




RESEARCH ARTICLE

Therapeutic efficacy of neuregulin 1-expressing human adipose-derived mesenchymal stem cells for ischemic stroke

Sun Ryu¹ , Jae-Min Lee^{1,2} , Cheong A. Bae^{1,2}, Chae-Eun Moon¹, Kyung-Ok Cho^{1,2,3,4} *

1 Department of Pharmacology, College of Medicine, The Catholic University of Korea, Seoul, South Korea, **2** Catholic Neuroscience Institute, College of Medicine, The Catholic University of Korea, Seoul, South Korea, **3** Department of Biomedicine & Health Sciences, College of Medicine, The Catholic University of Korea, Seoul, South Korea, **4** Institute of Aging and Metabolic Diseases, College of Medicine, The Catholic University of Korea, Seoul, South Korea

 These authors contributed equally to this work.

* kocho@catholic.ac.kr



OPEN ACCESS

Citation: Ryu S, Lee J-M, Bae CA, Moon C-E, Cho K-O (2019) Therapeutic efficacy of neuregulin 1-expressing human adipose-derived mesenchymal stem cells for ischemic stroke. PLoS ONE 14(9): e0222587. <https://doi.org/10.1371/journal.pone.0222587>

Editor: Giuseppe Pignataro, Università degli Studi di Napoli Federico II, ITALY

Received: January 13, 2019

Accepted: September 2, 2019

Published: September 27, 2019

Copyright: © 2019 Ryu et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This work was supported by a grant (14172MFDS974) from Ministry of Food and Drug Safety in 2016, the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) funded by the Ministry of Health & Welfare (HI15C2854), National Research Foundation of Korea (NRF) grants funded by the Korean government (NRF-

Abstract

Adipose-derived mesenchymal stem cells (AdMSCs) have been reported to ameliorate neurological deficits after acute ischemic stroke. As neuregulin 1 (NRG1, or heregulin 1), a growth factor with versatile functions in the central nervous system, has demonstrated protective effects against ischemic brain injuries, we have generated NRG1-overexpressing AdMSCs in order to investigate whether NRG1-AdMSCs could enhance therapeutic benefits of AdMSCs in ischemic stroke. After AdMSCs were infected with adenoviral NRG1, increased NRG1 secretion in NRG1-AdMSCs was confirmed with ELISA. At 1 d after ischemic stroke that was induced by the occlusion of middle cerebral artery (MCAo) for 60 min in Sprague Dawley (SD) rats, adenoviral NRG1, AdMSCs, NRG1-AdMSCs, or PBS were injected into the striatum and serial neurologic examinations were performed. Administration of NRG1-AdMSCs resulted in significant improvement of functional outcome following stroke compared to AdMSCs- or adenoviral NRG1-treated group, in addition to the reduction in the infarct size evaluated by hematoxylin and eosin staining. When NRG1 expression in the brain was examined by double immunofluorescence to human nuclei (HuNu)/NRG1 and ELISA, NRG1-AdMSCs demonstrated marked increase in NRG1 expression. Moreover, western blot analysis further showed that transplantation of NRG1-AdMSCs significantly increased both endogenous and adenoviral NRG1 expression compared to AdMSCs-treated group. To elucidate molecular mechanisms, NRG1-associated downstream molecules were evaluated by western blot analysis. Expression of ErbB4, a receptor for NRG1, was markedly increased by NRG1-AdMSCs administration, in addition to pMAPK and pAkt, crucial molecules of NRG1-ErbB4 signaling. Taken together, our data suggest that NRG1-AdMSCs can provide excellent therapeutic potential in ischemic stroke by activating NRG1-ErbB4 signaling network.

2019R1A2C1003958), and the Catholic Medical Center Research Foundation made in the program year of 2019 (awarded to KOC). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Stroke is an acute cerebrovascular disorder caused by insufficient blood supply to the brain due to ischemia or hemorrhage, resulting in extensive loss of neurons and their connections in the damaged brain [1]. Current approved therapies for ischemic stroke are restricted to fast recanalization of vascular obstruction and pharmacologic administration of thrombolytic agents, trying to remove inciting stimuli [2]. New approaches including cell-based therapy with multipotent stem cells have demonstrated great promises for functional recovery in patients with stroke [3, 4]. In particular, mesenchymal stem cells (MSCs) have shown substantial benefits due to their immunosuppressive properties and low immunogenicity [5]. Among various MSCs, adipose-derived mesenchymal stem cells (AdMSCs) show several advantages in clinical applications for stroke [6]. Considering abundance of adipose tissues, AdMSCs can be easily obtained by liposuction. Moreover, AdMSCs can proliferate and differentiate into neurons without significant adverse effects [7, 8], thereby alleviating ethical concerns. However, even although stem cell therapy is proven to be feasible for stroke [9] and vigorous research efforts have elucidated details of MSCs behaviors after brain injury, MSCs transplantation has achieved only modest success for the treatment of stroke. Therefore, it is no doubt that innovative approaches or functional improvements of MSCs are required to enhance therapeutic efficacy of current MSCs transplantation.

Neuregulin 1 (NRG1) is a trophic factor encoded by the NRG1 gene that can be alternatively spliced into six proteins (I–VI); these isoforms include Neu differentiation factor, heregulin, acetylcholine receptor inducing activity, glial growth factor, and sensory and motor neuron-derived factor [10–13]. All NRG1 isoforms contain an epidermal growth factor (EGF)-like domain and the majorities of NRG1 isoforms are transmembrane protein that requires additional proteolytic cleavage to produce a paracrine signal [10, 14, 15]. Once secreted, NRG1 can bind ErbB receptor tyrosine kinases, initiating receptor dimerization for the activation of downstream signaling [16–18]. NRG1 and ErbB tyrosine kinases are widely expressed in the brain, playing key roles in the regulation of neuronal survival, migration of precursor cells, and the expression of neurotransmitter receptors and ion channels [10, 18, 19]. Moreover, multiple brain diseases including multiple sclerosis, traumatic brain injury, and stroke can enhance NRG1-ErbB signaling networks [20–23]. NRG1 administration itself could improve neurological outcome in ischemic stroke [24, 25], implying additional therapeutic effects if MSCs can produce NRG1 more efficiently. In addition, as NRG1 could suppress stroke-induced pro-inflammatory and stress-related gene expressions, which are related with the molecular mechanisms of MSCs in ischemic stroke [24, 26], NRG1 can be an attractive target for MSCs modification to maximize therapeutic efficacies of cell-based therapy.

Therefore, we engineered AdMSCs to boost NRG1 secretion by adenoviral infection and investigated whether these NRG1-modified AdMSCs (NRG1-AdMSCs) could have better therapeutic potentials after ischemic stroke in rats. We found that transplantation of NRG1-AdMSCs could promote functional recovery with the reduction of infarct volume, compared to adenoviral NRG1-, AdMSCs- or PBS-injected groups. Moreover, NRG1-AdMSCs increased the expression of adenoviral and endogenous NRG1 expression in the ischemic hemisphere. Finally, NRG1-AdMSCs upregulated ipsilateral expressions of ErbB4, phosphorylated mitogen-activated protein kinase (pMAPK), and pAkt, suggesting NRG1-mediated activation of ErbB4 downstream signaling. Together, these findings highlight therapeutic benefits of NRG1-AdMSCs in ischemic stroke, possibly through the stimulation of NRG1-ErbB4 signaling networks.

Materials and methods

Preparation and adenoviral infection of AdMSCs

Human mesenchymal stem cells were isolated from waste adipose tissues during surgery after obtaining the written informed consent with the approval of the Institutional Review Board of Catholic University of Korea (Approval number: KC11TNSI0186). At passage 4, AdMSCs were grown in low glucose Dulbecco's modified Eagle's medium (DMEM) (Hyclon, GE Healthcare Life Sciences, UK) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin at 37°C. Adenovirus overexpressing NRG1 was produced as previously described [27]. For adenoviral infection, AdMSCs were incubated with adenovirus expressing a secreted form of EGF-like domain from NRG1 β at a multiplicity of infection (MOI) of 100 in 2% FBS-containing media for 2 h [27]. Medium was then replaced with fresh DMEM containing 10% FBS. AdMSCs were transplanted into the rat brain after 24 h of additional culture.

