Original Research

Activated Mesenchymal Stem Cells Induce Recovery Following Stroke Via Regulation of Inflammation and Oligodendrogenesis

Matthew K. Tobin, BSc; Terilyn K. L. Stephen, MSc; Kyra L. Lopez; Melissa R. Pergande, MSc; Amelia M. Bartholomew, MD, MPH; Stephanie M. Cologna, PhD; Orly Lazarov, PhD

Background: Brain repair mechanisms fail to promote recovery after stroke, and approaches to induce brain regeneration are scarce. Mesenchymal stem cells (MSC) are thought to be a promising therapeutic option. However, their efficacy is not fully elucidated, and the mechanism underlying their effect is not known.

Methods and Results: The middle cerebral artery occlusion model was utilized to determine the efficacy of interferon-γ–activated mesenchymal stem cells (aMSCγ) as an acute therapy for stroke. Here we show that treatment with aMSCγ is a more potent therapy for stroke than naive MSC. aMSCγ treatment results in significant functional recovery assessed by the modified neurological severity score and open-field analysis compared with vehicle-treated animals. aMSCγ-treated animals showed significant reductions in infarct size and inhibition of microglial activation. The aMSCγ treatment suppressed the hypoxia-induced microglial proinflammatory phenotype more effectively than treatment with naive MSC. Importantly, treatment with aMSCγ induced recruitment and differentiation of oligodendrocyte progenitor cells to myelin-producing oligodendrocytes in vivo. To elucidate the mechanism underlying high efficacy of aMSCγ therapy, we examined the secretome of aMSCγ and compared it to that of naive MSC. Intriguingly, we found that aMSCγ but not nMSC upregulated neuron-glia antigen 2, an important extracellular signal and a hallmark protein of oligodendrocyte progenitor cells.

Conclusions: These results suggest that activation of MSC with interferon-γ induces a potent proregenerative, promyelinating, and anti-inflammatory phenotype of these cells, which increases the potency of aMSCγ as an effective therapy for ischemic stroke.

Key Words: inflammation • ischemia • mesenchymal stem cells • oligodendrogenesis • stroke • therapy

Stroke is the fifth leading cause of death each year in the United States and is the number 1 cause of permanent disability in the world.1,2 There are ≈800,000 new stroke victims in the United States each year, and almost 130,000 mortalities.3 The vast majority, around 90%, of stroke patients suffer from an ischemic stroke caused by temporary or permanent occlusion of a blood vessel to the brain.4 Current therapy options for ischemic stroke patients in the acute setting are drastically limited, with only 1 FDA-approved drug, tissue plasminogen activator available. However, because of a number of limitations, mainly a therapeutic window of 3 to 4.5 hours, only 4% to 7% of patients are eligible for tissue plasminogen activator therapy.5 This therapeutic window can be increased to up to 24 hours with the addition of endovascular thrombectomy, but this type of therapy can only be administered in certain hospital settings and only after meeting a number of different treatment criteria.6,7 Therefore, it is crucial for new therapeutic options to be developed for the treatment of these patients in the acute setting.

Inflammatory responses after stroke involve both peripheral immune cells and microglia. How these
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CLINICAL PERSPECTIVE

What Is New?
- Activation of mesenchymal stem cells with interferon-γ promotes proregenerative characteristics and facilitates the anti-inflammatory characteristics of naive mesenchymal stem cells, making them more potent therapy following ischemic stroke.

What Are the Clinical Implications?
- There is an unmet need to develop proregenerative therapy for the treatment of ischemic stroke.
- Most clinical studies using mesenchymal stem cells use them in their naive form, which has limited anti-inflammatory effect and little proregenerative impact.
- Interferon-γ–activated mesenchymal stem cells injected at the acute phase of the insult exhibit both anti-inflammatory and proregenerative effects leading to functional recovery.

Nonstandard Abbreviations and Acronyms

| Abbreviation | Definition |
|--------------|------------|
| 7-AAD        | 7-aminoactinomycin D |
| aMSCy        | interferon-γ–activated mesenchymal stem cells |
| BDNF         | brain-derived neurotrophic factor |
| BMP1         | bone morphogenetic protein 1 |
| CNPase       | 2′,3′-cyclic-nucleotide 3′-phosphodiesterase |
| CSPG4        | chondroitin sulfate proteoglycan 4 |
| DCX          | doublecortin |
| DIC          | differential interference contrast |
| GDNF         | glial cell-derived neurotrophic factor |
| GFAP         | glial fibrillary acidic protein |
| IFN-γ        | interferon-γ |
| IGF-1        | insulin-like growth factor 1 |
| IL-4         | interleukin 4 |
| IL-6         | interleukin 6 |
| LDH          | lactate dehydrogenase |
| MBP          | myelin basic protein |
| MCAO         | middle cerebral artery occlusion |
| MG           | microglia |
| MRI          | magnetic resonance imaging |
| MSC          | mesenchymal stem cells |
| MTT          | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| N.D.         | not detected |
| nMSC         | naive mesenchymal stem cells |
| NPC          | neural progenitor cell(s) |
| NSC          | neural stem cell(s) |
| OGD          | oxygen-glucose deprivation |
| OPC          | oligodendrocyte progenitor cells |
| PDGFRα       | platelet-derived growth factor receptor α |
| SVZ          | subventricular zone |
| TNF-α        | tumor necrosis factor α |
| OGD          | oxygen-glucose deprivation |
| OPC          | oligodendrocyte progenitor cells |
| PDGFRα       | platelet-derived growth factor receptor α |
| SVZ          | subventricular zone |
| TNF-α        | tumor necrosis factor α |

processes affect long-term brain repair is still not fully understood. Many studies have demonstrated both detrimental and beneficial effects of brain inflammation. Furthermore, these changes, and whether they are good or bad, often depend on the time frame in which they are studied, with the beneficial effects of these cells attributed to the chronic phase of stroke. There have been numerous research studies and clinical trials looking into the efficacy of anti-inflammatory agents as stroke therapies; however, the majority of these studies reported either worse outcomes or no change from placebo, and all failed to make it beyond clinical trials.

Endogenous neurogenesis processes, long posited as therapeutic targets for brain repair, have largely been noncontributory in the setting of ischemic stroke. Although endogenous neural stem cells (NSC) do react acutely to ischemic stroke with increases in cell proliferation and cell migration, only 10% to 20% of these cells survive long term. These few surviving cells, however, do seem to mature into functional cells, with the majority of them becoming spiny neostriatal projection neurons or calretinin-positive interneurons. But despite these interesting data, it is still largely unknown why the vast majority of these new cells do not mature and survive long term and why they fail to support brain recovery.

Mesenchymal stem cells (MSC) have unique characteristics that make them an interesting tool to study brain repair after ischemic stroke. They have the ability to (1) reduce overall inflammation, thereby eliminating the potentially toxic environment that could be leading to NSC death, and (2) help support NSC survival and function via secretion of various neurotrophic factors. To date, there have been many preclinical studies as well as clinical trials demonstrating the efficacy of MSC therapy in stroke preclinical studies and safety of MSC treatment in clinical trials. Nevertheless, the extent of recovery following MSC treatment is not clear. Importantly, the mechanism by which MSC treatment conveys a functional benefit is not known, which impedes the development of an effective therapy. Additionally, most of the clinical trials using MSC have utilized this treatment in subacute and chronic stroke patients. Therefore, whether these cells can be an effective therapy acutely is largely unknown. Additionally, previous graft-versus-host studies
have demonstrated that when MSC are activated by interferon-γ (IFN-γ) ex vivo (aMSCy), their effect is greatly enhanced, and similar effect sizes can be seen using substantially fewer cells. However, the efficacy of aMSCy in brain repair is unknown.

In these studies we examined whether treatment with aMSCy acutely after ischemic stroke could enhance functional recovery and compared its efficacy and mechanism to treatment with naive MSC (nMSC). In addition, we investigated how microglia and NSC responded to hypoxia and ischemia and how treatment with aMSCy could regulate these cell types to promote long-term functional recovery after stroke. Finally, we analyzed the secretome of aMSCy and nMSC in an attempt to determine the factors underlying the extent of functional recovery that these treatments exert.

METHODS

Detailed materials and methods can be found in Data S1. Data sets generated or analyzed for these studies are available from the corresponding author upon reasonable request. Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the MassVE repository with the dataset identifier HYPERLINK "ftp://massive.ucsd.edu/MSV000082984" MSV000082984. Figure 6 and Supplemental Tables 3 and 4 were generated from these data.

