antagomiR-141-3p was able to ameliorate the detrimental effects of SI after a stroke. Moreover, our data suggest that the neuroprotective effects of antagomiR-141-3p might be mediated by several neuromodulatory cytokines and growth factors.

Several in vivo and in vitro studies have reported dysregulation in miRNA expression after stroke.[10,20,21] miRNAs can regulate target genes directly through interactions with both conserved and nonconserved target recognition elements, which can lead to both decrease and increase in transcript abundance.[22] Researchers have shown that miRNAs can be induced by proinflammatory stimuli[23] and can also induce proinflammatory responses.[24] The detrimental effects of poststroke SI are driven by several factors, including an enhanced inflammatory response, reduced anti-inflammatory cytokines and growth factors, changes in various gene expression that control long-term potentiation and long-term depression, as well as M1/M2-type pro- and anti-inflammatory switching.[3,5,25] With age, inflammatory cascades initiate more swiftly and aggressively after stroke in isolated mice compared with pair-housed mice.[3]

Among the 752 miRNAs evaluated in our study, 2 members (miR-200c-3p and miR-141-3p) of the miR-200 family were highly upregulated in mice subjected to poststroke SI. We focused our attention on miR-141-3p because of the persistent increase in its expression for 2 weeks after stroke in isolated mice and its known role in the regulation of pleiotropic cytokines including *Il-6* and *Tnf-α*.[26] Exacerbation of microglia activation and subsequent neuroinflammation mediate the detrimental effects of both SI[4,5,15] and aging[14,27,28] in stroke. We found that antagomiR-141-3p treatment reversed the reduced mRNA expression of several cytokines and cytokine receptors, including, Igf-1, Il-6, Tnf-α, and Tgfβr1 that were seen in isolated animals. Among these 4 genes, *Tgfβr1* and *Igf-1* are implicated in inhibition of inflammation and homeostasis.[29] Given that Tgfβr1 is a direct target of miR-141-3p and *Tgfβ* family genes modulate secretion and activity of many

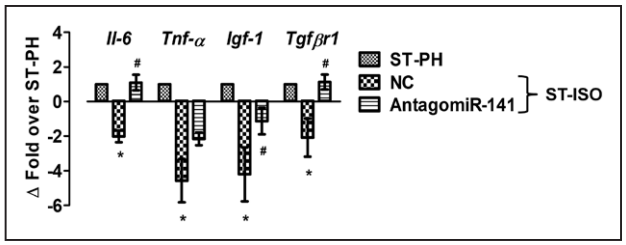


Figure 5. Effect of antagomiR-141-3p treatment on anti-inflammatory gene targets. Bar graphs show that poststroke social isolation decreases the expression of mRNA like Tgfβr1 (transforming growth factor beta receptor 1), Igf-1 (insulin-like growth factor 1), Il-6 (interleukin 6), and Tnf-α (tumor necrosis factor-α) as compared with ST-PH mice. AntagomiR treatment reinstated the expression of the anti-inflammatory gene in poststroke-isolated male mice at 7 days after stroke. Data are expressed as mean±SD and values on the *y* axis were presented as fold change in target gene expression of poststroke socially isolated mice (ST-ISO NC and ST-ISO antagomiR-141) mice at day 7 mice against pair-housed control stroked mice (ST-PH). ST-PH mRNA values were kept constant at 1 in determining fold change in respective genes expression of other groups (*P<0.05 ST-ISO NC vs ST-ISO antagomiR-141; n=8, n=6, and n=7 for ST-PH/SH-PH, ST-ISO NC, and ST-ISO antagomiR-141, respectively). ST-ISO indicates poststroke social isolation; and ST-PH, poststroke pair-housed mice.