Rat model of transient focal cerebral ischemia and injection of adenoviral NRG1, AdMSCs or NRG1-AdMSCs

Specific pathogen-free 6-week-old male Sprague-Dawley (SD) rats were obtained from the Orient Bio (Seoul, Korea) and used after 2 weeks of quarantine and acclimatization. The animals were housed in a room maintained at $22 \pm 3^\circ\text{C}$ under a relative humidity of $50 \pm 10\%$ with artificial lighting from 08:00 to 20:00. The animals were kept in individually ventilated plastic cages with wire mesh tops and allowed sterilized tap water and commercial rodent chow (TD2018S, Harlan Laboratories, USA) *ad libitum*. Animal health monitoring was performed twice a year using sentinel animals to check pathogenic viruses, bacteria, and protozoans. The vivarium was accredited by the Korea Excellence Animal Laboratory Facility from Korea Food and Drug Administration in 2017 and the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) in 2018. All surgical interventions and presurgical and postsurgical animal care were provided in accordance with the Laboratory Animals Welfare, the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) in School of Medicine, The Catholic University of Korea (Approval number: CUMS-2018-0089-01). An effort was made to minimize the pain and suffering of the animals such as buprenorphine administration when needed. Transient focal cerebral ischemia was induced by the intraluminal occlusion of the left middle cerebral artery (MCAo) as previously described with minor modifications [28–30]. Briefly, male SD rats, weighing 270–300 g, were anesthetized with 1.5% isoflurane in a 70% nitrous oxide and 30% oxygen mixture using a face mask. After midline neck incision, a 5–0 silicon-coated nylon monofilament (Doccol Corporation, USA) was inserted through the external carotid artery and into the internal carotid artery. At 1 h after the occlusion of the left MCA, the filament was withdrawn to allow reperfusion and animals were placed in the incubator. During cerebral ischemia, rectal temperature was maintained at $37 \pm 0.5^\circ\text{C}$ using a thermistor-controlled heating blanket. At one day after MCAo, rats were reanesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) cocktails and placed in a stereotaxic frame (David Kopf instruments, USA) for adenoviral NRG1 or AdMSCs injection. After a burr hole was made with a stereotaxic-attached drill, 2 μL of adenoviral NRG1 (1×10^{10} pfu/ml) or 5 μL of 1.2×10^5 AdMSCs/ μL in phosphate-buffered saline (PBS) were injected with a Hamilton syringe into the left lateral striatum (0.7 mm anterior to the bregma, 3.2 mm lateral to the midline, and 5.5 mm beneath the dura) for 5 min, and the needle was left for an additional 5 min after the injection. Control group was injected with PBS instead of stem cells. Subsequently,

dental cement was applied on the skull and the animals were returned to their cages once they become conscious. During the post-stroke care, animals were humanely euthanized with an overdose of ketamine and xylazine cocktails when paralysis, dysbasia, dyschezia, or the weight loss greater than 20% at 7 d after MCAo was observed. We did not find any mortality that occurred outside of planned euthanasia and humane endpoints we set.

Behavioral assessment

One of the most common neurological scales used in animal studies of stroke is the modified Neurologic Severity Score (mNSS), which can assess motor (muscle tone and abnormal movement, maximum 6 points), sensory (visual, tactile, and proprioceptive, maximum 2 points), reflex (pinna, corneal, startle, and myoclonus or myodystony, maximum 4 points), limb placement test (maximum 10 points), and balance tests (maximum 6 points) [31]. Using this mNSS system with minor modification, we gave scores to the rats when they showed functional deficits, grading the test results from 0 (normal) to 28 (maximal deficit score). In the morning at 1 d after MCAo, rats showing lower than 23 points in the mNSS test were excluded due to insufficient behavioral deficits (25 out of 126 rats) [31]. Only rats qualifying this criterion were further evaluated by mNSS at 7, and 14 d after MCAo. After the behavioral test was completed, some animals were perfused for the staining and the rest of the animals were sacrificed for western blotting and enzyme-linked immunosorbent assay (ELISA).

Measurement of infarct sizes

At 14 d after MCAo, animals were fully anesthetized with an overdose of ketamine and xylazine cocktails. After cardiac perfusion with normal saline and fixation with 4% paraformaldehyde in 0.1 M PBS, the brains were removed and cryoprotected with 30% sucrose solution. Then, the brains were cut into 40- μ m-thick coronal sections by using a cryostat microtome. Total of six sections with 2 mm intervals between each section were processed for hematoxylin and eosin (H&E) staining. Briefly, sections were stained in hematoxylin solution for 10 min, followed by washing with tap water. Then the sections were incubated with Eosin for 10 s, dehydrated, and mounted. Infarct region was defined as the area with eosinophilic neurons showing shrunken cell bodies, dark pyknotic nuclei, and the intense red cytoplasm [30, 32]. Using an image analysis program (Image-Pro Plus, USA), infarct area was measured by subtracting the area of the ipsilateral non-infarcted hemisphere from that of the contralateral tissue. The percentage of infarct volume was calculated by dividing the sum of the infarct areas by the areas of contralateral hemisphere.

Enzyme-linked immunosorbent assay (ELISA)

The secretion of adenoviral NRG1 into culture media and the brain NRG1 level after MCAo were measured using a commercial NRG1- β 1 ELISA system (R&D Systems, USA). 3×10^5 AdMSCs in a 35 mm culture dish were infected with NRG1-expressing adenovirus at 100 MOI in 2% FBS containing media for 2 h. Medium was replaced with fresh DMEM containing 10% FBS and the cells were cultured for an additional 12 h. Media was then removed and the cells were incubated for 48 h in 1 ml of serum-free DMEM. The culture supernatants were collected and stored at -70°C until analysis. Then, ELISA analysis was performed according to the manufacturer's instruction.

With regard to brain NRG1 expression, coronal brain tissue chunks ($-1.0 \sim 1.0$ mm to the bregma) were collected at 14 d after MCAO. Ischemic hemispheres (200 mg) were dissected on ice and were stored at -70°C until use. Subsequently, each tissue sample was resuspended in an equal weight of homogenization buffer and homogenized with a Dounce homogenizer. The

homogenate was centrifuged (10,000 g) for 10 min at 4°C and the supernatant (5 µg/µl) was processed for ELISA analysis.

Staining

For immunocytochemistry, AdMSCs and NRG1-AdMSCs were grown in four-well Lab-Tek II chamber slides (Nunc, USA) at a density of 2×10^4 cells/mL. Briefly, the cells were fixed with 4% paraformaldehyde for 30 min at 4°C. The cells were incubated in blocking solution (10% normal donkey serum) for 30 min and then with rabbit polyclonal anti-NRG1 (1:200; Santa Cruz Biotechnology, USA) for 16 h at 4°C. After washing with PBS, the cells were incubated with Cy3-conjugated donkey anti-mouse IgG (1:500; Jackson ImmunoResearch, USA) for 1 h at room temperature ($22 \pm 3^\circ\text{C}$). After final washing with PBS, the cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI).

For immunohistochemical staining, the 40-µm-thick coronal sections were incubated with 10% normal donkey serum for 1 h and then with the following primary antibodies for 16 h at 4°C: rabbit polyclonal anti-NRG1 (1:200; Santa Cruz Biotechnology), mouse monoclonal anti-human nuclei (1:200; Millipore Corporation, USA). After washing with PBS, the sections were incubated with the following secondary antibodies for 2 h at room temperature ($22 \pm 3^\circ\text{C}$): Cy3-conjugated donkey anti-mouse IgG (1:500; Jackson ImmunoResearch), Alexa488-conjugated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch). Then, after washing with PBS, the sections were mounted and observed using a confocal microscopy (LSM 510 Meta; Carl Zeiss Co., Germany).

Western blotting

Brain tissues were lysed in RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) added with protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, USA). Lysates were clarified by centrifugation and the supernatant was collected for protein extraction. Equal amounts of protein (60 µg) from each sample were separated on 8~15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membrane was then blocked with 5% bovine serum albumin in tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature and incubated overnight at 4°C with anti-Neuregulin-1 (1:500, Santa Cruz Biotechnology), anti-ErbB4 (1:500, Santa Cruz Biotechnology), p44/42 mitogen-activated protein kinase (MAPK; Cell signaling Technology, USA), anti-phospho MAPK (Cell signaling Technology), anti-Akt (Cell signaling Technology), or anti-phospho Akt (Cell signaling Technology) antibodies diluted 1:1000. The membrane was then incubated with horseradish peroxidase-conjugated secondary anti-rabbit, mouse antibody (1:1000, Santa Cruz Biotechnology) at room temperature for 1 h. Bands were detected using an enhanced chemiluminescence detection kit (Thermo Scientific, USA), after which these blots were re-probed with mouse monoclonal anti-GAPDH antibody (1:1000; Novus) as a loading control for all experiments. Relative quantification of the band immunoreactivity was performed by densitometric analysis using an ImageQuant LAS 4000 (Fujifilm, Japan).