Study Design

For all studies, sample sizes were predetermined using published data and our previous studies using G*Power 3.1 (Department of Psychology, Heinrich Heine Universität, Düsseldorf, Germany) assuming a 2-sided α level of 0.05, power of 80%, and homogeneous variances. Before the beginning of experiments, animals were randomly assigned to groups using a random number generator. Investigators responsible for data collection and analyses were blinded to group allocation throughout the experiments. End points were determined in advance as 24 hours, 1 week, or 3 weeks after reperfusion or for humane end points as determined by our institutional animal care and use committee. No data were excluded from these studies, and no outliers are reported.

Animals

All animal experiments were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee and are reported according to the ARRIVE (Animal Research Reporting of In Vivo Experiments) guidelines. For surgical experiments female Sprague-Dawley rats (age 14-16 weeks, weight 225-275 g) were purchased from Envigo (Huntingdon, Cambridgeshire, UK) and were group housed and maintained in standard housing conditions (14/10 hour light/dark cycle) with access to food and water ad libitum. Postoperatively, the animals were singly housed.

Middle Cerebral Artery Occlusion

Focal cerebral ischemia was induced using the middle cerebral artery occlusion model (MCAO) as detailed previously. Briefly, anesthesia was induced and maintained in isoflurane in 100% oxygen for the duration of the procedure, and body temperature was maintained at 37±0.4°C via rectal probe-monitored heating pad. Ventral midline neck dissection was performed, and the right middle cerebral artery was occluded via intraluminal filament for 90 minutes before the filament was removed, and the animal was allowed to recover. The model is described in detail in Data S1. Three hours following the onset of reperfusion after MCAO, the animals were reanesthetized with isoflurane, and MSC were administered intravenously via the retro-orbital sinus. Cells were given at a dose of 5×10⁶ cells/kg.

Behavioral Testing After MCAO

Animals were scored using the modified neurologi- cal severity score obtained at baseline before surgery and on postoperative days 1 to 7, 14, and 21, with higher scores reflecting worse impairment. Animals were also subjected to open-field testing for 15 minutes on postoperative days 1 and 7 using an open-field apparatus (Med Associates, Inc, Fairfax, VT). Center/periphery analysis and rotational analysis were done using predetermined configurations in the corresponding Activity Monitor Software package (Med Associates, Inc).

Magnetic Resonance Imaging

Image Acquisition

Forty-eight hours after surgery, animals were anesthetized using isoflurane, and magnetic resonance imaging was performed using an Agilent 9.4T magnetic resonance system (Agilent Technologies, Santa Clara, CA) with a 600 mT/m gradient insert, a 72-mm inside diameter active decoupling birdcage radiofrequency coil for transmission, and a 4-channel phase-array coil as the receiver (Rapid Biomed, Rimpar, Germany). In each animal T1-, T2-, and diffusion-weighted magnetic resonance images were acquired using spin-echo multislice sequences. For the T1 sequence, imaging settings were as follows: TR/TE=555/13 ms, field of view=40×40 mm, averages=6, matrix=256×256, slice thickness=1.0 mm, gap=0 mm. For the T2 sequence,
imaging settings were as follows: TR/TE=2000/45 ms, field of view=40×40 mm, averages=4, matrix=256×256, slice thickness=1.0 mm, gap=0 mm. For the diffusion-weighted imaging sequence, imaging settings were as follows: TR/TE=2000/45 ms, field of view=40×40 mm, averages=2, matrix=128×128, slice thickness=1.0 mm, gap=0 mm, 2 b-values of 0 and 650 s/mm², diffusion separation Δ=25 ms, diffusion gradient duration δ=5 ms.

**Infarct Volume Calculation**

Infarct volume and percentage infarction were calculated using the diffusion-weighted magnetic resonance imaging (MRI) sequences. With correction for edema, these were calculated as follows:

\[
\text{Infarct volume (mm}^2) = \sum_{n} \text{InV}_n - (\text{IpV}_n - \text{CoV}_n)
\]

\[
\text{Percent infarction (%)} = 100 \times \frac{\sum_{n} \text{InV}_n}{\sum_{n} (\text{IpV}_n + \text{CoV}_n)}
\]

where the individual slice infarct volume is InV, the individual slice ipsilateral hemisphere volume is IpV, individual slice contralateral hemisphere volume is CoV, and n is the slice number.

**MSC Isolation and Culture**

Female rats <4 weeks of age were euthanized via isoflurane overdose and decapitation. Femurs and tibias were dissected out, and bone marrow plugs were flushed out of the bones. The cell suspension was washed, filtered through a 70-μm cell strainer, spun, resuspended in warm growth medium, and plated at 37°C plus 5% CO₂. Medium was changed every 3 days thereafter. MSC were purified via magnetic bead negative selection using anti-CD11b and anti-CD45 antibodies to remove contaminating macrophages, and phenotype was confirmed via flow cytometry. Detailed methodologies are described in Data S1. For in vitro activation with IFN-γ as above.

Additionally, when assessed via modified neurological severity score, animals treated with both nMSC and aMSC showed a more rapid recovery, with animals returning to near baseline functional status by day 21 (Figure 1O). Although vehicle-treated animals still remained significantly more impaired compared with MSC-treated animals, and there were no differences between nMSC- and aMSC-treated animals. Last, MRI imaging was performed (Figure 1P and Video S1) to quantify lesion sizes in these animals. Both nMSC- and aMSC-treated animals had significantly smaller infarct volumes (Figure 1Q) and percentage infarction (Figure 1R) compared with vehicle-treated animals.
Figure 1. Mesenchymal stem cells (MSC) improve functional recovery and infarct volume following middle cerebral artery occlusion (MCAO).

A through D, Representative open field line tracking of sham+vehicle (A), MCAO+vehicle (B), MCAO+nMSC (C), and MCAO+aMSCy (D). E through I, Open-field measures from sham+vehicle (black), MCAO+vehicle (red), MCAO+nMSC (blue), and MCAO+aMSCy (green) animals of total distance traveled (E), ambulatory episodes (F), ambulatory time (G), average velocity (H), and clockwise rotations (I) 24 hours after surgery and treatment. J through N, Open-field measures of total distance traveled (J), ambulatory episodes (K), ambulatory time (L), average velocity (M), and clockwise rotations (N) 7 days after surgery and treatment. Data are mean±SEM; n=3 animals per group. Data were compared using nonparametric Kruskal-Wallis 1-way ANOVA. *Compared with Sham+Vehicle. #Compared with MCAO+Vehicle. *P≤0.05, #P≤0.05, ##P<0.01. O, Modified neurological severity score of sham+vehicle (black), MCAO+vehicle (red), MCAO+nMSC (blue), and MCAO+aMSCy (green) animals. Data were compared using repeated-measures 2-way ANOVA with Holm-Sidak multiple-comparison testing. *Sham+Vehicle vs MCAO+Vehicle; *MCAO+Vehicle vs MCAO+nMSC; ‡MCAO+Vehicle vs MCAO+aMSCy; †Day 1 vs Day 21. **P<0.01, ***P<0.001, ****P<0.0001, †††P<0.0001, ††††P<0.0001, ‡‡‡‡P<0.0001. P, Representative T1-weighted, T2-weighted, and diffusion-weighted imaging (DWI) from MCAO+vehicle (top), MCAO+nMSC (middle), and MCAO+aMSCy (bottom) animals 48 hours after surgery. Q, Infarct volume calculations from MCAO+vehicle (red), MCAO+nMSC (blue), and MCAO+aMSCy (green) animals. R, Percentage infarction calculations from MCAO+vehicle (red), MCAO+nMSC (blue), and MCAO+aMSCy (green) animals. Data are mean±SEM; n=3 animals per group. Data were compared using nonparametric Kruskal-Wallis 1-way ANOVA. *P≤0.05. aMSCy indicates interferon-γ–activated mesenchymal stem cells; and nMSC, naive mesenchymal stem cells.
and sham animals demonstrated no lesion on MRI (data not shown).