other cytokines including *Il-6*, *Tnf-α*, and various other interleukins[30] the neuroprotective of miR-141-3p may be mediated by *Tgfβ* signaling. Besides the *Tgfβ* family, increased microglial *Igf-1* expression also promotes recovery after an ischemic injury[31] possibly by increasing neurogenesis[32] which is consistent with observed neuroprotection seen in present study. Increases in both *Igf-1* and *Tgfβr1* from stroke patient-derived endothelial colony-forming cells promote proliferation and differentiation of these cells to form mature endothelial cells and lead to vascular repair,[33] and is consistent with a potential neuroprotective role after stroke. Il-6 and Tnf-α were also increased in brain tissue. Although *Il-6* and *Tnf-α* are generally considered proinflammatory cytokines and are markers of injury in plasma/peripheral tissues, their role in the brain is more complex and diverse, and is dependent on region and timing of expression.[30] For example, Il-6 has been widely implicated in poststroke SI, and Il-6 levels drop in the brain and increase in the plasma after isolation. Importantly, blocking the isolation-induced loss of brain Il-6 leads to improved outcome after stroke, suggesting that Il-6 signaling differs in the brain versus the peripheral tissues.[3,25] *Il-6* also influences the balance between M1 and M2-type phenotype of microglia and macrophages[34] and is an important determinant of alternative activation.[35] miR-141-3p inhibition by its antagomiR restored the decreased mRNA expression of Il-6 in the brain tissue of socially isolated mice, suggesting a possible indirect interaction between miR-141-3p and Il-6 as seen by others.[26] This suggests that miR-141-3p might be an indirect regulator for Il-6 as there was no direct miR-141-3p binding site at the 3′-UTR of Il-6. Tnf-α has a more complicated role in inflammation, as it has both pro- and anti-inflammatory effects.[36–38] A positive correlation between increased Il-6 and Tnf-α mRNA and neuroprotection after 7 days of stroke in aged mice suggest that the response of these cytokines might differ with age, tissue type, and duration of injury. The majority of experimental studies which have correlated increase inflammation/injury with elevated Tnf-α and Il-6 were conducted at acute time points (<3 days) in serum/plasma sample of young mice after stroke.[18,37,39] However, our study used aged mice and examined cytokine profiles in brain tissue at 7 days after stroke, a time point where the peak inflammatory phase is resolving and the resolution of inflammation occurs.[37,40] The temporal profile of any cytokine expression is likely a key factor in the response.[41] Moreover, it is also becoming increasingly clear that cytokine responses and their downstream effects are altered with aging.[42] The potential also exists that the neuroprotection is mediated in a cytokine-independent manner. In sum, our findings suggest the antagomiR-141-3p treatment ameliorates the detrimental effects of SI and that restoration of Tgfβr1 may contribute to this effect. Future studies will examine this pathway in aged female mice, as SI may differentially affect females,[19] but will require additional miRNAome analysis to identify targets.

In conclusion, using miRNAome analysis, we identified miR-141-3p as a unique miRNA that is significantly upregulated in a time-dependent manner up to 2 weeks after stroke in isolated mice. Systemic treatment with an in vivo, ready antagomiR of miR-141-3p at a clinically relevant time point reduced the isolation-induced increase in miR-141-3p to levels almost equal to that of pair-housed stroke controls. Posttreatment with this antagomiR significantly reduced mortality and infarct size after stroke, and increased the mRNA expression of neuroprotective Tgfβr1, a direct target of miR-141-3p, and modulated the responses of other cytokines implicated in SI. Overall, our data suggest a potential role for miR-141-3p in poststroke isolation and suggests that the time window for intervention is wide. Importantly, these therapeutic effects occurred in aged animals, the population most at risk for stroke and poststroke isolation.

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Disclosures

None.

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Inhibition of miR-141-3p Ameliorates the Negative Effects of Poststroke Social Isolation in Aged Mice

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SUPPLEMENTAL MATERIAL

Inhibition of miR-141-3p ameliorates the negative effects of post-stroke social isolation in aged mice

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Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☑ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☑ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☑ An overall study timeline is provided.
Inclusion and exclusion criteria	☑ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☑ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☑ Type and methods of randomization have been described. ☑ Methods used for allocation concealment have been reported.
Blinding	☐ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☑ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☑ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☑ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☑ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☐ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☑ Statistical methods used have been reported. ☐ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☑ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☐ Different sex animals have been used. If not, the reason/justification is provided. ☑ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☑ Statements on funding and conflicts of interests have been provided.