Statistical analysis

The data are presented as the mean \pm SEM. No statistical methods were used to determine sample sizes, but our sample sizes are similar to those reported in previous publications [9, 23, 24, 33, 34]. Statistical significance was assessed using SPSS software (version 21.0, IBM SPSS Corp.) for mNSS and western blot analysis, and GraphPad Prism 7 software (GraphPad Software Inc., USA) for the rest of statistical analysis. Student's unpaired t-test was performed to compare *in vitro* ELISA, and NRG1 expression between the groups as the data passed Shapiro-

Wilk normality test. As for behavioral test, repeated measures ANOVA was performed and for mNSS comparison at 14 d after stroke, one-way ANOVA followed by Tukey's post hoc test was carried out. In addition, infarct volume was assessed by Kruskal-Wallis test and uncorrected Dunn's post hoc test as the data failed to pass the normality test. Finally, *in vivo* ELISA and western blotting of downstream signaling were analyzed by one-way ANOVA followed by Duncan's or LSD post hoc test, respectively. $p < 0.05$ was considered statistically significant.

Results

AdMSCs infected with NRG1-expressing adenovirus release NRG1 protein *in vitro*

After we infected AdMSCs with NRG1-expressing adenovirus, we evaluated NRG1 expression with immunocytochemistry. NRG1 immunoreactivity was shown in both AdMSCs treated with or without adenovirus (Fig 1A). Since the adenovirus used in this study contained a secreted form of EGF-like domain from NRG1 β , we performed quantitative analysis of NRG1 secretion using ELISA (Fig 1B). Higher levels of NRG1 protein was detected in the media collected from NRG1-AdMSCs, compared to that from control AdMSCs ($t(6) = 7.81$, $p < 0.01$, $n = 4$ per group). Taken together, these findings demonstrated that AdMSCs engineered to produce NRG1 could have a greater capacity to release NRG1, although AdMSCs themselves could secrete low levels of NRG1.

NRG1-expressing AdMSCs improve neurological function with the reduction of infarct volume after ischemic stroke

We then assessed the functional impact of NRG1-AdMSCs transplantation after ischemic stroke using mNSS test. As we found that all of the MCAo-subjected animals showed comparable neurological deficits at 1 d after stroke with very low individual variations, we randomly assigned three groups for adenoviral NRG1, AdMSCs, NRG1-AdMSCs, or PBS injection and performed transplantation at 1 d post-stroke after we confirmed similar neurological outcomes by mNSS test. Then, motor function was further evaluated at 7 and 14 d post-stroke (Fig 2A).

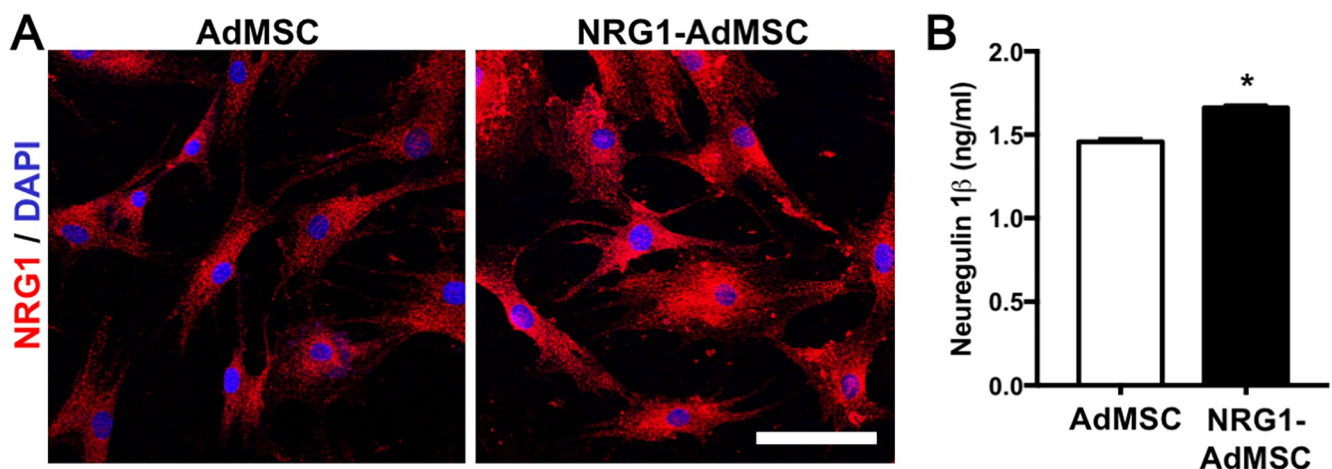


Fig 1. NRG1-expressing AdMSCs release NRG1 protein *in vitro*. (A) Immunocytochemistry to NRG1 shows NRG1 expression in both AdMSCs and NRG1-AdMSCs. Scale bar = 100 μ m. (B) NRG1 secretion by AdMSCs and NRG1-AdMSCs is measured with ELISA ($n = 4$ per group). NRG1 release into the media is significantly increased in NRG1-expressing AdMSCs. * $p < 0.05$ by Student's unpaired t-test. The data are presented as the mean \pm SEM.