**MSC Reduce Microglia Activation and Inflammatory Signaling**

A large portion of damage done following a stroke is a result of inflammatory mediators released by immune cells, especially activated microglia. This response results in an increase in toxic inflammatory mediators including but not limited to interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α). MSC have been demonstrated to preferentially polarize peripheral macrophages to a proregenerative phenotype as well as to reduce secreted levels of IL-12 and TNF-α and increase secretion of the proregenerative IL-10. Furthermore, it has been shown that in a mouse model of wound healing, both nMSC and, to a greater extent, aMSC enhance regeneration and wound healing demonstrated by increased wound tensile strength, with this effect being abrogated by depletion of macrophages. However, how MSC affects microglia following a stroke is not fully elucidated. Microglia are specialized cells originating from a hematopoietic lineage, similar to macrophages, and we hypothesized that these interactions between macrophages and MSC would hold. To investigate this, brain sections from vehicle-treated, nMSC-treated, and aMSC-treated rats were evaluated for cluster of differentiation 68 (CD68) immunoreactivity 1 week after MCAO (Figure 2A through 2C). CD68 is highly expressed in the monocyte lineage. We found that animals treated with both nMSC and aMSC had reduced immunoreactivity for CD68 (Figure 2D) compared with vehicle-treated animals, with sham animals demonstrating no CD68 immunoreactivity (for the latter, data not shown). Furthermore, when assessed at 3 weeks after MCAO, nMSC- and aMSC-treated animals maintained reductions in CD68 immunoreactivity (Figure 2F and 2G) compared with vehicle-treated animals (Figure 2E). Interestingly, at the 3-week time point, brains from vehicle-treated animals had undergone liquefactive necrosis with demonstration of a large cavity lesion (Figure 2E, outlined by dashed white line), which was not present in nMSC- and aMSC-treated animals (Figure 2F and 2G). In addition to quantifying total CD68 immunoreactivity, we also analyzed microglia morphology in the peri-infarct region from vehicle-treated (Figure 2H, H'), nMSC-treated (Figure 2I, I'), and aMSC-treated animals (Figure 2J, J') and found, that in vehicle-treated animals, microglia had morphologies consistent with activated microglia with large cell bodies and few processes, whereas microglia from MSC-treated animals exhibited morphology much more consistent with resting-state microglia with small cell bodies and numerous branching processes.

To further characterize how microglia respond to MSC treatment following hypoxia, primary microglia were primed in oxygen- and glucose-deprivation conditions (OGD) and then cultured in normal medium, conditioned medium from nMSC, or conditioned medium from aMSC. We found that when cultured in both nMSC- and aMSC-conditioned media, microglia have reduced secretion of proinflammatory cytokines IL-6 (Figure 2K) and TNF-α (Figure 2L) 48 hours later. Additionally, although not detectable in cells grown in normal medium, levels of anti-inflammatory IL-4 (Figure 2M) and IL-10 (Figure 2N) were detected at 7 days in cells grown in MSC-conditioned media, with aMSC having the largest effect. These data show that, similar to previous studies with peripheral macrophages, MSC have the ability to reduce microglial activation as well as convert them from a proinflammatory phenotype to a proregenerative phenotype. To validate the effect of hypoxia on cytokine production by microglia, we subjected primary microglial-cultured cells to OGD. Secreted levels of IL-6 and TNF-α (Figure 2O and 2P, respectively) were significantly increased 48 hours after OGD compared with levels at 24 hours. To determine whether alterations in cytokine production following OGD were the result of the increased number of cells, we examined the viability as well as the toxicity levels in culture using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], LDH (lactate dehydrogenase), and terminal deoxynucleotidyl transferase dUTP nick end labeling assays. We observed some reduction in viability based on the MTT assay but no sign of toxicity or apoptosis in the culture (Figures S1 and S2). These results support the notion that MSC reverse the effect of hypoxia on microglial phenotype following stroke.

**aMSCy Induce Oligodendrogenesis Following MCAO**

Another cell population that plays a role following stroke are neural progenitor cells (NPC) in the subventricular zone (SVZ). An estimated 10% of NPC survive after stroke. Hypotheses demonstrating the detrimental effects of inflammation on neurogenesis have been proposed. However, how inflammatory mediators released by immune cells, especially activated microglia, affect NSC and NPC is still largely unknown. To start to address that, we quantified the extent of cell proliferation and lineage markers in both the ipsilateral SVZ and ipsilateral striatum (Figure 3). We found that 24 hours after MCAO, vehicle-treated animals had a reduced number of proliferating cells, whereas aMSC-treated rats had similar number of proliferating cells to sham animals (Figure 3A through
Figure 2. Mesenchymal stem cells (MSC) reduce microglia activation and inflammatory signaling.

A through C, Representative images of CD68 staining of brain sections spaced 300 μm apart through the whole brain from middle cerebral artery occlusion (MCAO)+vehicle (A), MCAO+nMSC (B), and MCAO+aMSCγ (C) animals 1 week after surgery and treatment. D, Quantitation of CD68 staining 1 week after surgery and treatment. Data are means±SEM; n=3 to 4 animals per group. Data were analyzed using nonparametric Kruskal-Wallis 1-way ANOVA. E through G, Representative images of CD68 staining of brain sections spaced 300 μm apart through the whole brain from MCAO+vehicle (E), MCAO+nMSC (F), and MCAO+aMSCγ (G) animals 3 weeks after surgery and treatment. Dashed lines (E) approximate the location of the lateral border of the tissue in a normal brain. H through J, Representative confocal images of whole brain slices of MCAO+vehicle (H), MCAO+nMSC (I), and MCAO+aMSCγ (J) animals 1 week after surgery and treatment stained for Iba1 (green) and CD68 (red). Scale bar represents 1 mm. H’ through J’, Representative confocal images at high magnification from the white boxes in H through J stained for DAPI (blue), Iba1 (green), and CD68 (red). Scale bar represents 5 μm. K through N, Concentration of interleukin 6 (IL-6) (K), tumor necrosis factor α (TNF-α) (L), IL-4 (M), and IL-10 (N) secreted by primary microglia after priming in oxygen and glucose deprivation (OGD) and then growth in normal (black), nMSC-conditioned medium (blue), or aMSCγ conditioned medium (green). IL-6 and TNF-α were assayed at 48 hours, and IL-4 and IL-10 were assayed at 7 days. Data are means±SEM; n=3 independent experiments. Data were analyzed using a nonparametric Kruskal-Wallis 1-way ANOVA. N.D., not detected. *Compared with Normal, *P≤0.05. O and P, Concentration of IL-6 (O) and TNF-α (P) secreted by primary microglia after OGD. Data are means±SEM; n=3 independent experiments. Data were analyzed using a 2-tail, unpaired t test. *P≤0.05. aMSCγ indicates interferon-γ–activated mesenchymal stem cells; and nMSC, naive mesenchymal stem cells.
Further, after MCAO, vehicle-treated animals had no changes in the number of neuroblasts/immature neurons (Figure 3F). No change was observed in the number of proliferating neuroblasts (Figure 3G). This suggests that the increase in the number of proliferating cells observed in the MSC-treated animals does not lead to an increased number of cells differentiating into neurons. Thus, we asked whether proliferating cells differentiate into other cell types. For this purpose we examined the number of cells expressing platelet-derived growth factor receptor α (PDGFRα). These precursors give rise to myelin-producing oligodendrocytes.66 Interestingly, we observed a marked increase in the number of cells in the oligodendrocyte lineage in the SVZ of aMSCγ-treated rats compared with sham, vehicle-, or nMSC-treated MCAO rats (Figure 3H).
through 3R). The significant increase in the number of PDGFRα-positive cells in the SVZ of aMSCy-treated rats suggested that aMSCy induce an oligodendrogenic phenotype (Figure 3R).

These changes, however, were not sustained 1 week after MCAO (Figure S3). Additionally, when examining SVZ cell counts 3 weeks after MCAO, we found significant decreases in the numbers of neuroblasts/immature neurons (Figure S4A and S4B) with no changes in the number of mature oligodendrocytes in the SVZ (Figure S4C). This suggests that the increase in oligodendrocyte progenitors at 24 hours contributes to cells migrating out of the SVZ into the striatum toward the ischemic lesion. To investigate this, we assessed numbers of mature oligodendrocytes and oligodendrocyte progenitor cells in the ipsilateral striatum. We found that vehicle-treated animals had significantly fewer mature oligodendrocytes (Figure 3H, 3I, and 3L) based on the number of cells expressing the myelin-associated enzyme CNPase (2',3'-cyclic-nucleotide 3'-phosphodiesterase), and oligodendrocyte progenitor cells (PDGFRα+ cells, Figure 3M, 3N, and 3Q) compared with those in sham rats. Further, animals treated with either nMSC or aMSCy had significantly more mature oligodendrocytes (Figure 3J through 3L) as well as oligodendrocyte progenitor cells (PDGFRα+ cells; Figure 3O through 3Q) compared with vehicle-treated animals. Last, total protein levels of PDGFRα and myelin basic protein were quantified in tissue lysate of the ipsilateral hemisphere from animals 1 week after MCAO (Figure 3S through 3U). In animals treated with aMSCy there was a significant increase in the total amount of PDGFRα (Figure 3S and 3T) and myelin basic protein (Figure 3S and 3U), suggesting that aMSCy treatment not only induces oligodendrocyte differentiation but also causes these cells to become mature, myelinating oligodendrocytes.

