

MicroRNA-17-92 Cluster in Exosomes Enhance Neuroplasticity and Functional Recovery After Stroke in Rats

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Background and Purpose—Multipotent mesenchymal stromal cell (MSC) harvested exosomes are hypothesized as the major paracrine effectors of MSCs. In vitro, the miR-17-92 cluster promotes oligodendrogenesis, neurogenesis, and axonal outgrowth. We, therefore, investigated whether the miR-17-92 cluster-enriched exosomes harvested from MSCs transfected with an miR-17-92 cluster plasmid enhance neurological recovery compared with control MSC-derived exosomes.

Methods—Rats subjected to 2 hours of transient middle cerebral artery occlusion were intravenously administered miR-17-92 cluster-enriched exosomes, control MSC exosomes, or liposomes and were euthanized 28 days post-middle cerebral artery occlusion. Histochemistry, immunohistochemistry, and Golgi-Cox staining were used to assess dendritic, axonal, synaptic, and myelin remodeling. Expression of phosphatase and tensin homolog and activation of its downstream proteins, protein kinase B, mechanistic target of rapamycin, and glycogen synthase kinase 3 β in the peri-infarct region were measured by means of Western blots.

Results—Compared with the liposome treatment, both exosome treatment groups exhibited significant improvement of functional recovery, but miR-17-92 cluster-enriched exosome treatment had significantly more robust effects on improvement of neurological function and enhancements of oligodendrogenesis, neurogenesis, and neurite remodeling/neuronal dendrite plasticity in the ischemic boundary zone (IBZ) than the control MSC exosome treatment. Moreover, miR-17-92 cluster-enriched exosome treatment substantially inhibited phosphatase and tensin homolog, a validated miR-17-92 cluster target gene, and subsequently increased the phosphorylation of phosphatase and tensin homolog downstream proteins, protein kinase B, mechanistic target of rapamycin, and glycogen synthase kinase 3 β compared with control MSC exosome treatment.

Conclusions—Our data suggest that treatment of stroke with tailored exosomes enriched with the miR-17-92 cluster increases neural plasticity and functional recovery after stroke, possibly via targeting phosphatase and tensin homolog to activate the PI3K/protein kinase B/mechanistic target of rapamycin/glycogen synthase kinase 3 β signaling pathway. (*Stroke*. 2017;48:747-753. DOI: 10.1161/STROKEAHA.116.015204.)

Key Words: exosome ■ functional recovery ■ miR-17-92 cluster ■ neural plasticity ■ neurogenesis ■ oligodendrogenesis ■ stroke

Multipotent mesenchymal stromal cells (MSCs) are self-renewing, multipotent progenitor cells, which robustly release exosomes.¹⁻³ MSCs improve neurological outcome after stroke³⁻⁵ and may exert their therapeutic effects through exosomes.⁶⁻⁹ MSC-harvested exosomes are involved in cell-to-cell communication and are hypothesized as the paracrine effectors of MSCs by encapsulating and transferring a large number of functional factors, including regulatory RNAs, proteins, or lipids.^{6,10,11} In our previous studies, we have found that MSC-generated exosomes mediate therapeutic benefits of MSCs for stroke recovery.⁷ Intravenous administration

of cell-free MSC exosomes poststroke improves functional recovery and enhances neurite remodeling, neurogenesis, and angiogenesis. Thus, exosome therapy provides a novel treatment for stroke and has the potential to replace cell-based therapy.⁷

The miR-17-92 cluster gene is primarily transcribed into an 800 bp long polycistron and is subsequently processed into 6 individual miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a).¹² The miR-17-92 cluster is broadly expressed at every stage of development, with the mature miRNAs detectable in almost all tissues at variable

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levels.^{13,14} Our previous studies showed that the miR-17–92 cluster plays an important role in mediating neural progenitor cell function by increasing cell proliferation and inhibiting cell death.¹⁵ Axonal alteration of the miR-17–92 cluster expression contributes to axonal outgrowth of embryonic cortical neurons by locally modulating phosphatase and tensin homolog (PTEN) protein levels.¹⁶ In vitro, the tailored MSC exosomes containing elevated miR-17–92 cluster further enhance axonal growth compared with control MSC exosomes (Exo-Con).¹⁷ Collectively, these studies suggest that tailored MSC exosomes carrying the elevated miR-17–92 cluster enhance stroke recovery.

In the present study, for the first time, we use exosomes engineered with a specific miRNA cluster gene to treat stroke and demonstrate that these tailored exosomes provide an increased therapeutic effect on neurological recovery compared with the functional benefits derived from treatment with naive exosomes. Intravenously (IV) administered tailored MSC exosomes containing elevated miR-17–92 cluster were administered to rats subjected to middle cerebral artery occlusion (MCAO). We investigated the effects of these exosomes on the differential fate of the neural progenitor cells, as well as on the promotion of neurite remodeling, neural plasticity, and subsequently, functional recovery poststroke. We demonstrate that MSC exosomes containing elevated miR-17–92 cluster increase neural differentiation and plasticity and neurological recovery poststroke compared with the Exo-Con. The miR-17–92 cluster regulates PTEN expression and its downstream signaling pathway, which affects phosphorylation of protein kinase B (Akt), mechanistic target of rapamycin (mTOR), and glycogen synthase kinase 3 β (GSK-3 β), and may underlie the differential therapeutic benefit of miR-17–92 augmented exosomes for stroke.

Materials and Methods

All experimental procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Henry Ford Health System. All persons who performed the experiments, collected data, and assessed outcome were blinded to the treatment allocation throughout the course of the experiments.

Tailored MSC Exosomes Containing Elevated miR-17–92 Cluster

To generate tailored MSC exosomes containing elevated miR-17–92 cluster, an miR-17–92 cluster-contained plasmid (pCAG-GFP-miR-17–92, constructed according to our published protocol¹⁶) was transfected by electroporation into primary cultured MSCs isolated from rat bone marrow, with empty pcDNA3.1 expression plasmids (GenScript, Piscataway, NJ) transfection as control. Accordingly, the exosomes generated from the cultured media of those MSCs are referred to as miR-17–92 cluster-elevated MSC exosome (Exo-miR-17–92*) and Exo-Con, respectively. See methods in the online-only Data Supplement.

