Online Supplements

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 (μl) of Ingenio Electroporation Solution (Mirus Bio LLC., Madison, WI) with 2 μg 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 (αMEM) 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×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 amplifcation 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 mircon (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× objective. For evaluation of spine density, 10 neurons from each brain sample in layer III of cortex were digitized under an oil immersion 100× 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-glial 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.

1. Zhang Y, Ueno Y, Liu XS, Buller B, Wang X, Chopp M, et al. The microrna-17-92 cluster enhances axonal outgrowth in embryonic cortical neurons. *J Neurosci*. 2013;33:6885-6894
2. Wang S, Cesca F, Loers G, Schweizer M, Buck F, Benfenati F, et al. Synapsin i is an oligomannose-carrying glycoprotein, acts as an oligomannose-binding lectin, and promotes neurite outgrowth and neuronal survival when released via glia-derived exosomes. *J Neurosci*. 2011;31:7275-7290
3. Xin H, Li Y, Cui Y, Yang JJ, Zhang ZG, Chopp M. Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. *J Cereb Blood Flow Metab*. 2013;33:1711-1715
4. Xin H, Li Y, Buller B, Katakowski M, Zhang Y, Wang X, et al. Exosome-mediated transfer of mir-133b from multipotent mesenchymal stromal cells to neural cells contributes to neurite outgrowth. *Stem Cells*. 2012;30:1556-1564
5. Pols MS, Klumperman J. Trafficking and function of the tetraspanin cd63. *Exp Cell Res*. 2009;315:1584-1592
6. Suetsugu A, Honma K, Saji S, Moriwaki H, Ochiya T, Hoffman RM. Imaging exosome transfer from breast cancer cells to stroma at metastatic sites in orthotopic nude-mouse models. *Advanced drug delivery reviews*. 2013;65:383-390
7. Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, et al. Exosome secreted by msc reduces myocardial ischemia/reperfusion injury. *Stem cell research*. 2010;4:214-222
8. Guescini M, Genedani S, Stocchi V, Agnati LF. Astrocytes and glioblastoma cells release exosomes carrying mtdna. *J Neural Transm (Vienna)*. 2010;117:1-4
9. Atay S, Gercel-Taylor C, Kesimer M, Taylor DD. Morphologic and proteomic characterization of exosomes released by cultured extravillous trophoblast cells. *Exp Cell Res*. 2011;317:1192-1202
10. Orozco AF, Lewis DE. Flow cytometric analysis of circulating microparticles in plasma. *Cytometry A*. 2010;77:502-514
11. Liu X, Wang HW. Single particle electron microscopy reconstruction of the exosome complex using the random conical tilt method. *J Vis Exp*. 2011
12. Bhatnagar S, Schorey JS. Exosomes released from infected macrophages contain mycobacterium avium glycopeptidolipids and are proinflammatory. *J Biol Chem*. 2007;282:25779-25789
13. Xin H, Li Y, Buller B, Katakowski M, Zhang Y, Wang XL, et al. Exosome mediated transfer of mir-133b from multipotent mesenchymal stromal cells to neural cells contributes to neurite outgrowth. *Stem Cells*. 2012
14. Ekanger LA, Ali MM, Allen MJ. Oxidation-responsive eu(2+/3+)-liposomal contrast agent for dual-mode magnetic resonance imaging. *Chemical communications*. 2014;50:14835-14838
15. Villarroya-Beltri C, Gutierrez-Vazquez C, Sanchez-Cabo F, Perez-Hernandez D, Vazquez J, Martin-Cofreces N, et al. Sumoylated hnrnpa2b1 controls the sorting of mirnas into exosomes through binding to specific motifs. *Nature communications*. 2013;4:2980
16. Ueno Y, Chopp M, Zhang L, Buller B, Liu Z, Lehman NL, et al. Axonal outgrowth and dendritic plasticity in the cortical peri-infarct area after experimental stroke. *Stroke*. 2012;43:2221-2228

17. Li Y, Sharov VG, Jiang N, Zaloga C, Sabbah HN, Chopp M. Ultrastructural and light microscopic evidence of apoptosis after middle cerebral artery occlusion in the rat. *Am J Pathol*. 1995;146:1045-1051