Figure 4. Changes in microglia (MG) and neural stem cells (NSC) after hypoxia.
A, In vitro proliferation of subventricular zone (SVZ)-derived NSC after exposure to oxygen and glucose deprivation (OGD) measured via bromodeoxyuridine (BrdU) uptake and represented as percentage of normoxia. Data are mean±SEM; n=5 independent experiments. Normoxia vs OGD data within a given time were compared using a 2-tail unpaired t test. Data across time were compared using nonparametric Kruskal-Wallis 1-way ANOVA. *Compared with normoxia; †Compared with 24 hours OGD. *P≤0.05, †P≤0.05, ‡P<0.01.
B through D, mRNA fold change levels of glia-derived neurotrophic factor (GDNF) (B), brain-derived neurotrophic factor (BDNF) (C), and insulin-like growth factor (IGF-1) (D) from SVZ-derived NSC after exposure to OGD. Data are mean±SEM; n=3 independent experiments. Data were analyzed using a 2-way ANOVA with Holm-Sidak multiple-comparison testing. **P<0.01, ***P<0.001, ****P<0.0001.
E and F, In vitro proliferation of SVZ-derived NSC at 48 hours (E) and 72 hours (F) after exposure to normal medium (black), conditioned medium from normoxia-primed microglia (blue), or hypoxia-primed microglia (red) measured via BrdU uptake and represented as percentage of normal. Data are mean±SEM; n=3 independent experiments. Data were analyzed using a nonparametric Kruskal-Wallis 1-way ANOVA. *P<0.05, **P<0.01.
Changes in Microglia and NSC After Hypoxia

To further investigate the effect of reduced oxygen on neural progenitor cells, neurosphere cultures were subjected to in vitro OGD. We found that in contrast to the in vivo conditions, OGD induced an increase in NPC proliferation, measured via bromodeoxyuridine uptake, 24 hours after exposure to OGD, but this increase was not sustained beyond 24 hours (Figure 4A). Additionally, mRNA levels of glia-derived neurotrophic factor (Figure 4B), brain-derived neurotrophic factor (Figure 4C), and IGF-1 (insulin-like growth factor 1; Figure 4D) were all significantly increased in neurosphere extracts 24 hours after OGD, but by 48 hours these mRNA levels were significantly decreased to almost nonexistent levels. To determine whether a change in neurotrophic factor production results from a change in cell number or viability of the culture, we counted the number of NPC at different time points after OGD and performed MTT, LDH, and terminal deoxynucleotidyl transferase dUTP nick end labeling. We observed that the number of NPC was not significantly changed 48 hours after OGD, although the viability of the culture was reduced (Figure S5). No sign of toxicity or apoptosis was observed in culture following OGD (Figures S5 and S6). Thus, increased production of neurotrophic factors 24 hours following OGD is not simply due to an increased number of cells in culture. We next asked whether the effect on NPC proliferation in vivo reflects a combined effect of OGD and microglia. Previous studies have implicated TNF-α secreted by activated microglia as an inhibitor of NPC function. To address this, neurospheres were grown in normal medium, conditioned medium from normoxia-primed microglia, and conditioned medium from OGD-primed microglia, and proliferation was measured via bromodeoxyuridine uptake. We found that when grown in medium from OGD-primed microglia, NPC had a small but significantly reduced proliferation compared with NPC grown in normal medium or medium from normoxia-primed microglia at both 48 hours (Figure 4E) and 72 hours (Figure 4F). This agrees with our result that secreted levels of IL-6 and TNF-α were significantly increased 48 hours after OGD compared with levels at 24 hours (Figure 2O and 2P, respectively).

MSC Promote NSC Function and Differentiation

To further investigate the effect of MSC on NPC phenotype, we examined the effect of MSC on trophic factor production level in OGD conditions. As shown earlier, NSC demonstrate trophic factor loss following OGD; however, IFN-γ activation causes significant increases in MSC production of glia-derived neurotrophic factor and IGF-1 (Figure S7, Table S1), suggesting that MSC might aid in sustaining trophic factor levels after OGD. We found that, after OGD-priming, NPC grown in a coculture system with aMSC have significantly increased production of mRNA for glia-derived neurotrophic factor (Figure 5A), brain-derived neurotrophic factor (Figure 5B), and IGF-1 (Figure 5C) after both 24 and 48 hours.

Normally, NSC grow as neurospheres in suspension (Figure 5D). However, after culture in conditioned medium from either nMSC or aMSC, these cells adhere to the plate and put out fine processes (Figure 5E and 5F), similar to their morphology while undergoing differentiation. To determine how MSC-conditioned medium affected the NPC cell cycle, bromodeoxyuridine flow cytometry was performed to determine the percentage of cells in each of the cell cycle stages (Figure 5G through 5L). We found that when grown in nMSC-conditioned medium and, to a larger extent, aMSC-conditioned medium, NPC had significantly fewer cells in the S phase of the cell cycle and significantly more cells in the G0/1 phase of the cell cycle at 24 hours (Figure 5J), 48 hours (Figure 5K), and 72 hours (Figure 5L), suggesting that these cells are no longer proliferating and are exiting the cell cycle—a process that occurs when these cells undergo differentiation.

To determine what types of cells these NPC were becoming, they were grown for 6 days in either normal medium, nMSC-conditioned medium, or aMSC-conditioned medium, at which point lineage-specific markers were analyzed by Western blot (Figure 5M through 5R, Table S2). We found that, when the cells were grown in nMSC-conditioned medium and, to a significant extent, aMSC-conditioned medium, there was reduced expression of the astrocyte marker glial fibrillary acidic protein (Figure 5N), the neuron marker neurofilament L (Figure 5O), and the stem-cell marker Sox2 (Figure 5R), with significantly increased expression of the oligodendrocyte lineage marker PDGFRα (Figure 5O). Together, these data support our in vivo observations and demonstrate that conditioned medium from MSC, with a larger effect from aMSC, causes NPC to differentiate toward an oligodendrocyte lineage.

Identification of the MSC Secretome

The protein secretome from MSC (for MSC identification and characterization see Figures S8 and S9) has been described under various conditions; however, it is unknown what factors secreted by MSC induce NPC oligodendrogenesis and phenotype change in microglia subjected to hypoxic conditions. In addition, the differences in secreted proteins between nMSC and aMSC have never been described before. Based on the data presented in this study, aMSC exert a more potent effect on microglial phenotype and induce...
oligodendrogenesis and myelination. Therefore, in order to determine what potential protein factors secreted by the aMSCγ were responsible for the changes we observed in vitro and in vivo, proteomics analyses of the MSC and aMSCγ secretome were performed via mass spectrometry (Figure 6; for the full list of proteins see Tables S3 and S4). From this approach, 244 proteins were identified, with 16 proteins being unique to nMSC, 27 proteins being unique to aMSC, and the remaining 201 proteins being secreted by both MSC types (Figure 6A). Additionally, secreted proteins belong to many different biologic processes and protein classes with almost a quarter of proteins (21.6%) belonging to the signaling molecule or extracellular matrix classes (Figure 6B). Further, the secretome between both experimental and biological replicates remains consistent with very little variability between replicates both in the number of secreted proteins and the amount of those proteins (Figure 6C). Furthermore, with ex vivo IFN-γ activation, there are 18 proteins that are significantly upregulated (>2-fold increase, P<0.008) and 21 proteins that are significantly downregulated (>2-fold decrease, P<0.008) compared with nMSC (Figure 6D). Notably, aMSCγ but not nMSC induce upregulation of CSPG4 (chondroitin sulfate proteoglycan 4), an important extracellular signal and a hallmark protein of oligodendrocyte precursor cells (OPC).72 In addition, we observed upregulation of BMP1 (bone morphogenetic protein 1), which promotes the generation of mature myelinating oligodendrocytes in vivo.73 MetaCore (Thomson Reuters, Toronto, Ontario, Canada) pathway analysis of these differential proteins resulted in 24 significantly enriched pathways (Tables S3 and S4, false discovery rate, FDR<0.05).
DISCUSSION

This study reports several important observations. We first showed that, when administered 3 hours after ischemic injury, MSC treatment leads to a significant functional recovery of treated animals as a direct result of preservation of brain parenchyma. In that regard, for various reasons, including delay to...
treatment and lack of recognition of stroke symptoms, no stroke therapy can be developed to completely regenerate infarcted tissue or to prevent the formation of an infarct core. Consequently, therapies aimed at limiting the size of the infarct and infarct penumbra are most clinically relevant. Related to this, preservation of the support structure within the penumbra is crucial for long-term functional recovery after stroke. Thus, the observation that MSC treatment as administered in this study can minimize the infarct and penumbra is highly significant. The feasibility of this treatment is similar to that of tissue plasminogen activator, the only treatment currently available for acute ischemic stroke within 3 hours of onset. Because MSC do not induce a host immune response, they could be banked as a blood-bank product and should be available for treatment on thawing.