<https://doi.org/10.1371/journal.pone.0222587.g001>

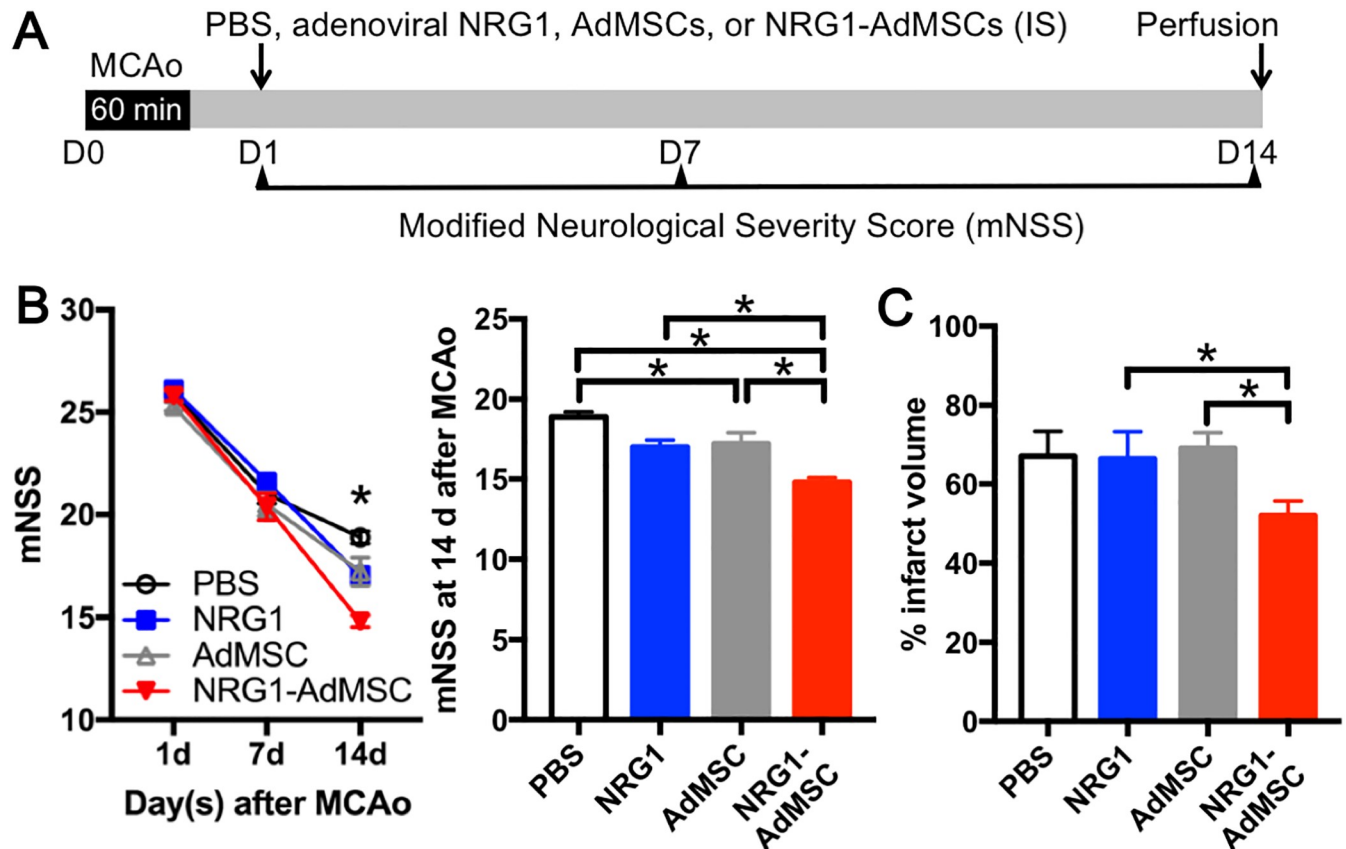


Fig 2. NRG1-expressing AdMSCs enhance neurological functional recovery and reduce infarct volume. (A) Experimental design for ischemic stroke and MSC transplantation. (B) mNSS is performed at 1, 7, and 14 days after MCAo. Rats treated with NRG1-AdMSCs ($n = 26$) show significantly improved neurological function compared to PBS- ($n = 19$), adenoviral NRG1- ($n = 10$), or AdMSCs-treated rats ($n = 18$). $*p < 0.05$ by repeated measures ANOVA for the left graph. $*p < 0.05$ by one-way ANOVA, followed by Tukey's post hoc test for the right graph. (C) Rats transplanted with NRG1-AdMSCs ($n = 10$) show a significant reduction in infarct volume compared to PBS- ($n = 6$), adenoviral NRG1- ($n = 4$), or AdMSCs-treated rats ($n = 7$) at 14 days after MCAo. $*p < 0.05$ by Kruskal-Wallis test followed by uncorrected Dunn's test. The data are presented as the mean \pm SEM.

<https://doi.org/10.1371/journal.pone.0222587.g002>

Temporal improvement of neurological function after MCAo was observed regardless of the groups ($F_{2,138} = 418.06$, $p < 0.01$; Fig 2B). However, rats transplanted with NRG1-AdMSCs ($n = 26$) demonstrated significant lower mNSS scores, compared to PBS- ($n = 19$), adenoviral NRG1 ($n = 10$), or AdMSCs-treated groups ($n = 18$) ($F_{3,69} = 5.55$, $p < 0.01$). When we further assessed neurological outcomes among 4 groups at 14 d after MCAo, NRG1-AdMSCs-treated rats showed greater functional recovery than adenoviral NRG1- or AdMSCs-treated group, suggesting excellent therapeutic efficacy of NRG1-expressing AdMSCs (Fig 2B). In support of mNSS results, infarct size was markedly reduced in rats transplanted with NRG1-AdMSCs ($n = 10$) compared to PBS-injected controls ($n = 6$), adenoviral NRG1- ($n = 4$) or AdMSCs-treated rats ($n = 7$) ($H = 8.46$, $p = 0.04$; Fig 2C).

NRG1-expressing AdMSCs show an increased NRG1 expression in the ischemic hemisphere

To determine NRG1 expression in the transplanted AdMSCs and NRG1-AdMSCs, we performed double immunofluorescence with antibodies specific to human nuclei (HuNu) and NRG1 (Fig 3A). HuNu-positive AdMSCs were mainly located in the infarct area co-expressing

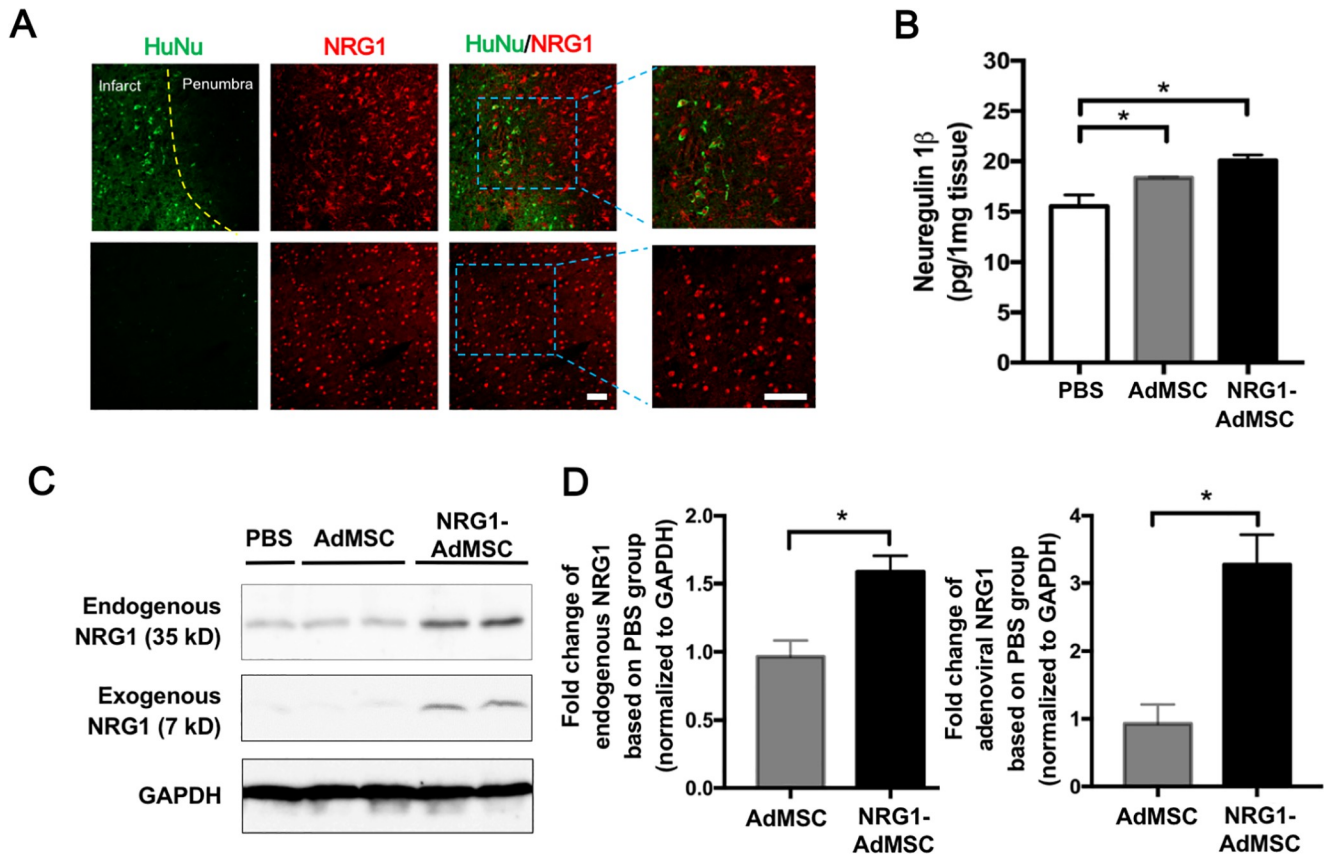


Fig 3. Transplantation of NRG1-expressing AdMSCs increases NRG1 expression in the ipsilateral hemisphere. (A) Immunohistochemical staining shows that NRG1 immunoreactivity (red) is co-localized with human nuclei (HuNu, green) in the ipsilateral hemisphere whereas no double-labeled cells are detected in the contralateral hemisphere. Scale bar in the low magnified image = 100 μm. Scale bar in the high magnified image = 100 μm. (B) Protein level of NRG1 determined by ELISA is significantly increased by NRG1-AdMSCs transplantation (n = 3 for each group). *p < 0.05 by one-way ANOVA followed by Duncan's post hoc test. (C) Representative western blot of three independent experiments of exogenous and endogenous NRG1. (D) Graphs showing relative expression levels of endogenous and exogenous NRG1 between AdMSCs- (n = 4) and NRG1-AdMSCs- (n = 6) treated groups. Both endogenous NRG1 (35 kD) and exogenous adenoviral NRG (7 kD) are increased by the transplantation of NRG1-AdMSCs. *p < 0.05 by Student's unpaired t-test. The data are presented as the mean ± SEM.