Exosome Isolation and Quantification

Exosome isolation from the cell-cultured media was performed at 4°C via multistep centrifugation, as previously described.^{7,8} See methods in the online-only Data Supplement.

MCAO Model, Exosome Treatment, and Behavioral Tests

Adult male Wistar rats (weighing 270–300 g) purchased from Charles River (Wilmington, MA) were subjected to 2 hours MCAO, using a method of intraluminal vascular occlusion, as modified in our laboratory.^{4,18} Exo-miR-17–92* or Exo-Con dissolved into 0.5 mL phosphate-buffered solution were IV administered to the rats (100 μ g total exosome protein per rat, respectively) at 24 hours after induction of stroke. Rats subjected to MCAO treated with 0.5 mL phosphate-buffered solution–diluted synthetic liposomes (3 \times 10¹¹ particles, which is comparable to the particle number of 100 μ g total protein exosomes, as determined in our laboratory.) were used as vehicle control (n=8/group, respectively). To mimic the MSC exosomal lipid layer, we prepared liposomes consisting of the 3 primary fatty acids that we identified in MSC exosomal lipid analysis via the thin-film hydration technique (see methods in the online-only Data Supplement).¹⁹ To label cell proliferation, rats received intraperitoneal injections of the 5-bromodeoxyuridine (BrdU; 50 mg/kg) daily starting 24 hours after MCAO for 14 days.

A modified neurological severity score and Foot-fault tests were performed by a blinded investigator before MCAO and at 1, 3, 7, 14, 21, and 28 days after MCAO, as previously described.²⁰ All rats were euthanized at 28 days post-MCAO. Randomly selected 5 rat brains per group were snap frozen in liquid nitrogen after saline perfusion, and the frozen coronal sections were cut (40 μ m thickness for molecular studies, eg, Western blot, 8 μ m thickness for immunohistochemical staining) and stored in –80°C for later analysis. The remaining 3 rat brains per group were removed and rinsed with distilled water after saline perfusion, followed by Golgi–Cox staining.

Histochemistry and Immunohistochemistry

Bielschowsky silver combined with Luxol fast blue histochemistry staining as well as immunostaining with antibodies against the phosphorylated epitope of neurofilament heavy polypeptide (NF-H) and synaptophysin were used, respectively. To detect neurogenesis and oligodendrogenesis in the IBZ, we double stained the specific differentiation markers of neurons and progenitor and mature oligodendrocytes with BrdU. See methods in the online-only Data Supplement.

Golgi–Cox Staining

To investigate the changes of neuronal dendrites and dendritic spines in the ischemic brain after treatment, a Golgi–Cox impregnation-based FD Rapid GolgiStain Kit (PK401; FD Neuro-Technologies, Inc, Columbia, MD) was used to stain the neurons and glia following the manufacturer's protocol with modifications in our laboratory.²¹ See methods in the online-only Data Supplement.

Western Blot Assay

To detect PTEN expression and subsequent activation of PTEN downstream proteins, Akt, and mTOR, as well as inhibition of GSK-3 β activity, the total protein extracted from the IBZ area of frozen brain section was used for Western blot assay following the standard protocol (Molecular Clone, Edition II). See methods in the online-only Data Supplement.

Statistical Analysis

Data are summarized and presented using mean \pm standard error (SE). The differences between mean values were evaluated with the 1-way analysis of variance (ANOVA) and Dunnett's post hoc test. The Global test using the Generalized Estimating Equation was used to evaluate the MSC exosome treatment effects influenced by miR17–92 cluster enrichment on functional recovery.²² All statistical analyses were conducted using SAS software (version 9.2; SAS Institute, Cary, NC).

Results

MiR-17-92 Cluster-Elevated MSC Exosomes Improve Neurological Outcome After Stroke

We used a single harvest of miR-17-92 exosomes for the treatment of all animals. Real-time reverse transcriptase polymerase chain reaction analysis with TaqMan miRNA assay kit revealed that levels of individual members of the miR-17-92 cluster in exosomes harvested from MSCs transfected by the miR-17-92 vector were increased compared with miRNAs in exosomes harvested from MSCs transfected by an empty vector (7.4-folds of miR-17, 1.3-folds of miR-18a, 3.6-folds of miR-19a, 4.8-folds of miR-19b, 29.2-folds of miR-20a, and 3.0-folds of miR-92, respectively).

Compared with the liposome treatment, both exosome treatment groups exhibited significant reduction in neurological deficits in the modified neurological severity score test (Figure 1A) and Foot-fault test (Figure 1B). Significant improvement in neurological function was detected 7 days after MCAO in the Exo-miR-17-92⁺ and in the Exo-Con treatment groups on the modified neurological severity score test; however, for the Foot-fault test, significant improvement was apparent 7 days and 21 days after MCAO in the Exo-miR-17-92⁺ and in the Exo-Con treatment groups, respectively. Compared with the Exo-Con, the Exo-miR-17-92⁺ treatment significantly increased functional improvement, indicated by modified neurological severity score test ($P < 0.05$ after day 21; Figure 1A) and Foot-fault test results ($P < 0.05$ after day 14; Figure 1B).

MiR-17-92 Cluster-Elevated MSC Exosomes Increase Neurite Remodeling in the IBZ

To identify the histological basis underlying the enhanced functional recovery of tailored MSC exosomes containing elevated miR-17-92 cluster, we used a set of methods to assess dendritic, axonal, synaptic, and myelin remodeling. To evaluate the axon–myelin bundles in the white matter in the IBZ, Bielshowsky silver and Luxol fast blue were used for detecting axons and myelin, respectively. Our data show that the exosome treatment significantly increased axon–myelin bundle density along the IBZ compared with the liposome control 28 days after MCAO (Figure 2A; $P < 0.05$, respectively), whereas Exo-miR-17-92⁺ further increased the axonal density compared with the Exo-Con (Figure 2A; $P < 0.05$).