Second, we demonstrated that MSC reverse the proinflammatory phenotype of microglia induced by hypoxia and ischemia. Additionally, treatment of stroke animals by both nMSC and aMSCγ induces a significant reduction in CD68+ monocytes and microglia, which may contribute to the preservation of brain tissue and the reduction in infarct size. This observation suggests that MSC are effective modifiers of microglial or monocyte phenotype, and their effect may be applicable to other brain insults characterized by increased inflammation. It should be noted that lba-1 and CD68 recognize both microglia and infiltrating monocytes, which can present a different phenotype. Thus, we cannot exclude the possibility that the detected effect reflects both cell types.

Third, we showed that aMSCγ are more potent than nMSC. Although both nMSC and aMSCγ lead to equivalent levels of functional recovery, aMSCγ exert 2 main molecular and cellular advantages that we believe make them a better therapeutic option. We showed (1) that, compared with nMSC, aMSCγ cause significant increases in anti-inflammatory cytokine secretion by microglia with corresponding decreases in proinflammatory cytokines, and (2) that treatment with aMSCγ in vivo more effectively induces oligodendrocyte differentiation and myelination. Thus, we propose that aMSCγ would be a significantly more effective therapeutic agent than nMSC.

Fourth, in contrast to previous reports, we observed that ischemia suppresses the proliferation of neural progenitor cells in the SVZ and that neurogenesis is not induced either by ischemia or by MSC treatment. Therefore, future therapeutic avenues should test the regenerative efficacy of aMSCγ in combination with proneurogenic approaches.

Fifth, we showed that treatment with aMSCγ after stroke induced a significant increase in the number of oligodendrocyte progenitor cells (OPC) in the SVZ, ultimately leading to their differentiation and increased myelin production. We also demonstrated a significant increase in the number of OPC and mature oligodendrocytes in the striatum of stroke animals treated with aMSCγ. To start to address the mechanism underlying aMSCγ-induced oligodendrogenesis, we compared the secretome of aMSCγ and nMSC. We unraveled a set of proteins that may be involved in the differential effect exerted by aMSCγ, which may underlie the robust pro-oligodendrogenic response seen with aMSCγ treatment. For example, we observed upregulation of BMP1, which promotes the generation of mature myelinating oligodendrocytes in vivo. Further, we observed upregulation of chondroitin sulfate proteoglycan 4 (CSPG4, also known as neuron-glia antigen 2), an important extracellular signal and a hallmark protein of the OPC. Mutations in CSPG4 cause OPC dysfunction and are implicated in familial schizophrenia. Interestingly, CSPG4 is highly expressed by brain pericytes, an important component of the neurovascular unit. Thus, it is possible that aMSCγ affect the composition of the blood-brain barrier. This may have implications for the integrity of the blood-brain barrier and for brain function. In support of this notion, the administration of MSC-derived extracellular vesicles following spinal cord injury enhanced the integrity of the blood–spinal cord barrier by affecting pericyte signaling. Another important extracellular component upregulated in aMSCγ is collagen α-2(VI), 1 of the 3 α-chains of type VI collagen, a protein known to have a protective effect in the central nervous system under stress conditions, including enhancement of neural cell viability and reduction of oxidative stress. Importantly, an earlier study suggested that collagen VI regulates proper myelination in the peripheral nervous system. Interestingly, we observed that Svec1 (sushi, von Willebrand factor type A, EGF, and pentraxin domain-containing 1) and SRPX2 (sushi repeat containing protein X-linked 2) are differentially upregulated in the aMSCγ. SRPX2 is implicated in angiogenesis and is a ligand of the urokinase plasminogen activator surface receptor. In addition, it was shown to promote synapse formation. On the other hand, we observed downregulation of the immunomodulator SEMA7A (Semaphorin 7A) involved in inflammatory responses. SEMA7A is also known as the John-Milton-Hagen blood group antigen, an 80-kDa glycoprotein expressed on activated lymphocytes and erythrocytes. Interestingly, SEMA5A is an inhibitor of axonal growth expressed by oligodendrocytes and their precursor cells in the developing optic nerve and is thought to contribute to the inhibition of CNS axon growth by oligodendrocyte lineage cells after injury. In addition, we observed downregulation of...
olfm13- expressed on microglia. Of note, Q6AYQ9 peptidyl-prolyl cis-trans isomerase is expressed in MSC, but its role and the significance of its levels are yet to be understood.

One limitation of this study is the small sample size. A prerequisite for the successful translation of this approach would be the examination of this treatment in a large cohort. In addition, further studies are warranted in order to determine whether post-stroke treatment with aMSC promotes neuronal replacement. In that regard the efficacy of a combined therapy of aMSC with other stem cell–based promising treatments that promote neuronal production is an avenue worth exploring. For example, vascu-loprotective drugs, such as activated protein C, a serine protease known to promote cytoprotective signaling in the ischemic brain endothelium at the blood-brain barrier, have been shown to stimulate neuronal production by transplanted human NSC, promote circuit restoration, and improve functional recovery.

In summary, our study reveals important information that advances our understanding of the efficacy and therapeutic potential of MSC for the treatment of ischemic stroke. It provides a potent alternative in the form of aMSC and unravels its mechanism of action, which may facilitate drug development.

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Affiliations
From the Departments of Anatomy and Cell Biology (M.K.T., T.K.L.S., K.L.L., O.L.), Chemistry (M.R.P., S.M.C.), and Surgery (A.M.B.), University of Illinois at Chicago, IL.

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Disclosures
None.

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DATA S1.

SUPPLEMENTAL METHODS

Chemicals and Reagents

Chemicals and reagents used are listed below with vendors listed in parenthesis. Primers used for qRT-PCR are listed in Table S1 and antibodies are listed in Table S2.

Animals

All animal experiments were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee. For surgical experiments, female Sprague-Dawley rats (age 14 to 16 weeks, weight 225-275 grams) were purchased from Envigo (Huntingdon, Cambridgeshire, UK) and were group housed and maintained in standard housing conditions (14/10 hour light/dark cycle) with access to food and water ad libitum. Post-operatively, animals were singly housed. For neonatal mixed glial cell isolations, breeding pairs of Sprague-Dawley rats (age 8 weeks) were purchased from Charles River Laboratories and were maintained as above. Female breeders were used for up to 3 litters before being replaced as breeders. Animals used for mesenchymal stem cell and subventricular neural stem cell isolation were obtained from an in-house breeding pair.