<https://doi.org/10.1371/journal.pone.0222587.g003>

NRG1 (Fig 3A), consistent with AdMSCs and NRG1-AdMSCs *in vitro*. Meanwhile, there were no HuNu-labeled cells in the contralateral hemisphere in spite of many NRG1-expressing cells (Fig 3A). Ipsilateral ischemic brain tissues transplanted with NRG1-AdMSCs showed a significant increase in NRG1 expression when assessed by ELISA (Fig 3B, $F(2,6) = 9.88$, $p = 0.01$, $n = 3$ per group). In order to further identify the source of increased NRG1 expression after NRG1-AdMSCs transplantation, western blot analysis was conducted (Fig 3C). Since we introduced short EGF-like domain of NRG1 to adenovirus, adenoviral NRG1 could be identified by 7 kD of molecular weight, which was only expressed in NRG1-infected AdMSCs (Fig 3C). Interestingly, in addition to exogenous NRG1 expression, endogenous NRG1 (35 kD) was also increased in NRG1-AdMSCs (Fig 3C and 3D). Relative quantitative analysis of both endogenous and exogenous NRG1 expression based on the value of PBS group revealed that transplantation of NRG1-AdMSCs (n = 6) significantly upregulated NRG1 compared to AdMSCs administration (n = 4) ($t(8) = 3.61$, $p < 0.01$ for endogenous NRG1, $t(8) = 3.92$, $p < 0.01$ for exogenous NRG1; Fig 3D). These results suggest that NRG1-AdMSCs can release adenoviral NRG1, stimulating endogenous NRG1 upregulation in the ischemic hemisphere.

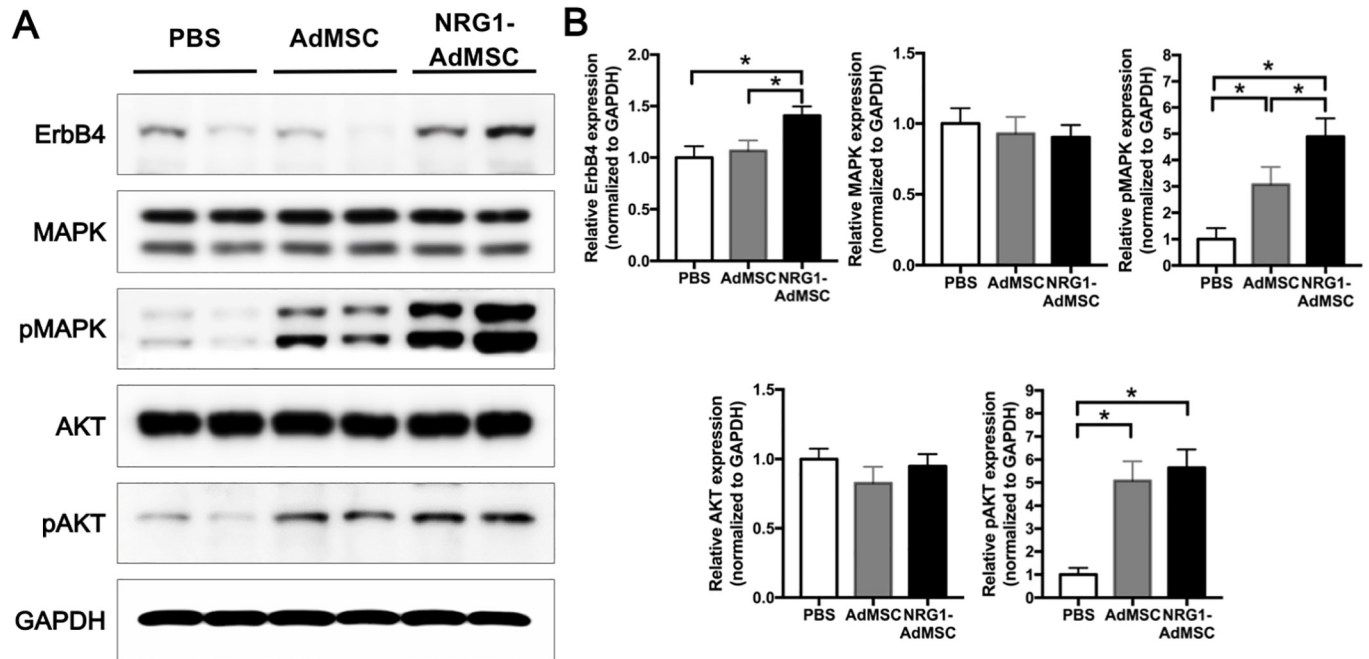


Fig 4. Transplantation of NRG1-AdMSCs activates ErbB4-associated downstream signaling pathways. (A) Representative western blot of three independent experiments results of ErbB4, MAPK, pMAPK, Akt, and pAkt. (B) Graphs showing relative expression levels of ErbB4, MAPK, pMAPK, AKT, and pAKT among PBS-, AdMSCs-, and NRG1-AdMSCs-treated groups. Expression of ErbB4, a receptor of NRG1, was markedly increased by NRG1-AdMSCs treatment (PBS, $n = 7$; AdMSCs, $n = 8$; NRG1-AdMSCs, $n = 8$). Moreover, pMAPK and pAkt expressions were significantly increased by NRG1-AdMSCs transplantation, compared to PBS-treated controls (PBS, $n = 7$; AdMSCs, $n = 10$; NRG1-AdMSCs, $n = 10$). * $p < 0.05$ by one-way ANOVA followed by LSD post hoc test. The data are presented as the mean \pm SEM.

<https://doi.org/10.1371/journal.pone.0222587.g004>

Transplantation of NRG1-expressing AdMSCs activates MAPK and Akt, with the upregulation of ErbB4 expression

We then sought to explore the underlying molecular mechanisms of beneficial effects of NRG1-AdMSCs after ischemic stroke. To do that, we performed western blot to investigate NRG1-associated downstream signaling pathways. Despite there was no difference in the expression of the total MAPK and Akt among the groups treated with PBS, AdMSCs, and NRG1-AdMSCs, phosphorylated MAPK and Akt were markedly increased in the ischemic hemisphere injected with NRG1-AdMSCs (Fig 4, $F(2,24) = 8.31$, $p < 0.01$ for pMAPK, $F(2,24) = 9.67$, $p < 0.01$ for pAKT, $n = 7$ (PBS), $n = 10$ (AdMSCs), $n = 10$ (NRG1-AdMSCs)), demonstrating activated MAPK and Akt pathways by NRG1-AdMSCs transplantation. Moreover, at 14 d after MCAo, ErbB4, a receptor for NRG1, was significantly increased in NRG1-AdMSCs-treated group (Fig 4, $F(2,20) = 4.80$, $p = 0.02$, $n = 7$ (PBS), $n = 8$ (AdMSCs), $n = 8$ (NRG1-AdMSCs)). Collectively, our findings suggest that NRG1-AdMSCs transplantation after ischemic stroke can activate MAPK and Akt pathways, possibly through the upregulation of the NRG1 receptor, ErbB4.

Discussion

In the present study, we demonstrated therapeutic potentials of AdMSCs engineered to produce NRG1 in ischemic stroke. We first confirmed that NRG1-AdMSCs could release higher levels of NRG1 compared to AdMSCs. Transplantation of NRG1-expressing AdMSCs into the ipsilateral striatum after ischemic stroke significantly reduced the brain infarct size and enhanced functional recovery, which was accompanied with the upregulation of both exogenous and endogenous NRG1 expression. Moreover, a NRG1 receptor, ErbB4, was upregulated

in the ischemic hemisphere by NRG1-AdMSCs administration, in addition to the increased pMAPK and pAkt, crucial downstream molecules activated by NRG1-ErbB4 stimulation.