Assignment of experimental groups.

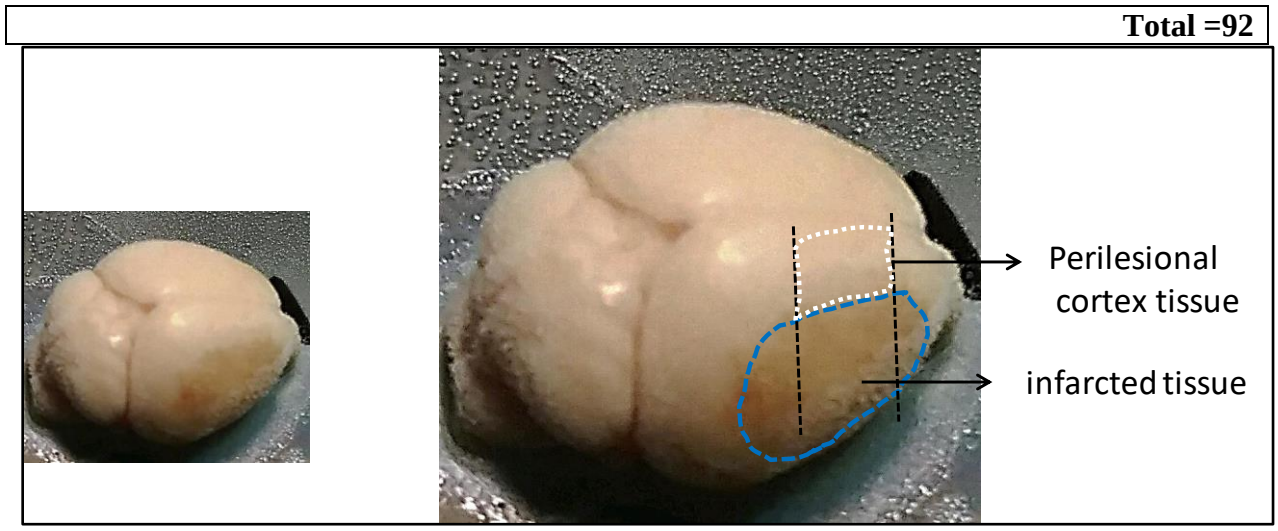
Aged C57BL/6 male mice (18-20) months; 40 ± 2 g; National Institute on Aging, Bethesda, MD) were acclimatized for at least two months in the animal care facility at ambient temperature and humidity with free access to food and water. We randomly pair housed (two mice/cage) 144 mice for three weeks with a daily compatibility examination (e.g., weight gain and absence of fight wounds); we excluded ten pairs of mice due to incompatibility. Thus, 124 mice were randomly assigned to stroke or sham surgery. Immediately after surgery, mice were randomly assigned to one of four groups using a two-way factorial design. Surgical condition: sham (SH) versus stroke (ST)) was the first between-subjects factor and housing condition: housed with sham (SH), housed with stroke (ST) or housed in isolation (ISO), was the second between-subjects factor. The following groups were generated; Sham Isolated (SH ISO), Stroke Isolated (ST-ISO), Sham paired with Stroke (SH-PH) and Stroke paired with Sham (ST-PH). The latter two groups are the mice of same pair (one was subjected to sham and another to stroke surgery) The assigned housing conditions were maintained throughout the reperfusion period (3, 7 or 14 days depending on the cohort) until sacrifice. If either mouse among the pair died, the partners were excluded from the study.

Treatment with antagomiR-141-3p:

mirVana in vivo ready miR-141 antagomiR (antagomiR-141) or negative control (NC) (Ambion, Life Technology) were complexed with Invivofectamine 3.0 (Invitrogen, Life Technology) reagent according to the manufacturer's instruction, and were injected via the tail vein in aged male C57BL/6 mice. A daily dose of 7 mg/kg i.v in 200 µl volume was given for 3 days starting 24 hrs after stroke.