Immunohistochemistry staining with antiphosphorylated NF-H (p-NF-H) antibody identifies the accumulation of p-NF-H in axons and dendrites after stroke and reflects the axonal plasticity in the peri-infarct region. Our data show that the exosome treatment significantly increased the p-NF-H immunoreactive area in the IBZ compared with the liposome treatment 28 days after MCAO (Figure 2B; $P < 0.05$, respectively), and similarly, Exo-miR-17-92⁺ further increased the p-NF-H immunoreactivity compared with the Exo-Con (Figure 2B; $P < 0.05$).

To measure synaptic plasticity, synaptophysin was stained by immunohistochemistry. Immunostaining data show that compared with the liposome treatment group, the MSC exosome treatment significantly increased synaptophysin immunoreactivity in the IBZ 28 days after MCAO (Figure 2C; $P < 0.05$, respectively), while Exo-miR-17-92⁺ further increased

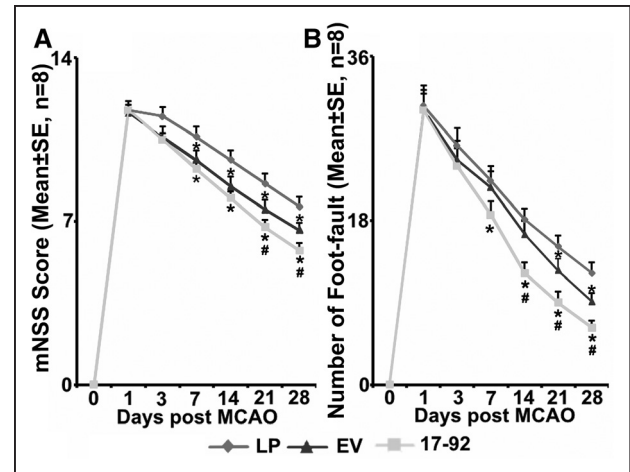


Figure 1. MiR-17-92 cluster elevated multipotent mesenchymal stromal cell (MSC) exosomes augments the improvement of neurological outcome post-middle cerebral artery occlusion (MCAO). Modified neurological severity score (mNSS) score (A) and Foot-fault test (B) data show that compared with the liposome treatment, both MSC exosome treatment groups exhibited significant improvement of functional recovery. Compared with the control MSC-derived exosomes (Exo-Con), miR-17-92 cluster-enriched exosomes (Exo-miR-17-92⁺) significantly increased functional improvement. LP, MCAO rats treated with liposomes; EV, MCAO rats treated with Exo-Con; 17-92, MCAO rats treated with Exo-miR-17-92⁺. * $P < 0.05$ compared with LP; # $P < 0.05$ compared with EV, respectively. Mean ± SE, n=8/group.

the synaptophysin immunoreactivity compared with the Exo-Con (Figure 2C; $P < 0.05$).

MiR-17-92 Cluster-Elevated MSC Exosomes Increase Neuronal Dendritic Plasticity

Dendrites and dendritic spines provide support for axonal outgrowth,²³ and axonal outgrowth accompanied with dendritic plasticity is present in the cortical peri-infarct area after experimental stroke.²¹ We analyzed dendritic plasticity by means of Golgi silver impregnation and found that compared with liposome treatment, the exosome treatment significantly increased primary and secondary neurite branching as well as spine density, and Exo-miR-17-92⁺ further increased dendritic plasticity compared with the Exo-Con (Figure 3B through 3D; $P < 0.05$, respectively).

MiR-17-92 Cluster-Elevated MSC Exosomes Increase Neurogenesis and Oligodendrogenesis in the IBZ

The miR-17-92 cluster increases neural progenitor cell proliferation and reduces neural progenitor cell death¹⁵ and regulates oligodendrogenesis during development.²⁴ Our present data show that newly generated neurons (identified by cells with BrdU and neurons-positive staining), oligodendrocyte progenitor cells (identified by cells with BrdU and progenitor-positive staining), and mature oligodendrocytes (identified by cells with BrdU and mature oligodendrocytes-positive staining) were significantly increased after exosome treatment compared with liposome treatment. Treatment with Exo-miR-17-92⁺ significantly increased neurogenesis and oligodendrogenesis, respectively (Figure 4A through 4C; $P < 0.05$, respectively) compared with the Exo-Con treatment.