Middle cerebral artery occlusion

Anesthesia was induced and maintained in isoflurane in 100% oxygen for the duration of the procedure and body temperature was maintained at 37 ± 0.4°C via rectal probe-monitored heating pad. The ventral neck was shaved and remaining hair was removed with depilatory cream. The skin was prepped with 3 alternating wipes of isopropanol wipes and povidone-iodine swabs and an approximately 3.5 cm incision was made. Glandular tissue and fascia was dissected using
a combination of blunt and sharp dissection until the underlying muscles on the right side were visualized. The right omohyoid muscle was resected to expose the common carotid artery (CCA) and the carotid bifurcation. A 0.5 cm section of the proximal common carotid artery was dissected free from surrounding adventitia taking care to not disturb the adjacent vagus nerve. Following CCA dissection, the carotid bifurcation, external carotid artery (ECA), superior thyroid artery, occipital artery, and the internal carotid artery (ICA) were dissected free from surrounding adventitia. The superior thyroid artery and occipital artery were both coagulated and divided to allow for free movement of the ECA. The ECA was then dissected as distally as possible, coagulated, and divided and a loosely tied 5-0 silk suture was tied around the proximal ECA stump. The remaining ICA was dissected distally to expose the bifurcation of the ICA and the pterygopalatine artery. Temporary vessel clips were placed first on the previously dissected 0.5 cm segment of the CCA and then on the ICA as distally as possible. A small arteriotomy was made in the ECA stump and a silicone-coated 4-0 nylon filament (Doccol #404156PK10Re) was inserted through the arteriotomy into the ICA. The 5-0 silk tie was tightened to secure the filament, the ICA vessel clip was removed and the filament was inserted into the ICA until resistance was felt. At this time, the animal received a single BrdU injection (100 mg/kg IP) and the filament was left in place to occlude the middle cerebral artery for 90 minutes. After 90 minutes, the filament was withdrawn, a temporary vessel clip was placed on the ICA, the ECA stump was coagulated to close the arteriotomy, the two vessel clips were removed and the skin was closed. The animal was then placed into a recovery chamber maintained at 30°C and received 2.0 mL of warm saline intraperitoneally. Following a 3-hour recovery period, the animal was re-anesthetized and was administered either vehicle (1X PBS) treatment, 5.0 X 10^6 nMSC/kg, or 5.0 X 10^6 aMSCγ/kg intravenously.
**Mesenchymal stem cell isolation and culture**

Female rats less than 4 weeks of age were euthanized via isoflurane overdose and decapitation. Femurs and tibias were dissected out into ice cold dissection media (50:50 MEMα/F-12, 10% FBS) and washed three times in cold dissection media. The ends of the bones were cut off and bone marrow plugs were flushed out with 10 mL of cold dissection media. Bone marrow plugs were pipetted 10X with a 10 mL serological pipet and were passed through a 70 µm cell strainer. Using the plunger from a 10 mL syringe, bone marrow plugs were pushed through the cell strainer which was then wash twice with 10 mL ice cold dissection media. The cell suspension was spun at 300g for 10 minutes and the pellets were resuspended in warm growth media (50:50 MEMα/F-12, 10% FBS, 1% antibiotic-antimycotic) and plated at 37°C plus 5% CO₂. After 24 hours, and then every 3 days thereafter, the media was replaced until the cells reached confluency. At that point, cells were trypsinized and underwent magnetic bead purification. Trypsinized cells were counted and 200,000 cells were set aside to use as a “pre-purification” sample. The remaining cells were spun at 300g for 5 minutes and were resuspended in 50µL flow buffer (1X DPBS without calcium and magnesium, 2% BSA, 25 mM HEPES, 5 mM EDTA) per 10⁶ cells containing 10 µg/mL each of biotin anti-rat CD45 and biotin anti-rat CD11b/c (Table S2) and were incubated for 30 minutes at 4°C. Cells were washed with 10X volume of flow buffer and spun at 500g for 10 minutes. The cell pellet was resuspended in 80 µL flow buffer plus 10 µL anti-biotin microbeads (Miltenyi Biotec #130-090-485) per 10⁷ cells and incubated for 15 minutes at 4°C. Cells were washed with 10X volume of flow buffer and spun at 500g for 10 minutes. While spinning, an LS column (Miltenyi Biotec #120-000-475) was washed with 3 mL of flow buffer. The cell pellet was resuspended in 3 mL flow buffer and added to the LS column attached to a
QuadroMACS separator (Miltenyi Biotec #130-090-976) and allowed to flow through into a 15 mL conical tube. The column was washed three times with 3 mL flow buffer and collected into the same conical tube, which contains purified mesenchymal stem cells. 5 mL of flow buffer was added to the LS column and the supplied plunger was used to plunge the bound cells (macrophages) into a new 15 mL conical tube. The purified MSCs were counted and $10^5$ cells/per tube were taken for phenotype analysis by flow cytometry and the remainder of the cells were put back into culture for continued use. Pre-purification cells, bead-bound macrophages, and purified MSCs were stained and analyzed by flow cytometry to determine the purity of the MSC population. Cells were stained for CD11b, MHC-II (RT1D), CD29, CD45, CD90 or isotype controls (Figure S8). Cell events were analyzed on a Fortessa cell analyzer (Becton Dickinson, Franklin Lakes, NJ, USA; gating strategy demonstrated in Figure S9) and analyzed using FlowJo Software (FlowJo, LLC, Ashland, OR, USA).

**Mixed glial cell culture and microglia isolation**

Rat pups (P0-5, mixed gender) were decapitated and the brains were dissected into ice cold HBSS. Meninges were removed and neocortex was dissected out into ice cold HBSS. Tissue was then combined and incubated in 0.25% trypsin plus 50 µg DNase I for 7 minutes at room temperature and then washed with DMEM plus 10% FBS. Tissue was then washed twice with HBSS and then passed 5 times each through decreasing size serological pipet (10 mL, 5 mL, glass Pasteur pipet) to dissociate the tissue. Tissue suspension was filtered through a 70 µm cell strainer, spun at 300g for 10 minutes and plated in DMEM plus 10% FBS plus 1% antibiotic-antimycotic (3 brains per T-75 cm² flask) and cultured at 37°C plus 5% CO₂. Media is changed the next day and every 3 days afterwards until confluent. Once confluent, microglia are isolated from mixed
glial population by sealing flasks and shaking at 280 rpm for 30 minutes at 37°C. Microglia-containing media is collected and utilized for downstream applications.

**Subventricular zone neural stem cell isolation**

4-6-week-old female rats were euthanized via isoflurane overdose and decapitation and the brains were dissected into ice cold HBSS. Brains were cut at the level of the optic chiasm and the subventricular zone was dissected out. Tissue was minced using a scalpel and was transferred to 3 mL of warm media (DMEM/F-12, 20% B27 supplement, 10% N2 supplement, 1% penicillin-streptomycin, 1 mM glutamine, 20 mM KCl, 2 µg/mL heparin) and allowed to settle. Media was aspirated and tissue was resuspended in 1.25 mL 0.1% trypsin-EDTA plus 50 µg DNase I and incubated with agitation for 7 minutes at 37°C. Following incubation, 3 mL of trypsin inhibitor (Sigma-Aldrich #T6522; 139 µg/mL plus 10 µg/mL DNase I in HBSS) and spun at 300g for 5 minutes. Tissue pellet is resuspended in 1 mL of warm media and pipetted 25X with a P1000 pipet set to 800 µL. Media is added to 5 mL and tissue suspension is filtered through a 70 µm cell strainer and spun at 300g for 5 minutes. Cell pellet is resuspended in warm media plus 20 ng/mL EGF + 10 ng/mL bFGF. Growth factors are added every 2 days and media is changed once a week. Neurospheres were passaged using Accumax (Innovative Cell Technologies #AM105) by incubating cells in 1 mL of Accumax for 10 minutes at room temperature on an orbital rotator. Cells were then pipetted 25X with a P1000 pipet set to 900 µL and added to 9 mL of growth media. Cells were then passed through a 70 µm cell strainer, spun at 300g for 5 minutes, and plated for either culture maintenance or for individual experiments.

**Oxygen-glucose deprivation**
For oxygen-glucose deprivation, cells were dissociated into single cell suspensions, resuspended in 1X HBSS + 1% antibiotic, and plated at appropriate densities. Cells were cultured in 1% oxygen for 4 hours and then transferred back to normal growth conditions.

**Oxygen-glucose deprivation effects on cell viability**

*NSC Cell Counts*

NSC were singly dissociated, plated at a concentration of 1000 cells/μL and subjected to OGD conditions as described in earlier. After each time point, cells were singly using Accumax (Innovative Cell Technologies #AM105) and counted via hemocytometer.

*MTT Assay*

For NSCs, cells were plated at a concentration of 1000 cells/μL and subjected to OGD conditions as described in earlier. After OGD conditions, 100 μL of cells were transferred to a 96-well plate. At each time point post OGD, 10 μL of a 12 mM MTT in PBS solution was added to each well and cells were incubated at 37°C for 48 hours. After incubation, all media was removed, 50 μL of DMSO was added and incubated at 37°C for 10 mins. Absorbance was read at 595 nm.

For Microglia, 96 well plates were coated with 10 μg/mL Poly-L-Lysine in sterile tissue culture water for 5 mins and then rinsed 3X with 1X PBS prior to seeding cells. Microglia were plated at concentration 1000 cells/μL and let adhere to wells overnight. Microglia were subjected to OGD conditions as described in earlier and after, fresh media was added to wells. At each time point post OGD, 10 μL of a 12 mM MTT in PBS solution was added to each well and cells
were incubated at 37°C for 24 hours. After incubation, all media was removed, 50 μL of DMSO was added and incubated at 37°C for 10 mins. Absorbance was read at 595 nm.