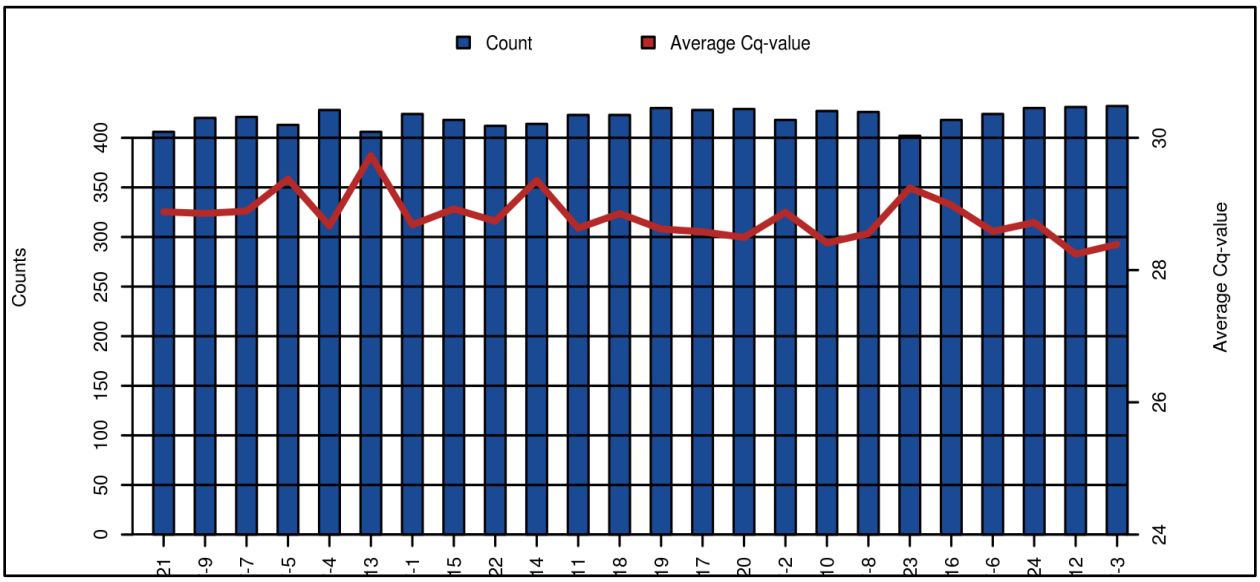
Supplementary Table II: *List of total number of animals used for the study.*

S. No.	Name of Cohorts	Terminal time points of experiments	ST-PH	SH-PH	SH-ISO	ST-ISO (NC)	ST-ISO 141 anta
1	**miRNAome assay**	**Day 15**	4	4	4	4	
2	**Temporal expression profiling**	**Day 3**	4	4	-	4	4
		Day 7	4	4	-	4	4
3	**Treatment efficacy and target validation**	**Day 7**	4	4	-	6	7
4	**Neuroprotection group**	**Day 7**	5	5	-	6	7



Supplementary Figure I: Post stroke mouse brain shows visible infract damage (shown inside blue dotted line) after quick perfusion with ice cold PBS. Perilesional cortex (shown by white dotted line; part of parieto- temporal and frontal lobe) is carefully dissected for total mRNA isolation purpose.