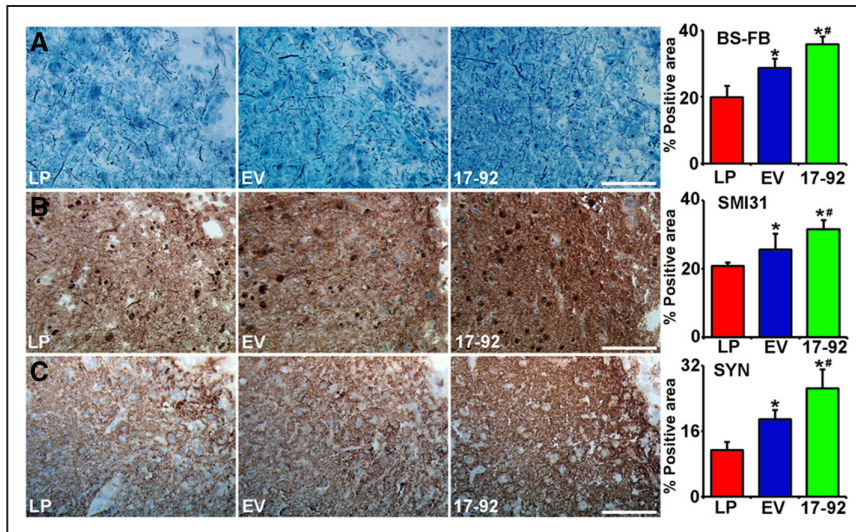


Figure 2. MiR-17-92 cluster-elevated multipotent mesenchymal stromal cell (MSC) exosomes increase neurite remodeling in the ischemic boundary zone (IBZ). Bielshowsky silver and Luxol fast blue double-staining (A), SMI-31 immunostaining (B), and synaptophysin immunostaining (C) show that based on the increase of neurite remodeling and synaptic plasticity in the IBZ by the control MSC-derived exosomes (Exo-Con) treatment, the miR-17-92 cluster-enriched exosomes (Exo-miR-17-92⁺) treatment further increased neurite remodeling and synaptic plasticity in the IBZ. LP, MCAO rats treated with liposome; EV, MCAO rats treated with Exo-Con; 17-92, MCAO rats treated with Exo-miR-17-92⁺. MCAO indicates middle cerebral artery occlusion. **P*<0.05 compared with LP; #*P*<0.05 compared with EV, respectively. Mean±SE, n=5/group. Scale bar =50 μm.

MiR-17-92 Cluster-Elevated MSC Exosomes Downregulate PTEN Level and Subsequently Activate the PI3K/Akt/mTOR Signaling Pathway

PTEN is a validated target of the miR-17-92 cluster, and its downstream PI3K/Akt/mTOR signaling pathway controls neurite remodeling,^{16,25} cell proliferation, and differentiation.^{15,26} By using Western blot, we measured protein level of PTEN and the phosphorylated downstream effectors in the IBZ. The data show that compared with liposome and Exo-Con treatment, Exo-miR-17-92⁺ treatment significantly reduced the PTEN level (Figure 5A-1) and increased the phosphorylation of Akt (Figure 5A-2) and mTOR (Figure 5A-3) and the phosphorylation of GSK-3β (Figure 5A-4). Double immunofluorescence staining shows that p-GSK-3β was localized in the

IBZ neurons (Figure 5B). Because GSK-3β inhibits axonal outgrowth and phosphorylation of GSK-3β inactivates its function, our data suggest that Exo-miR-17-92⁺ treatment targets PTEN and subsequent activation of the downstream PI3K/Akt/mTOR signaling pathway to inactivate GSK-3β in neurons.

Discussion

MSC exosomes are hypothesized as the major paracrine effectors of MSCs because they are involved in cell-to-cell communication by encapsulating and transferring a large number of functional factors, including regulatory RNAs, proteins, or lipids,^{6,10,11} to the recipient cells. In our previous studies, we found that MSC-generated exosomes mediate therapeutic benefits of

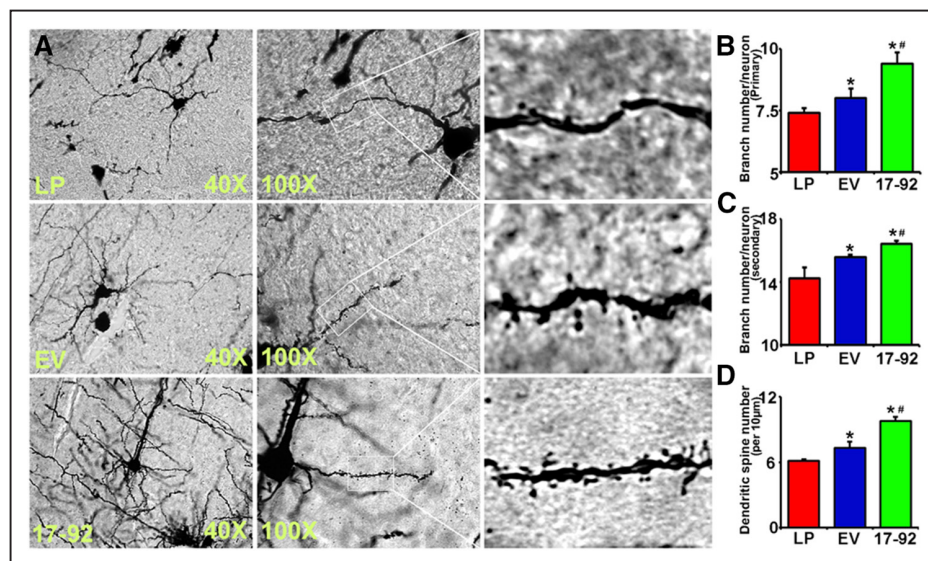


Figure 3. MiR-17-92 cluster-elevated multipotent mesenchymal stromal cell (MSC) exosomes increase neuronal dendritic plasticity. A, Representative optical microscopy images show the morphology of Golgi silver impregnation-stained neurons and their dendrites (individual row represents for LP, EV, and 17-92 treatment, respectively). The primary (B) and secondary (C) neurite branching as well as the spine density (D) were significantly increased after exosome treatment, and miR-17-92 cluster-enriched exosomes (Exo-miR-17-92⁺) further increased dendritic plasticity compared with the control MSC-derived exosomes (Exo-Con; *P*<0.05, respectively). LP, MCAO rats treated with liposome; EV, MCAO rats treated with Exo-Con; 17-92, MCAO rats treated with Exo-miR-17-92⁺. MCAO indicates middle cerebral artery occlusion. **P*<0.05 compared with LP. #*P*<0.05 compared with EV. Mean±SE n=3/group.