**LDH Assay**

For NSCs, cells were plated at a concentration of 1000 cells/μL and subjected to OGD conditions as described in earlier. For LDH media experiments, 50 μL of media was taken at each time point and analyzed as described in the Pierce™ LDH Cytotoxicity Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). For LDH max experiments, 100 μL of media containing cells was collected at each time point and lysed with 10X lysis buffer provided by kit. 50 μL of lysate was then analyzed as described by the kit. Absorbance was read at 450 nm and 620 nm and absorbance were corrected by subtracting 620 nm values from 450 nm.

For Microglia, 96 well plates were coated with 10 μg/mL Poly-L-Lysine in sterile tissue culture water for 5 mins and then rinsed 3X with 1X PBS prior to seeding cells. Microglia were plated at concentration 1000 cells/μL and let adhere to wells overnight. Cells were subjected to OGD conditions as described in earlier. At each time point, 50 μL of media was collected for LDH media experiments and remaining cells were and lysed with 10X lysis buffer provided by kit. Samples were then analyzed according to kit instruction.

**TUNEL Assay**

For NSCs, 1x10^6 cells were subjected to OGD, collected at each time point and stained with slight modification of NeuroTACS™ II In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA). Streptavidin-488 at a 1:1000 concentration in 1X PBS was used instead of Streptavidin-HRP and DAB solution. Slides were incubated in Streptavirdin-488 for
20 minutes at RT, washed 3X in 1X PBS for 10 minutes, stained with DAPI at 1:10,000 in 1X PBS and mounted in PVA-DABCO.

For Microglia, 8-well chamber slides (MilliporeSigma, Burlington, MA, USA) were coated with 10 μL/mL Matrigel Matrix (Corning, New York, NY, USA) in DMEM for 1 hour at RT followed by 1X PBS wash prior to use. Microglia were plated at 166 cells/μL and let adhere to wells overnight. Cells were subjected to OGD and stained as described for the NSC TUNEL experiments.

Quantitative reverse transcription polymerase chain reaction

RNA was extracted from cultured cells using the ISOLATE II RNA Mini Kit (Bioline USA, Inc, Taunton, MA, USA) according to manufacturer’s instructions and was quantified using a Nanodrop and stored at -80°C until used. cDNA synthesis was performed using the SuperScript™ III First-Strand Synthesis SuperMix (ThermoFisher Scientific) according to manufacturer’s instructions starting with 1 μg total RNA per sample and using oligo(dT) priming and stored at -20°C until used. qPCR was performed using SensiFAST™ SYBR® & Fluorescein Kit (Bioline USA, Inc, Taunton, MA, USA). Each reaction was done using 1 μL of starting cDNA. Relative expression was calculated using the ΔΔCt method using RPLP0 as the housekeeping gene. Primer sets are listed in Table S1.

Western blot analysis

Cells were collected and spun at 300g for 5 minutes. The cell pellet was resuspended in RIPA buffer containing protease inhibitor cocktail (1:100, Sigma-Aldrich #P8340) and was sonicated once for 15 seconds at 40% amplitude and then spun at 14,000g for 10 minutes.
Supernatants were collected, quantified via the BCA method, and diluted to 1 µg/µL with lysis buffer and 2X Laemmli sample buffer and boiled for 10 minutes at 95°C with leftover protein being stored at -80°C. 15 µg of each sample was loaded into a 10% Mini-PROTEAN TGX precast gel, run in 1X Tris/Glycine/SDS buffer for 30 minutes at 200V at room temperature and then transferred to nitrocellulose membrane at 100V for 1 hour at 4°C. The membrane was stained with Ponceau S for 5 minutes to confirm protein transfer, destained with 0.1N NaOH and was then washed three times in PBST to remove any trace stain or NaOH. The membrane was then blocked in 5% nonfat milk in 1X PBS plus 0.01% Tween 20 (PBST) for 1 hour at room temperature and incubated for 1 hour at room temperature in primary antibody (Table S2) diluted in PBST. The membrane was then washed three times in PBST and then incubated for 1 hour at room temperature in appropriate secondary antibody (Table S2) diluted in 5% BSA in PBST plus 0.1% NaN₃. The membrane was then washed three times in PBST followed by once in PBS and imaged using an Odyssey CLx (700 nm or 800 nm channel set to Auto) and protein expression levels were quantified using Image Studio Lite (version 5.2.5; Li-Cor). All protein levels were normalized to levels of actin.

For Western blot analysis of tissue extract, rat brain hemispheres were lysed using the Bio-Plex Cell Lysis Kit (Bio-Rad, Inc) according to manufacturer’s instructions, quantified using the BCA method, and diluted to 3 µg/µL with lysis buffer and 2X Laemmli sample buffer and boiled for 10 minutes at 95°C with leftover protein being stored at -80°C. SDS-PAGE and Western blot was performed as above using 30 µg of total protein per sample.

**BrdU Flow Cytometry**
Cell cycle analysis was done utilizing a FITC BrdU Flow Kit (BD Biosciences) according to manufacturer’s instructions. Briefly, neural stem cells were singly dissociated and plated in either normal growth media, nMSC conditioned media, or aMSCγ conditioned media and allowed to grow for 24, 48, or 72 hours. 4 hours prior to fixation, BrdU (10 µM final concentration) was added to each well to label dividing cells. At appropriate time points, cells were fixed and permeabilized, and treated with DNase I to expose the incorporated BrdU. Cells were then incubated with a FITC-conjugated anti-BrdU antibody and labeled with 7-AAD. Cell events were analyzed on a Fortessa cell analyzer (Becton Dickinson, Franklin Lakes, NJ, USA; gating strategy demonstrated in Figure S9) and analyzed using FlowJo Software (FlowJo, LLC, Ashland, OR, USA).

Magnetic resonance imaging

Image Acquisition

48 hours after surgery, animals were anesthetized using isoflurane and magnetic resonance imaging was performed using an Agilent 9.4T MR system (Agilent Technologies, Santa Clara, CA), with a 600 mT/m gradient insert, a 72 mm ID active decoupling birdcage RF coil for transmission, and a 4-channel phase array coil as the receiver (Rapid Biomed, Ripmar, Germany). In each animal, T1-, T2-, and diffusion-weighted MR images were acquired, using spin echo multi slice (SEMS) sequences. For the T1 sequence, imaging settings were as follows: TR/TE = 555/13 ms, FOV = 40X40 mm, Averages = 6, matrix = 256X256, slice thickness = 1.0 mm, gap = 0 mm. For the T2 sequence, imaging settings were as follows: TR/TE = 2000/45 ms, FOV = 40X40 mm, Averages = 4, matrix = 256X256, slice thickness = 1.0 mm, gap = 0 mm. For the diffusion weighted imaging (DWI) sequence, imaging settings were as follows: TR/TE = 2000/45 ms, FOV = 40X40
Infarct Volume calculation

Infarct volume and percent infarction were calculated using the DWI MRI sequences. Correcting for edema, these were calculated as follows:

\[
\text{Infarct Volume (mm}^3\text{)} = \sum_{1}^{n} \text{InV}_n - (\text{IpV}_n - \text{CoV}_n)
\]

\[
\text{Percent Infarction (\%)} = 100 \times \frac{\sum_{1}^{n} \text{InV}_n}{\sum_{1}^{n}(\text{IpV}_n + \text{CoV}_n)}
\]

Where the individual slice infarct volume is InV, the individual slice ipsilateral hemisphere volume is IpV, individual slice contralateral hemisphere volume is CoV, and n is the slice number.

Functional testing after MCAO

Modified Neurological Severity Score

Animals were scored using the modified neurological severity score (mNSS) as previously described\(^60\). Scores were obtained at baseline before surgery and on postoperative days 1-7, 14, and 21. The mNSS is a cumulative scoring system that combines motor, sensory, balance, and reflex deficits. One point is given for an inability to perform a motor or sensory task and one point is given for the absence of one of the reflexes. The section scores are summed for a maximum score of 18, with higher scores indicating a more impaired animal.

Open Field Testing
Animals were tested in an open field apparatus (Med Associates, Inc) for 15 minutes 24 hours and 7 days after surgery. Center/periphery analysis and rotational analysis were done using predetermined configurations in the corresponding Activity Monitor Software package (Med Associates, Inc).