Average miRNA numbers per brain tissue sample:

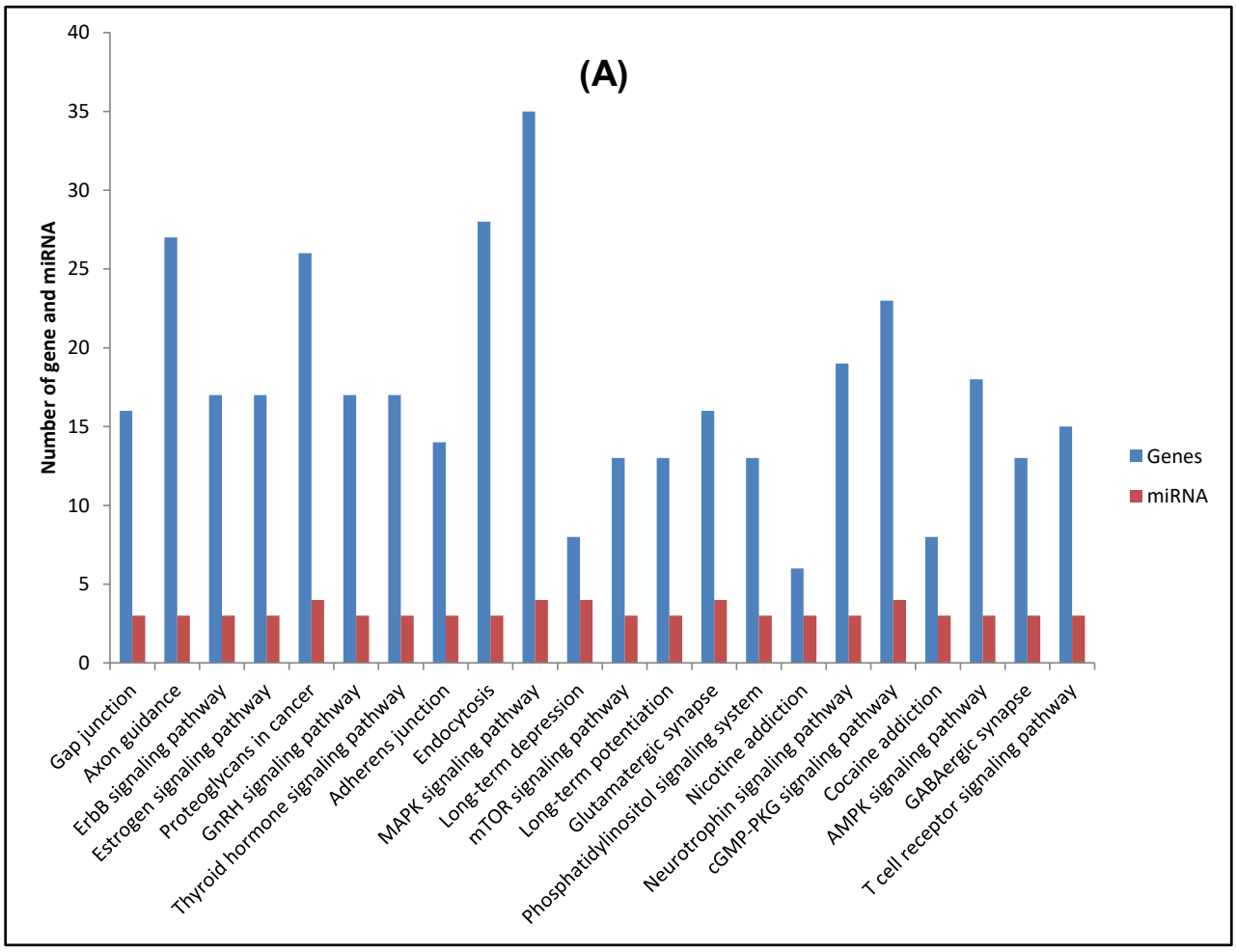


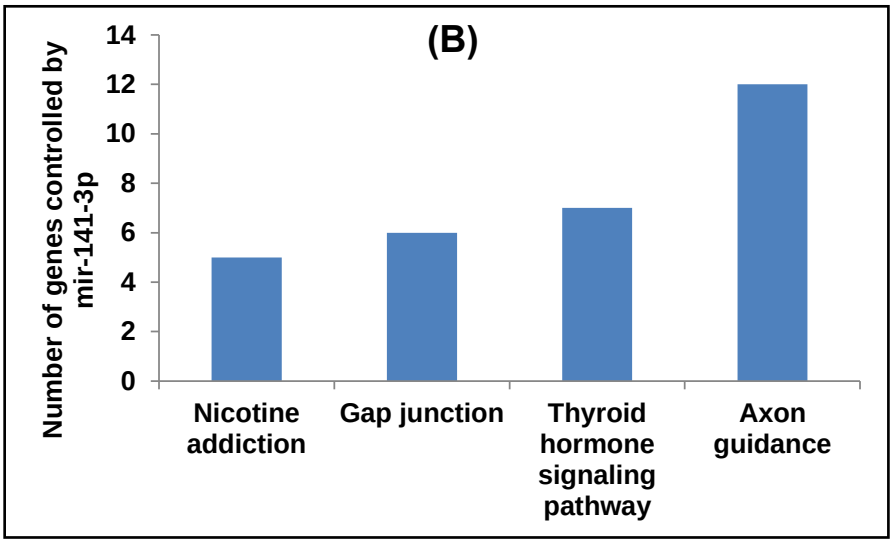
Supplementary Figure II: Graphical illustration of the microRNA content. The blue bars represent number of microRNAs detected and the red line shows the average Cq value for the commonly expressed microRNAs. On average, we detected 421 microRNAs per sample.

Bioinformatics analyses of significantly modulated miRNAs

The DIANA mirPath tool identified the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways targeted by the validated miRNAs[1]. Ingenuity Pathway Analysis software (Qiagen)

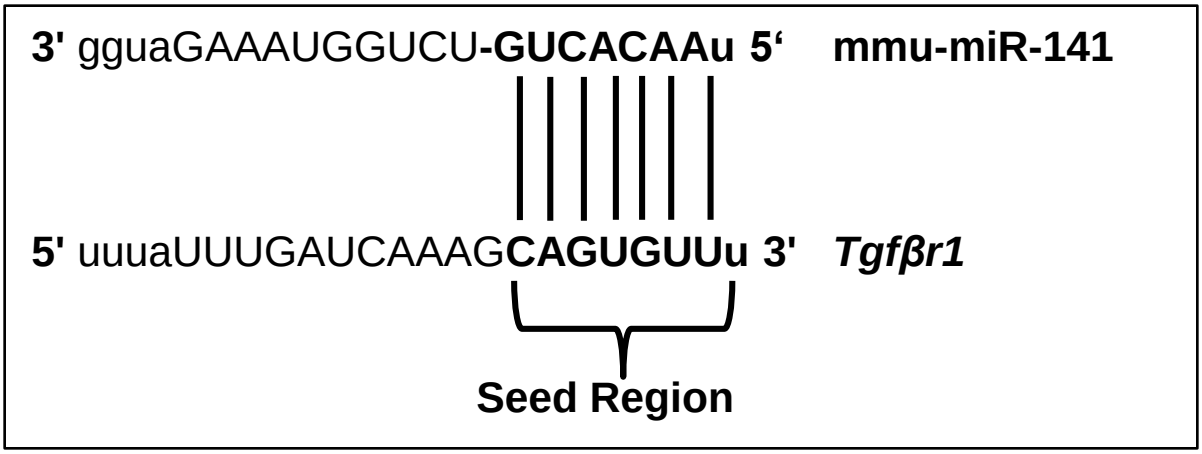
evaluated functional pathways of common miRNAs that were altered 15 days post-MCAo and generated networks.





Supplementary Figure III. The top KEGG pathways of biological function of the targets of miRNAs altered after post-stroke SI. A) Only pathways that were significantly altered in at least three of total 4 miRNAs (shown in red bar here) are presented here. B) The top 4 KEGG pathways targeted by miR-141-3p. X axis shows the name of the pathway (blue) and the y axis shows the number of total genes involved in those pathways.

***Tgfβr1* 3'UTR is a direct target of mouse miR-141-3p:**



Supplementary Figure IV: *Tgfβr1* is a predicted target of miR-141-3p. Seed sequence of mature miRNA is indicated in bold solid lines shows perfect Watson–Crick complementarity**.**

Reference:

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