Therapeutic efficacies of transplanted AdMSCs after ischemic stroke have been demonstrated in numerous studies [35]. Consistent with previous reports, we also demonstrated functional improvements by AdMSCs administration. However, in spite of the significant amelioration of stroke-induced motor dysfunction, infarct size was not affected by AdMSC transplantation in our study, which is also shown in a couple of reports [36, 37]. AdMSCs-mediated functional improvements in ischemic stroke had been thought to be attributed to their subsequent differentiation into parenchymal cells replacing damaged tissues [7]. However, many studies later reported that MSCs could release trophic factors and other signaling molecules such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factors (VEGF), and transforming growth factor (TGF) β , contributing to the functional recovery following stroke [38–40]. Moreover, the capacity of MSCs to release neurotrophins and growth factors was markedly enhanced when MSCs were exposed to MCAO-subjected tissue homogenates [41], suggesting active interactions between MSCs and endogenous ischemic brain tissues. In line with these reports, secretome analysis of MSCs stimulated by TGF α revealed that NRG1 was one of the most enriched genes [42], implying that NRG1 may be responsible for the paracrine actions of MSCs following stroke. However, given the limitations of current stem cell therapy that cannot guarantee complete reversal of stroke-induced motor dysfunction, boosting the production of important growth factors in MSCs can be an attractive option to enhance therapeutic potential of MSCs. To prove this hypothesis, researchers have tried to upregulate various trophic factors such as BDNF, VEGF, glial cell-derived neurotrophic factor (GDNF), hepatocyte growth factor (HGF), placental growth factor (PIGF), and fibroblast growth factor 2 (FGF2) in human MSCs and found functional improvement after stroke, compared to naïve MSCs [33, 38–40, 43–47]. In accordance with prior reports, our NRG1-expressing AdMSCs, which was confirmed to release high levels of NRG1, could result in improved post-stroke motor function. Collectively, these data including our findings suggest that gene-modified stem cell therapy can be a useful, new approach for the treatment of ischemic stroke.

Interestingly, our NRG1-AdMSCs transplantation markedly elevated endogenous NRG1 expression in the ischemic cerebral issues, not to mention adenoviral NRG1 secretion. Since NRG1 itself can prevent stroke-induced pro-inflammatory and stress gene expressions, promoting neuronal survival against ischemic stroke [24, 48, 49], NRG1-AdMSCs-mediated high NRG1 expression during the critical post-ischemic period, together with beneficial roles of AdMSCs, may lead to better functional recovery and reduction in infarct volume, compared to adenoviral NRG1-, AdMSCs- or PBS-treated groups. It is also noteworthy that with the addition of NRG1-secreting AdMSCs, host ischemic brain tissues increased the endogenous NRG1 production, along with the NRG1 receptor, ErbB4, suggesting an enhanced NRG1-ErbB4-NRG1 autocrine loop by the transplantation of NRG1-AdMSCs. In support of this hypothesis, ischemic stroke can induce ErbB4 expression in neurons [50] and NRG1 ligand, possibly secreted from neuronal presynaptic terminals can positively regulate ErbB4 signaling [51]. Moreover, there is another report showing that ErbB4-expressing MSCs could increase NRG1 production and secretion, supporting positive feedback relationships of NRG1-ErbB4 signaling.

At the molecular level, NRG1 can activate multiple intracellular signal transduction pathways through interactions with ErbB receptor tyrosine kinases [16–18]. Our study demonstrated significant upregulations of phosphorylated MAPK and Akt in the ischemic brain of NRG1-AdMSCs-treated rats, with the increase of ErbB4 expression. Supporting our findings, activation of NRG1-ErbB4 signaling was neuroprotective, which was mediated by enhancing GABAergic transmissions in ischemic brain injury and schizophrenia [52–54]. These data

suggest that ErbB4 activation can be crucial in NRG1-associated protective effects. With regard to downstream signaling pathways, ErbB4 is known to be linked to Ras-MAPK and phosphoinositide 3-kinase (PI3K)-Akt pathways [55]. In accordance with our findings showing increased pMAPK and pAkt by NRG1-AdMSCs transplantation, increased NRG1 expression by lipopolysaccharide-induced neuroinflammation could alter phosphorylation of Akt in the mouse brain [56]. Moreover, administration of NRG1 β to autonomic ganglion neurons could also increase the expressions of pAkt and pMAPK [57], in line with our results. Interestingly, when compared to AdMSCs, transplantation of NRG1-AdMSCs significantly increased pMAPK expression in the ischemic hemisphere, while pAKT expression was upregulated in both AdMSCs- and NRG1-AdMSCs-treated groups. These data suggest that enhanced MAPK signaling can play a role in additional functional benefits of NRG1-AdMSCs compared to AdMSCs, whereas AKT signaling can serve to promote AdMSCs-mediated functional improvements after ischemic stroke. Further studies exploring differential molecular mechanisms of AdMSCs and NRG1-AdMSCs after ischemic stroke are warranted.

Conclusion

Cell-based therapies have emerged as a new approach for the treatment of ischemic stroke. Various trophic factor-modified MSCs could enhance therapeutic potentials in the ischemic stroke by boosting secretory function of stem cells. In this study, we showed that transplantation of NRG1-expressing AdMSCs could significantly improve neurologic outcomes against MCAo-induced ischemic brain injury, compared to naïve AdMSCs or NRG1 overexpression. We also demonstrated that neuroprotective effects of NRG1-AdMSCs administration could be mediated by the upregulation of ErbB4. Finally, transplantation of NRG1-AdMSCs significantly increased the expression of pMAPK and pAkt compared to PBS-injected controls, suggesting the activation of MAPK and AKT signaling. As neuroprotective mechanisms of gene-modified MSCs transplantation for treating stroke are complex and are still not fully understood, additional investigations are necessary for deciphering complicated repair processes after ischemic brain injury.

Supporting information

S1 File. The ARRIVE guidelines checklist.
(PDF)

Acknowledgments

We thank Ms. Hyosun Lim and Dr. In-Young Choi for their technical support. The adipose stem cells supplied by Seoul St. Mary's hospital of Korea were derived from human adipose tissue donated.

Author Contributions

Conceptualization: Sun Ryu, Kyung-Ok Cho.

Formal analysis: Sun Ryu, Jae-Min Lee, Cheong A. Bae, Chae-Eun Moon, Kyung-Ok Cho.

Funding acquisition: Kyung-Ok Cho.

Investigation: Sun Ryu, Jae-Min Lee, Cheong A. Bae, Chae-Eun Moon.

Methodology: Sun Ryu, Jae-Min Lee.

Supervision: Kyung-Ok Cho.

Validation: Jae-Min Lee, Cheong A. Bae.

Visualization: Jae-Min Lee, Cheong A. Bae.

Writing – original draft: Sun Ryu, Kyung-Ok Cho.

Writing – review & editing: Kyung-Ok Cho.