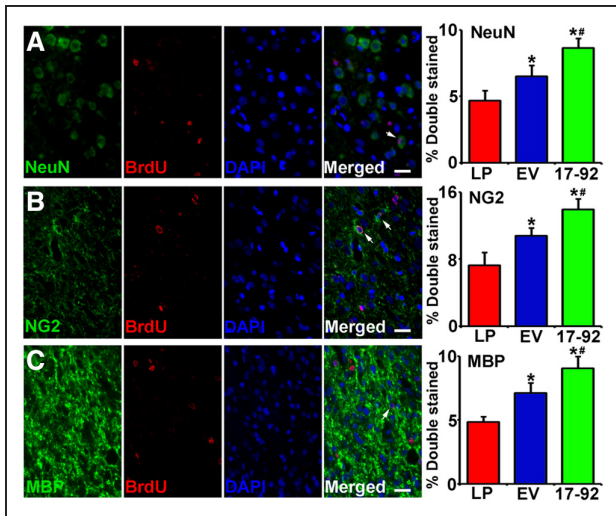


Figure 4. MiR-17-92 cluster-elevated multipotent mesenchymal stromal cell (MSC) exosomes increase neurogenesis and oligodendrogenesis in the ischemic boundary zone (IBZ). Representative micrographs show the double stained cells with neuron (NeuN) and 5-bromodeoxyuridine (BrdU; **A**), progenitor (NG2) and BrdU (**B**), or mature oligodendrocytes (MBP) and BrdU (**C**). Compared with liposome treatment, exosome treatment significantly increased the percentage of BrdU-NeuN-stained cells, BrdU-NG2-stained cells, and BrdU-MBP-stained cells in the IBZ in rats after stroke, and miR-17-92 cluster-enriched exosomes (Exo-miR-17-92*) further increased these double stained cells compared with the control MSC-derived exosomes (Exo-Con; $P < 0.05$, respectively). LP, MCAO rats treated with liposome; EV, MCAO rats treated with Exo-Con; 17-92, MCAO rats treated with Exo-miR-17-92*. MCAO indicates middle cerebral artery occlusion. * $P < 0.05$ compared with LP. ** $P < 0.05$ compared with EV. Mean \pm SE, $n = 5$ /group. Scale bar = 25 μ m.

MSC therapy for stroke,⁷ and the therapeutic impact of MSC exosomes may be attributed in part to their miRNA contents.^{6,8} Functional benefits derived from MSC treatment of stroke may also, in part, be attributed to the induction of the sonic hedgehog pathway in brain parenchymal cells,²⁷ and we and others have demonstrated that sonic hedgehog stimulates the miR-17-92 cluster.^{15,28} Based on our *in vitro* data that overexpression of the miR-17-92 cluster promotes axonal outgrowth of primary cortical neurons cultured in a microfluidic chamber,¹⁶ and the important role of the miR-17-92 cluster in mediating neural progenitor cell function, including cell proliferation and cell death,¹⁵ we selected the miR-17-92 cluster as a target for our studies of using tailored exosomes with modified miRNA content for the promotion of brain plasticity and enhancing functional recovery from stroke. Our *in vitro* studies then demonstrated the tailored MSC exosomes containing elevated miR-17-92 cluster further enhances axonal growth compared with native MSC exosomes.¹⁷ Here, our *in vivo* study demonstrates for the first time that MSC-harvested exosomes engineered to elevate the miR-17-92 cluster augment the Exo-Con-mediated improvement of neurological outcome for stroke recovery and increase neural remodeling, including neurogenesis, oligodendrogenesis, and neurite plasticity. Because frozen sections of the cerebral tissue were obtained and primarily used for specific Western blot and histological analyses, H&E staining for analysis of volume of cerebral infarction was not performed in the present study. Although we and others have demonstrated

that volumes of cerebral infarction are not altered by the MSC and MSC exosome treatments when treatment was initiated 24 hours poststroke,^{7,29,30} we cannot exclude the possibility that treatment with miR-17-92-enriched MSC exosomes at 24 hours poststroke also evokes some neuroprotective benefits in the ischemic boundary region.

In the present study, we found that the miR-17-92 members increased in the MSC exosomes after transfection, and our previous studies have demonstrated that miRNA content in the MSC exosome transferred to recipient cells *in vitro* and *in vivo*.^{8,9,17,31} Also, we have found that a validated miR-17-92 target, PTEN, was downregulated in the brain tissue, which suggests that the miR-17-92 cluster members transferred to cerebral tissue after treatment with the miR-17-92-enriched MSC exosomes and, thereby, contributed to recovery. The increase of miR-17-92 cluster level in MSCs may alter profiles of the proteins, lipids, and the RNAs in the MSCs, as well as their exosome cargos. We also do not exclude the possibility that enriching the exosome miR-17-92 cluster likely alters the myriad of protein/RNA/lipid content of exosomes, and secondary changes in exosome content induced by introducing of miR-17-92 may contribute to the enhancement of plasticity and neurological recovery post stroke, possibly, via direct modification of the recipient cells or indirectly by affecting

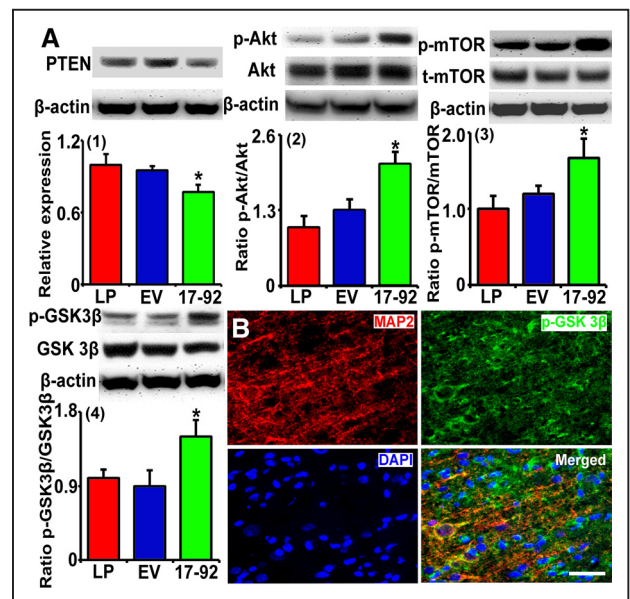


Figure 5. MiR-17-92 cluster-elevated multipotent mesenchymal stromal cell (MSC) exosomes negatively regulate the phosphatase and tensin homolog (PTEN) expression and subsequently activate the PI3K/Akt/mTOR signaling pathway. **A**, Compared with liposome and control MSC-derived exosomes (Exo-Con) treatment, miR-17-92 cluster-enriched exosomes (Exo-miR-17-92*) treatment significantly downregulated the PTEN protein level in the ischemic boundary zone (IBZ); 1) and subsequently increased the phosphorylation of Akt (2) and mTOR (3), and then increased the phosphorylation of glycogen synthase kinase 3 β (GSK-3 β); 4) in the IBZ. Double immunofluorescence staining shows p-GSK-3 β locates in the IBZ neurons (**B**). LP, MCAO rats treated with liposome; EV, MCAO rats treated with Exo-Con; 17-92, MCAO rats treated with Exo-miR-17-92*. Akt indicates protein kinase B; MCAO, middle cerebral artery occlusion; and mTOR, mechanistic target of rapamycin. * $P < 0.05$ compared with EV. Mean \pm SE, $n = 3$ /group. Scale bar = 50 μ m.