References

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, et al. Executive summary: heart disease and stroke statistics—2014 update: a report from the American Heart Association. *Circulation*. 2014; 129(3):399–410. Epub 2014/01/22. <https://doi.org/10.1161/01.cir.0000442015.53336.12> PMID: [24446411](https://pubmed.ncbi.nlm.nih.gov/24446411/)
2. Hacke W, Kaste M, Bluhmki E, Brozman M, Davalos A, Guidetti D, et al. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *N Engl J Med*. 2008; 359(13):1317–29. <https://doi.org/10.1056/NEJMoa0804656> PMID: [18815396](https://pubmed.ncbi.nlm.nih.gov/18815396/)
3. Gutierrez-Fernandez M, Otero-Ortega L, Ramos-Cejudo J, Rodriguez-Frutos B, Fuentes B, Diez-Tejedor E. Adipose tissue-derived mesenchymal stem cells as a strategy to improve recovery after stroke. *Expert Opin Biol Ther*. 2015; 15(6):873–81. <https://doi.org/10.1517/14712598.2015.1040386> PMID: [25959243](https://pubmed.ncbi.nlm.nih.gov/25959243/)
4. Napoli E, Borlongan CV. Recent Advances in Stem Cell-Based Therapeutics for Stroke. *Translational stroke research*. 2016. Epub 2016/08/16. <https://doi.org/10.1007/s12975-016-0490-6> PMID: [27515852](https://pubmed.ncbi.nlm.nih.gov/27515852/)
5. Castillo M, Liu K, Bonilla L, Rameshwar P. The immune properties of mesenchymal stem cells. *Int J Biomed Sci*. 2007; 3(2):76–80. PMID: [23675026](https://pubmed.ncbi.nlm.nih.gov/23675026/)
6. Chang KA, Lee JH, Suh YH. Therapeutic potential of human adipose-derived stem cells in neurological disorders. *J Pharmacol Sci*. 2014; 126(4):293–301. <https://doi.org/10.1254/jphs.14R10CP> PMID: [25409785](https://pubmed.ncbi.nlm.nih.gov/25409785/)
7. Parker AM, Katz AJ. Adipose-derived stem cells for the regeneration of damaged tissues. *Expert Opin Biol Ther*. 2006; 6(6):567–78. <https://doi.org/10.1517/14712598.6.6.567> PMID: [16706604](https://pubmed.ncbi.nlm.nih.gov/16706604/)
8. Ra JC, Shin IS, Kim SH, Kang SK, Kang BC, Lee HY, et al. Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem Cells Dev*. 2011; 20(8):1297–308. <https://doi.org/10.1089/scd.2010.0466> PMID: [21303266](https://pubmed.ncbi.nlm.nih.gov/21303266/)
9. Bang OY, Jin KS, Hwang MN, Kang HY, Kim BJ, Lee SJ, et al. The Effect of CXCR4 Overexpression on Mesenchymal Stem Cell Transplantation in Ischemic Stroke. *Cell Med*. 2012; 4(2):65–76. <https://doi.org/10.3727/215517912X647172> PMID: [26858855](https://pubmed.ncbi.nlm.nih.gov/26858855/)
10. Falls DL. Neuregulins: functions, forms, and signaling strategies. *Exp Cell Res*. 2003; 284(1):14–30. [https://doi.org/10.1016/s0014-4827\(02\)00102-7](https://doi.org/10.1016/s0014-4827(02)00102-7) PMID: [12648463](https://pubmed.ncbi.nlm.nih.gov/12648463/)
11. Steinthorsdottir V, Stefansson H, Ghosh S, Birgisdottir B, Bjornsdottir S, Fasquel AC, et al. Multiple novel transcription initiation sites for NRG1. *Gene*. 2004; 342(1):97–105. <https://doi.org/10.1016/j.gene.2004.07.029> PMID: [15527969](https://pubmed.ncbi.nlm.nih.gov/15527969/)
12. Harrison PJ, Law AJ. Neuregulin 1 and schizophrenia: genetics, gene expression, and neurobiology. *Biol Psychiatry*. 2006; 60(2):132–40. <https://doi.org/10.1016/j.biopsych.2005.11.002> PMID: [16442083](https://pubmed.ncbi.nlm.nih.gov/16442083/)
13. Tan W, Wang Y, Gold B, Chen J, Dean M, Harrison PJ, et al. Molecular cloning of a brain-specific, developmentally regulated neuregulin 1 (NRG1) isoform and identification of a functional promoter variant associated with schizophrenia. *J Biol Chem*. 2007; 282(33):24343–51. <https://doi.org/10.1074/jbc.M702953200> PMID: [17565985](https://pubmed.ncbi.nlm.nih.gov/17565985/)
14. Buonanno A, Fischbach GD. Neuregulin and ErbB receptor signaling pathways in the nervous system. *Curr Opin Neurobiol*. 2001; 11(3):287–96. [https://doi.org/10.1016/s0959-4388\(00\)00210-5](https://doi.org/10.1016/s0959-4388(00)00210-5) PMID: [11399426](https://pubmed.ncbi.nlm.nih.gov/11399426/)
15. Fischbach GD, Rosen KM. ARIA: A neuromuscular junction neuregulin. *Annu Rev Neurosci*. 1997; 20:429–58. <https://doi.org/10.1146/annurev.neuro.20.1.429> PMID: [9056721](https://pubmed.ncbi.nlm.nih.gov/9056721/)
16. Bublil EM, Yarden Y. The EGF receptor family: spearheading a merger of signaling and therapeutics. *Curr Opin Cell Biol*. 2007; 19(2):124–34. <https://doi.org/10.1016/j.ceb.2007.02.008> PMID: [17314037](https://pubmed.ncbi.nlm.nih.gov/17314037/)
17. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J*. 2000; 19(13):3159–67. <https://doi.org/10.1093/emboj/19.13.3159> PMID: [10880430](https://pubmed.ncbi.nlm.nih.gov/10880430/)
18. Britsch S. The neuregulin-I/ErbB signaling system in development and disease. *Adv Anat Embryol Cell Biol*. 2007; 190:1–65. PMID: [17432114](https://pubmed.ncbi.nlm.nih.gov/17432114/)

19. Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S, et al. Neuregulin 1 and susceptibility to schizophrenia. *Am J Hum Genet.* 2002; 71(4):877–92. <https://doi.org/10.1086/342734> PMID: 12145742
20. Cannella B, Pitt D, Marchionni M, Raine CS. Neuregulin and erbB receptor expression in normal and diseased human white matter. *J Neuroimmunol.* 1999; 100(1–2):233–42. [https://doi.org/10.1016/s0165-5728\(99\)00201-5](https://doi.org/10.1016/s0165-5728(99)00201-5) PMID: 10695733
21. Erlich S, Shohami E, Pinkas-Kramarski R. Closed head injury induces up-regulation of ErbB-4 receptor at the site of injury. *Mol Cell Neurosci.* 2000; 16(5):597–608. <https://doi.org/10.1006/mcne.2000.0894> PMID: 11083921
22. Tokita Y, Keino H, Matsui F, Aono S, Ishiguro H, Higashiyama S, et al. Regulation of neuregulin expression in the injured rat brain and cultured astrocytes. *J Neurosci.* 2001; 21(4):1257–64. PMID: 11160396
23. Parker MW, Chen Y, Hallenbeck JM, Ford BD. Neuregulin expression after focal stroke in the rat. *Neurosci Lett.* 2002; 334(3):169–72. [https://doi.org/10.1016/s0304-3940\(02\)01126-6](https://doi.org/10.1016/s0304-3940(02)01126-6) PMID: 12453622
24. Xu Z, Croslan DR, Harris AE, Ford GD, Ford BD. Extended therapeutic window and functional recovery after intraarterial administration of neuregulin-1 after focal ischemic stroke. *J Cereb Blood Flow Metab.* 2006; 26(4):527–35. <https://doi.org/10.1038/sj.jcbfm.9600212> PMID: 16136057
25. Wang S, Li Y, Paudyal R, Ford BD, Zhang X. Spatio-temporal assessment of the neuroprotective effects of neuregulin-1 on ischemic stroke lesions using MRI. *J Neurol Sci.* 2015; 357(1–2):28–34. Epub 2015/07/18. <https://doi.org/10.1016/j.jns.2015.06.055> PMID: 26183085
26. Xu Z, Ford GD, Croslan DR, Jiang J, Gates A, Allen R, et al. Neuroprotection by neuregulin-1 following focal stroke is associated with the attenuation of ischemia-induced pro-inflammatory and stress gene expression. *Neurobiol Dis.* 2005; 19(3):461–70. <https://doi.org/10.1016/j.nbd.2005.01.027> PMID: 16023588
27. Joung I, Yoo M, Woo JH, Chang CY, Heo H, Kwon YK. Secretion of EGF-like domain of heregulinbeta promotes axonal growth and functional recovery of injured sciatic nerve. *Mol Cells.* 2010; 30(5):477–84. <https://doi.org/10.1007/s10059-010-0137-5> PMID: 20957456
28. Cho KO, Kim YS, Cho YJ, Kim SY. Upregulation of DSCR1 (RCAN1 or Adapt78) in the peri-infarct cortex after experimental stroke. *Exp Neurol.* 2008; 212(1):85–92. <https://doi.org/10.1016/j.expneurol.2008.03.017> PMID: 18485347
29. Ryu S, Lee SH, Kim SU, Yoon BW. Human neural stem cells promote proliferation of endogenous neural stem cells and enhance angiogenesis in ischemic rat brain. *Neural Regen Res.* 2016; 11(2):298–304. <https://doi.org/10.4103/1673-5374.177739> PMID: 27073384
30. Lim H, Park SH, Kim SW, Cho KO. Therapeutic Potential of Human Turbinate-Derived Mesenchymal Stem Cells in Experimental Acute Ischemic Stroke. *Int Neurourol J.* 2018; 22(Suppl 3):S131–8. <https://doi.org/10.5213/inj.1836220.110> PMID: 30396262
31. Chen J, Li Y, Wang L, Zhang Z, Lu D, Lu M, et al. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke.* 2001; 32(4):1005–11. <https://doi.org/10.1161/01.str.32.4.1005> PMID: 11283404
32. Zille M, Farr TD, Przesdzing I, Muller J, Sommer C, Dirnagl U, et al. Visualizing cell death in experimental focal cerebral ischemia: promises, problems, and perspectives. *J Cereb Blood Flow Metab.* 2012; 32(2):213–31. <https://doi.org/10.1038/jcbfm.2011.150> PMID: 22086195
33. Zhao MZ, Nonoguchi N, Ikeda N, Watanabe T, Furutama D, Miyazawa D, et al. Novel therapeutic strategy for stroke in rats by bone marrow stromal cells and ex vivo HGF gene transfer with HSV-1 vector. *J Cereb Blood Flow Metab.* 2006; 26(9):1176–88. <https://doi.org/10.1038/sj.jcbfm.9600273> PMID: 16421510
34. Xu ZF, Jiang J, Ford G, Ford BD. Neuregulin-1 is neuroprotective and attenuates inflammatory responses induced by ischemic stroke. *Biochem Biophys Res Commun.* 2004; 322(2):440–6. <https://doi.org/10.1016/j.bbrc.2004.07.149> PMID: 15325249
35. Chan TM, Harn HJ, Lin HP, Chiu SC, Lin PC, Wang HI, et al. The Use of ADSCs as a Treatment for Chronic Stroke. *Cell Transplant.* 2014; 23(4–5):541–7. <https://doi.org/10.3727/096368914X678409> PMID: 24816449
36. Kang SK, Lee DH, Bae YC, Kim HK, Baik SY, Jung JS. Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. *Exp Neurol.* 2003; 183(2):355–66. [https://doi.org/10.1016/s0014-4886\(03\)00089-x](https://doi.org/10.1016/s0014-4886(03)00089-x) PMID: 14552877
37. Mu J, Bakreen A, Juntunen M, Korhonen P, Oinonen E, Cui L, et al. Combined Adipose Tissue-Derived Mesenchymal Stem Cell Therapy and Rehabilitation in Experimental Stroke. *Front Neurol.* 2019; 10:235. <https://doi.org/10.3389/fneur.2019.00235> PMID: 30972000
38. Kurozumi K, Nakamura K, Tamiya T, Kawano Y, Kobune M, Hirai S, et al. BDNF gene-modified mesenchymal stem cells promote functional recovery and reduce infarct size in the rat middle cerebral artery

- occlusion model. *Mol Ther*. 2004; 9(2):189–97. <https://doi.org/10.1016/j.ymthe.2003.10.012> PMID: 14759803
39. Nomura T, Honmou O, Harada K, Houkin K, Hamada H, Kocsis JD. I.V. infusion of brain-derived neurotrophic factor gene-modified human mesenchymal stem cells protects against injury in a cerebral ischemia model in adult rat. *Neuroscience*. 2005; 136(1):161–9. <https://doi.org/10.1016/j.neuroscience.2005.06.062> PMID: 16229956
 40. Jeong CH, Kim SM, Lim JY, Ryu CH, Jun JA, Jeun SS. Mesenchymal stem cells expressing brain-derived neurotrophic factor enhance endogenous neurogenesis in an ischemic stroke model. *Biomed Res Int*. 2014; 2014:129145. <https://doi.org/10.1155/2014/129145> PMID: 24672780
 41. Chen X, Li Y, Wang L, Katakowski M, Zhang L, Chen J, et al. Ischemic rat brain extracts induce human marrow stromal cell growth factor production. *Neuropathology*. 2002; 22(4):275–9. PMID: 12564767
 42. De Luca A, Roma C, Gallo M, Fenizia F, Bergantino F, Frezzetti D, et al. RNA-seq analysis reveals significant effects of EGFR signalling on the secretome of mesenchymal stem cells. *Oncotarget*. 2014; 5(21):10518–28. <https://doi.org/10.18632/oncotarget.2420> PMID: 25344915
 43. Liu H, Honmou O, Harada K, Nakamura K, Houkin K, Hamada H, et al. Neuroprotection by PIGF gene-modified human mesenchymal stem cells after cerebral ischaemia. *Brain*. 2006; 129(Pt 10):2734–45. <https://doi.org/10.1093/brain/awl207> PMID: 16901914
 44. Chen B, Zhang F, Li QY, Gong A, Lan Q. Protective Effect of Ad-VEGF-Bone Mesenchymal Stem Cells on Cerebral Infarction. *Turk Neurosurg*. 2016; 26(1):8–15. <https://doi.org/10.5137/1019-5149.JTN.11488-14.3> PMID: 26768863
 45. Lai T, Li M, Zheng L, Song Y, Xu X, Guo Y, et al. Over-expression of VEGF in marrow stromal cells promotes angiogenesis in rats with cerebral infarction via the synergistic effects of VEGF and Ang-2. *J Huazhong Univ Sci Technolog Med Sci*. 2012; 32(5):724–31. <https://doi.org/10.1007/s11596-012-1025-3> PMID: 23073804
 46. Ikeda N, Nonoguchi N, Zhao MZ, Watanabe T, Kajimoto Y, Furutama D, et al. Bone marrow stromal cells that enhanced fibroblast growth factor-2 secretion by herpes simplex virus vector improve neurological outcome after transient focal cerebral ischemia in rats. *Stroke*. 2005; 36(12):2725–30. <https://doi.org/10.1161/01.STR.0000190006.88896.d3> PMID: 16282547
 47. Miki Y, Nonoguchi N, Ikeda N, Coffin RS, Kuroiwa T, Miyatake SI. Vascular endothelial growth factor gene-transferred bone marrow stromal cells engineered with a herpes simplex virus type 1 vector can improve neurological deficits and reduce infarction volume in rat brain ischemia. *Neurosurgery*. 2007; 61(3):586–94. <https://doi.org/10.1227/01.NEU.0000290907.30814.42> PMID: 17881973
 48. Guo WP, Wang J, Li RX, Peng YW. Neuroprotective effects of neuregulin-1 in rat models of focal cerebral ischemia. *Brain Res*. 2006; 1087(1):180–5. <https://doi.org/10.1016/j.brainres.2006.03.007> PMID: 16616052
 49. Shyu WC, Lin SZ, Chiang MF, Yang HI, Thajeb P, Li H. Neuregulin-1 reduces ischemia-induced brain damage in rats. *Neurobiol Aging*. 2004; 25(7):935–44. <https://doi.org/10.1016/j.neurobiolaging.2003.10.012> PMID: 15212847
 50. Xu Z, Ford BD. Upregulation of erbB receptors in rat brain after middle cerebral arterial occlusion. *Neurosci Lett*. 2005; 375(3):181–6. <https://doi.org/10.1016/j.neulet.2004.11.039> PMID: 15694257
 51. Li B, Woo RS, Mei L, Malinow R. The neuregulin-1 receptor erbB4 controls glutamatergic synapse maturation and plasticity. *Neuron*. 2007; 54(4):583–97. <https://doi.org/10.1016/j.neuron.2007.03.028> PMID: 17521571
 52. Guan YF, Wu CY, Fang YY, Zeng YN, Luo ZY, Li SJ, et al. Neuregulin 1 protects against ischemic brain injury via ErbB4 receptors by increasing GABAergic transmission. *Neuroscience*. 2015; 307:151–9. <https://doi.org/10.1016/j.neuroscience.2015.08.047> PMID: 26318331
 53. Chen YJ, Zhang M, Yin DM, Wen L, Ting AN, Wang P, et al. ErbB4 in parvalbumin-positive interneurons is critical for neuregulin 1 regulation of long-term potentiation. *Proc Natl Acad Sci U S A*. 2010; 107(50):21818–23. <https://doi.org/10.1073/pnas.1010669107> PMID: 21106764
 54. Woo RS, Li XM, Tao Y, Carpenter-Hyland E, Huang YZ, Weber J, et al. Neuregulin-1 enhances depolarization-induced GABA release. *Neuron*. 2007; 54(4):599–610. <https://doi.org/10.1016/j.neuron.2007.04.009> PMID: 17521572
 55. Iwakura Y, Nawa H. ErbB1-4-dependent EGF/neuregulin signals and their cross talk in the central nervous system: pathological implications in schizophrenia and Parkinson's disease. *Front Cell Neurosci*. 2013; 7. ARTN 410.3389/fncel.2013.00004.
 56. Yang Z, Jiang Q, Chen SX, Hu CL, Shen HF, Huang PZ, et al. Differential changes in Neuregulin-1 signaling in major brain regions in a lipopolysaccharide-induced neuroinflammation mouse model. *Mol Med Rep*. 2016; 14(1):790–6. <https://doi.org/10.3892/mmr.2016.5325> PMID: 27220549

57. Kim HG, Lee CK, Cho SM, Whang K, Cha BH, Shin JH, et al. Neuregulin 1 up-regulates the expression of nicotinic acetylcholine receptors through the ErbB2/ErbB3-PI3K-MAPK signaling cascade in adult autonomic ganglion neurons. *J Neurochem*. 2013; 124(4):502–13. <https://doi.org/10.1111/jnc.12109> PMID: [23199222](https://pubmed.ncbi.nlm.nih.gov/23199222/)