recipient cell targeting or uptake. However, it is evident that the enrichment of the miR-17-92 cluster as a perturbation to the complex web of exosome cargo facilitates recovery, given that PTEN, a major target of the miR-17-92, is concomitantly altered with miR-17-92. The importance of our current study is that it demonstrates, for the first time, that tailoring the exosome content with targeted miRNAs may be a viable option to promote restorative neurological processes after stroke. Investigation of the contributions of secondary changes of (possibly hundreds of) proteins, lipids, and RNAs as a result of selectively enriching the miR-17-92 cluster is warranted.

Exosome therapy, by minimizing potential adverse effects of administering potentially replicating and thrombosis-mediating cells,³² provides a novel treatment for stroke, with the possibility to replace cell-based therapy.^{6,33,34} We have previously demonstrated that exosomes harvested from cells likely provide the therapeutic effects of cell-based therapies.^{7,35} However, the complexities and questions associated with therapeutic action present in cell-based therapy are also evident in exosome therapy, for example, the distribution, localization, and half-life of administered exogenous exosomes, as well as questions of potential systemic and immune/inflammatory system modulating effects. Exosomes penetrate the blood-brain barrier^{36,37}; however, the detailed mechanisms of doing so are unclear. Given the difficulty in measurements, the cellular and organ distribution of the IV-administered exosomes were not determined in the current study. It is likely, as with the use of cell-based therapies, that most of the exosomes are distributed in other organs, like the liver.^{38,39} However, given that the molecular target of the miR-17-92 in brain, that is, PTEN, responds to the exosome administration, it is likely that exosomes do enter brain. We do not exclude the possibility that like cell-based therapies, exosome therapy modulates the immune/inflammatory system, which in concert with the induction of neuroplasticity or independently contributes to the therapeutic efficacy. Investigation of the exosome contributions to systemic effects and the modulation of immune/inflammatory system by enriching the miR-17-92 cluster in exosomes is warranted. Although angiogenesis after ischemic stroke correlates with improvement of functional outcome in both animal models and in human patients with stroke,⁴⁰ our previous data show that treatment with naïve MSC exosomes increases angiogenesis⁷; in the current study, we did not observe angiogenesis induced by Exo-miR-17-92⁺. A possible explanation is that individual members of the miR-17-92 cluster are either proangiogenic or antiangiogenic, and the net effect of the cluster may be context dependent.^{41,42} Thus, with the miR-17-92 enrichment, there is a balanced proangiogenic or antiangiogenic effect, which may mitigate the net induction of angiogenesis.

Our present study only focused on the miR-17-92 cluster targeting the PTEN signaling pathway because PTEN is a validated target of miR-17-92 cluster and the miR-17-92/PTEN axis controls neurite remodeling,^{16,25} cell proliferation, and differentiation.^{15,26} Our previous in vitro data demonstrated that overexpression of the miR-17-92 cluster promoted axonal outgrowth of primary cortical neurons cultured in a microfluidic chamber, via downregulating of PTEN and subsequent activation of the PI3K/Akt/mTOR signaling pathway.¹⁶ In vivo, stroke induces

limited axonal outgrowth in the peri-infarct region, which was closely associated with an increase in myelin proteins.²⁴ The miR-17-92 cluster also regulates oligodendrogenesis during development²⁴ and promotes stroke-induced neurogenesis. The PTEN/PI3K/mTOR signaling pathway mediates axonal regeneration after spinal cord injury.⁴³ GSK-3 β plays an essential role in axon regeneration,⁴⁴ and inactivation of GSK-3 β promotes axonal growth and recovery in the central nervous system.⁴⁵ Expression of PTEN and activation of its downstream proteins, Akt, mTOR, and GSK-3 β in the peri-infarct region were measured by means of Western blots and immunohistochemistry. We found that exosomes encapsulating the elevated miR-17-92 cluster downregulated PTEN, as well as activated the PTEN downstream proteins, Akt, and mTOR and inhibited GSK-3 β activity by increasing the phosphorylation of GSK-3 β , the inactivated form of GSK-3 β . Further studies on the quantification of mRNA levels of PTEN in neurons, and connective tissue growth factor and Tsp1 in astrocytes, are warranted.

Summary

We demonstrate, for the first time, that IV treatment of stroke with exosomes tailored to encapsulate an increase of a specific miRNA cluster gene, the miR-17-92 cluster, significantly increases neurogenesis, oligodendrogenesis, and neural plasticity and significantly augments the therapeutic benefits for stroke treated with control exosomes. The molecular bases for these restorative changes may in part be attributed to the miR-17-92 cluster downregulation of PTEN expression and subsequent activation of PTEN downstream proteins, Akt, and mTOR, as well as inhibition of GSK-3 β activity. This study demonstrates that stroke may be treated with exosomes whose content is engineered to amplify neural plasticity and enhance functional recovery. This novel therapeutic approach may find application for other forms of neural injury or disease.

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Disclosures

None.

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MicroRNA-17–92 Cluster in Exosomes Enhance Neuroplasticity and Functional Recovery After Stroke in Rats

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Correction to: MicroRNA-17-92 Cluster in Exosomes Enhance Neuroplasticity and Functional Recovery After Stroke in Rats

In the article by Xin et al, “MicroRNA-17-92 Cluster in Exosomes Enhance Neuroplasticity and Functional Recovery After Stroke in Rats,” which published online ahead of print February 23, 2017, and appeared in the March 2017 issue of the journal (*Stroke*. 2017;48:747–753. DOI: 10.1161/STROKEAHA.116.015204), a correction is needed.

On page 747, in the title, “MicroRNA cluster miR-17-92 Cluster in Exosomes Enhance Neuroplasticity and Functional Recovery After Stroke in Rats,” has been changed to read “MicroRNA-17-92 Cluster in Exosomes Enhance Neuroplasticity and Functional Recovery After Stroke in Rats.”

This correction has been made to the current online version of the article, which is available at <http://stroke.ahajournals.org/content/48/3/747>.

Supplemental Methods:

Production of tailored MSC exosomes containing elevated miR-17-92 cluster

Prior to these experiments, we performed PCR on both naïve and empty plasmid transfected exosomes (ddCT, normalized to U6), and found no significant difference in the expression of the 17-92 miRNAs between exosomes from empty-plasmid-transfected and exosomes from non-transfected cells. To generate tailored MSC-exosomes containing elevated miR-17-92 cluster, a miR-17-92 cluster contained plasmid (pCAG-GFP-miR-17-92, constructed according to our published protocol ¹) was transfected by electroporation into primary cultured MSCs isolated from rat bone marrow, with empty pcDNA3.1 expression plasmids (GenScript, Piscataway, NJ) transfection as control. Briefly, 2×10^6 MSCs were suspended in 150 micro liter (μ l) of Ingenio Electroporation Solution (Mirus Bio LLC., Madison, WI) with 2 μ g of plasmid DNA. Program A-33 was used for electroporation in an Amaxa Nucleofector Device (Lonza Group Ltd., Walkersville, MD). Transfected cells were resuspended in 10ml complete culture medium, followed by centrifugation, and then plated for exosome production. 2×10^6 MSCs were seeded in 10 ml alpha Modified Eagle Medium (α MEM) supplemented with 20% Exo-free Fetal Bovine Serum (System Biosciences, Inc., Palo Alto, CA) and cultured for 24 hours (h). The culture medium was then replaced with fresh medium, and cells were cultured for an additional 48h, at which point exosomes in the medium were isolated by ultracentrifugation. Media collected were centrifuged at $3,000 \times g$ for 10 minutes (min) to remove floating dead cells, and then the supernatants were stored in -80°C for future use. Accordingly, the exosomes generated are referred to as, miR-17-92 cluster elevated MSC exosome (**Exo-miR-17-92⁺**) and control MSC exosome (**Exo-Con**), respectively.

For the measurement of the miR-17-92 cluster members in exosomes from transfected MSCs, samples were lysed in Qiazol reagents and the total RNA was isolated using the miRNeasy Mini kit (Qiagen, Valencia, CA). Using RT-PCR, we detected the miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a levels. Briefly, miRNAs were reversely transcribed with the miRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and PCR amplification was performed with the individual TaqMan miRNA assay kit (Applied Biosystems, which is specific for mature miRNA sequences) according to the manufacturer's protocols, with U6 snRNA as an internal control.

Exosome isolation and quantification

Exosome isolation from the cell cultured media was performed at 4°C via multi-step centrifugation, as previously described ²⁻⁴. Briefly, the stocked cell cultured media were thawed and followed by vortex mixing for 1 min before centrifugation, then centrifuged at 10,000×g for 30 min to remove large debris. The supernatants were then filtered through a 0.22 micron (µm) filter to remove small cell debris, and the resulting media were further centrifuged at 100,000×g for 2h. By this step, the pellets primarily contained exosomes ². Exosomes were then identified by the marker proteins, CD63 ^{5,6} or Alix ⁷⁻⁹, using Western blot, as well as by electron microscopy to verify the exosome presence ^{10,11}. The quantity of the exosomes were obtained by measuring the total protein concentration with the micro Bicinchoninic Acid (BCA) protocol (Pierce, Rockford, IL) ¹². The final exosome pellets were identified, as previously described ¹³, resuspended in PBS and stored at -80°C for further use.

Liposome Preparation

In order to mimic the MSC exosomal lipid layer, we prepared liposomes consisting of the three primary fatty acids that we identified in MSC exosomal lipid analysis. We extracted lipids from exosomes, and prepared in fatty acid methyl esters, then the lipids were analyzed by gas chromatography (Mylnefield Lipid Analysis, UK). Liposomes were prepared via the thin-film hydration technique ¹⁴. Briefly, 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (14.0 mg, 19 µmol), 1, 2-distearoyl-*sn*-glycero-3-phosphocholine (4.0 mg, 5

μmol), 1, 2-dioleoyl-*sn*-glycero-3-phosphocholine (4.0 mg, 5 μmol), cholesterol (8.0 mg, 2.1 μmol), and chloroform (1 ml) were added to a 4ml vial to produce a clear, colorless solution. Solvent was removed under reduced pressure to afford a visible film on the bottom of the vial. The hydration solution, PBS (1.15 ml) and vial containing the lipid thin film were placed in a water bath at 60°C for 30 min, and then the hydration solution was added to the vial containing the thin film. The resulting white suspension was stirred at 60°C for 1h. Extrusion of the suspension was accomplished using a mini-extruder and heating block (Avanti Polar Lipids, Alabaster, AL) heated to 60°C (4 passes through a 0.2 μm polycarbonate filter followed by 15 passes through a 0.1 μm polycarbonate filter). After extrusion, the suspension was allowed to cool to ambient temperature. Liposome samples were prepared for light scattering experiments by diluting liposome suspensions in PBS. Dynamic light scattering (DLS) data were obtained using a Malvern Zetasizer Nano-ZS instrument (ZEN3600, Malvern Instruments Ltd, United Kingdom) operating with a 633 nm wavelength laser. Dust was removed from samples by filtering through 0.2 μm hydrophilic filters (Millex-LG, SLLGR04NL, EMD Millipore, Billerica MA). The size distribution of the prepared liposome was determined by DLS and the effective diameter was approximately 134 nm, which is in agreement with the previous report of exosomal size ¹⁵.

Golgi-Cox staining

To investigate the changes of neuronal dendrites and dendritic spines in the ischemic brain after treatment, a Golgi-Cox impregnation based FD Rapid GolgiStain™ Kit (PK401, FD Neuro-Technologies, Inc., Columbia, MD 21046) was employed to stain the neurons and glia, following the manufacture's protocol with modifications in our lab ¹⁶. Briefly, three rat brains per group were removed and rinsed with distilled water after saline perfusion, then immersed in the impregnation solution (A/B=1:1, total 15 ml/rat) in a 50 ml plastic tube and covered with aluminum foil, stored at room temperature in the dark. Two weeks later, brains were transferred into fresh Solution C, and stored at 4°C in the dark for 24h. Solution C was refreshed and the brains were stored and maintained at 4°C for an additional 72h. Brains were then placed in a 30% sucrose solution in distilled water (20 ml/rat brain) for 7d in the dark at 4°C. Brains were cut on a vibratome

into 100 μm sections in the presence of cold 6% sucrose under dim dark light and then each section was transferred to a gelatin-coated slide, and the performed staining and washing procedures followed manufacture`s protocols.

To measure neurite branching, 10 intact neurons uniformly dispersed within the layer III of cortex that showed complete Golgi impregnation were randomly selected, and primary and secondary branching counted under a 40 \times objective. For evaluation of spine density, 10 neurons from each brain sample in layer III of cortex were digitized under an oil immersion 100 \times objective. Ten random stretches of dendrites of at least 10 μm in length were chosen, and 2 point length measurement was used to measure the length of the dendrite segment, and the number of spines was counted along this chosen length. The spine density is presented as number of spines/10 μm dendritic length.

Histochemistry and Immunohistochemistry

To determine neurite remodeling in the IBZ, adjacent frozen coronal sections of rat brains were used for staining the following markers. Bielschowsky silver (stains neuronal processes) combined with Luxol fast blue (stains myelin sheath) histochemistry staining as well as immunostaining with antibodies against the phosphorylated epitope of neurofilament heavy polypeptide (NF-H), Clone SMI 31 (SMI 31, reacts broadly with thick and thin axons and some dendrites) and synaptophysin (a marker for synapses, since synaptophysin is ubiquitously present at the synapses) were employed, respectively. Briefly, for immunostaining, adjacent frozen brain sections were incubated with the primary antibodies against SMI 31 (1:500, Cat# ab82259, Abcam, Cambridge, MA) and synaptophysin (1:100, Cat# MAB5258, EMD Millipore), followed with corresponding horseradish peroxidase (HRP) conjugated to secondary antibodies and 3, 3'-diaminobenzidine developing, respectively. To detect neurogenesis and oligodendrogenesis in the IBZ, we double stained the specific differentiation markers of neurons, and progenitor and mature oligodendrocytes with bromodeoxyuridine (BrdU). Double immunofluorescent staining for BrdU (1:100, Cat# Ab1893 from Abcam or Cat# M0744 from Dako, Carpinteria, CA) with Hexaribonucleotide Binding

Protein-3 (NeuN, neuronal nuclear antigen commonly used as a biomarker for neurons , 1:500; Cat# MAB377, EMD Millipore), neuron-glia antigen 2 (NG2, indicator of oligodendrocyte precursor cells (OPCs) , 1:300, Cat# AB5320, EMD Millipore) and myelin basic protein (MBP, exclusively expressed in mature oligodendrocytes , 1:400, Cat# A0623, Dako) followed by their corresponding second antibody staining (fluorescein isothiocyanate (FITC)-labeled for NeuN, NG2 and MBP, and Cy3-labeled for BrdU) were employed.

Positive staining within 9 areas (4 from the cortex, 4 from the striatum and 1 from the corpus callosum) were randomly selected along the IBZ in these groups, and were digitized under a 40X objective (BX40; Olympus Optical) using a 3-CCD color video camera (DXC-970MD, Sony) interfaced with the MCID™ software (Imaging Research Inc., St. Catharines, Ontario, Canada)¹⁷. For the analysis of neurite remodeling, the area percentage of positive staining signals within the IBZ were analyzed using the MCID software based on an average of 3 histology slides (8 µm thick, every 10 slide interval) from the standard block of each animal. For the quantification of newly generated cells and double staining for neurogenesis and oligodendrogenesis, the BrdU labeled cells in each field and the percentage of double stained cells were counted and calculated to present indices of neurogenesis and oligodendrogenesis.

Western blot assay

The total protein extracted from the IBZ area of frozen brain section was used for Western blot assay following the standard Western blotting protocol (Molecular Clone, Edition II). The following concentrations of the primary antibodies employed were: PTEN (1:1000, Cat# 9559, Monoclonal antibody, Cell Signaling Technology, Danvers, MA), p-Akt (Ser473) (1:500, Cat# 3787, Monoclonal antibody, Cell Signaling Technology), p-mTOR (Ser2448) (1:1000, Cat# 2971, Polyclonal antibody, Cell Signaling Technology), p-GSK-3β (Ser9) (1:1000, Cat# 9323, Monoclonal antibody, Cell Signaling Technology), GSK-3β (1:1000, Cat# 9315, Monoclonal antibody, Cell Signaling Technology) and beta actin (1:10000, Cat# ab6276, Monoclonal antibody, Abcam). Respective HRP- labeled secondary antibodies were applied

and enhanced chemiluminescence (ECL) detection was used according to the manufacturer's instructions (Pierce, Rockford, IL). The integrated density mean grey value of the band was analyzed under ImageJ software and the corresponding relative phosphorylation ratio were compared to non-phosphorylated protein and corresponding expression ratio of PTEN was compared to β actin.

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