**Brain processing**

At appropriate time points, rats were euthanized via isoflurane overdose followed by transcardial perfusion with ice cold heparinized saline (10 U/mL) for 12 minutes and with ice cold 4% paraformaldehyde for 15 minutes. Brains were then removed and post-fixed in PFA for 16 hours at 4°C, washed once in 1X PBS, and transferred to 30% sucrose until they sunk. Brains were then sectioned at 50 µm and stored in cryoprotectant (47.6% 1X PBS, 28.6% ethylene glycol, 23.8% glycerol) at -20°C.

**Immunofluorescence staining**

Tissue sections were washed three times at room temperature in 1X TBS for 5 minutes each. Tissue was then blocked in blocking buffer (1X TBS, 0.3M glycine, 5% normal donkey serum, 0.25% Triton X-100) for 2 hours at room temperature and then incubated in primary antibody (Table S2), diluted in blocking buffer, for 72 hours at 4°C. Tissue was then washed three times at room temperature in 1X TBS for 5 minutes each and incubated in secondary antibody (Table S2), diluted in blocking buffer, for 2 hours at room temperature. Tissue was then washed three times at room temperature in 1X TBS for 5 minutes each and incubated in DAPI (0.5 µg/mL; ThermoFisher Scientific #D1306) or TO-PRO-3 Iodide (1 µM; ThermoFisher Scientific #T3605) for 5 minutes at room temperature and then mounted and stored at 4°C. For sections that were
stained for BrdU, tissue was washed three times at room temperature in 1X TBS for 5 minutes each then incubated in 2N HCl at 37°C for 30 minutes. Afterwards, tissue was incubated in 0.1M borate buffer for 10 minutes at room temperature, washed six times at room temperature in 1X TBS for 5 minutes each, and stained as above.

**Stereological quantification**

Cell counts were performed using design-based stereology (StereoInvestigator, MBF Biosciences). For the analysis, every sixth section of brain tissue was quantified using the optical fractionator workflow of StereoInvestigator. Regions of interest were traced under 5X magnification with counting performed under 63X magnification with a counting frame of 225 µm x 145 µm and sampling grid of 100 µm x 100 µm with 12.5 µm top and bottom guard zones.

**Quantification of CD68 staining**

To quantify level of microglia activity, tissue sections were stained for CD68 and were imaged using a Zeiss AxioImager with a 10X objective and were tiled in AxioVision (Carl Zeiss). Intensity analysis was performed in ImageJ. On each section, the intensity of the region of interest was measured as well as three background regions. CD68 staining was background corrected and normalized to measured area according to the following formula:

\[
CD68\ \text{Intensity} = \frac{\sum_{i=1}^{n}(Int_{ROI})_{n} - (\bar{Int}_{Back})_{n}}{\sum_{i=1}^{n}\text{Area}_{n}}
\]

Where \(Int_{ROI}\) is the intensity of the defined region of interest, \(\bar{Int}_{Back}\) is the average of the background intensity, Area is the measured area of the region of interest, and \(n\) is the section number.
Quantification of striatal CNPase and PDGFRα

For each animal, 3 to 4 randomly selected fields (280 x 280 µm) from the striatum in 4 sections with 300 µm separating each section were acquired using a Zeiss LSM 880 confocal microscope with Airyscan detector. Forty-eight micron maximum projection z-stacks were reconstructed and the number of CNPase-positive and PDGFRα-positive cells per mm² were determined using the ImageJ Cell Counter plugin analysis tool.

In vitro cytokine analysis

100,000 primary rat microglia were plated in triplicate per well of a 96 well plate and grown in oxygen-glucose deprivation or normal growth conditions for 4 hours. After 4 hours, media was replaced with the normal growth media, nMSC conditioned media, or aMSCγ conditioned media. Supernatants were collected 24 hours, 48 hours, 4 days, or 7 days later and stored at -80°C until analyzed. Cytokines levels were determined using the Rat Proinflammatory Panel 2 V-PLEX Assay (Meso Scale Discovery) according to manufacturer’s instructions.

Mass spectrometry

Sample preparation, TMT labeling, and fractionation

15 mL of conditioned media was collected as above and concentrated using an Amicon Ultra-15 centrifugal filter unit with a 3 kDa molecular weight cutoff (Millipore, Burlington, MA, USA). Concentrated protein was quantified using a BCA protein assay kit (ThermoFisher Scientific, Waltham, MA, USA) and 100 µg of total protein from each sample was processed by filter aided sample preparation. Proteins were diluted in 8 M urea, reduced in 5 mM DTT in 8 M urea for 20 minutes at 55°C and then alkylated in 10 mM iodoacetamide for 20 minutes at room
temperature in the dark. The samples were then urea exchanged with 50 mM triethylammonium bicarbonate (TEAB) and trypsin (2 µg per sample) was added and samples were incubated overnight at 37°C. Peptides were eluted with 80 µL of 50 mM TEAB and then digested peptides were labeled using 2 tandem mass tag (TMT) kits (ThermoFisher Scientific) according to manufacturer’s instructions where samples were labeled using 126-131 tags (one replicate per mass tag). Subsequently, the individual samples where combined, lyophilized, and resuspended in 10 mM ammonium hydroxide and fractionated (20 fractions) via high pH reverse phase chromatography. Each fraction was then lyophilized and resuspended in 20 µL 0.1% formic acid prior to nano-LC-MS/MS

**Nano-LC-MS/MS analysis and data analysis**

Mass detection occurred with an Orbitrap Velos Pro (ThermoFisher Scientific) equipped with an Agilent HPLC system. Chromatographic separation of peptides was accomplished using a Zorbax 300SB-C18 column (3.5 µm ID x 150 mm, particle size 5 µm, pore size 100 Å, Agilent Technologies). The peptides were loaded onto a Zorbax 300SB-C18 trap cartridge at a flow rate of 3 µL/minute for 10 minutes. After washing with 0.1% formic acid, the peptides were eluted using a 5-40% B gradient for 60 minutes at a flow rate of 250 nL/minute acid (mobile phase A = 0.1% formic acid; mobile phase B = 0.1% formic acid in acetonitrile). The flow-through was analyzed using DDA settings to select the top 10 most abundant ions for HCD fragmentation. Proteins were identified by searching MS/MS spectra against the Human SwissProt database using the Mascot search engine employing a reversed decoy database to calculate the false discovery rate (FDR). Trypsin was set as the protease with two missed cleavages and searches were performed with precursor and fragment mass error tolerances were set at 15 ppm and 0.4 Da,
respectively. Peptide precursors of +2, +3, and +4 were considered. Peptide variable modifications allowed during the search were: oxidation (M) and deamination (NQ), whereas carbamidomethyl (C) and TMT6plex (K and N-term) were set as fixed modifications. To calculate the FDR, the search was performed using the “decoy” option in Mascot. Scaffold Q+ was used to quantitate TMT-labeled peptides from the samples. Peptide identifications were accepted if they could be established at a greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at a greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Normalization was performed iteratively (across samples and spectra) on intensities. Medians were used for averaging. Spectra data were log-transformed, pruned of those matched to multiple proteins, and weighted by an adaptive intensity weighting algorithm. Pathway analyses were conducted using the PANTHER database (www.pantherdb.org) and the DAVID Gene Functional Classification Tool (https://david.ncifcrf.gov). Differentially expressed proteins for MetaCore (Thomson Reuters) pathway enrichment were determined by applying Permutation Test with unadjusted significance level p < 0.05 corrected by Benjamini-Hochberg (new p < 0.008) and a minimum fold change of ±1 (Table S3,S4).

**Data availability**

Datasets generated or analyzed for these studies are available from the corresponding author upon reasonable request. Mass spectrometry proteomics data have been deposited to the
ProteomeXchange Consortium via the MassIVE repository with the dataset identifier MSV000082984. Figure 6 and Tables S3 and S4 were generated from these data.
Table S1. Primer sequences for qRT-PCR.

| Gene   | Forward                  | Reverse                  | RefSeq No.     |
|--------|--------------------------|--------------------------|----------------|
| GDNF   | CGAAGAGAGAGGAACCG        | TAGCCCAAACCCAAGTC        | NM_019139.1    |
| IGF-1  | TACTTCAACAAGCCCACAG     | TACATCTCCAGCCTCCTC      | NM_001082478.1 |
| BDNF   | ACCCTGAGTTCCACCAG        | AAGTTGCTTTGTCGTG         | NM_001270630.1 |
| NTF3   | TAATGATGAGTGCTGCAG       | CCGTATGACTATCCAGGG       | NM_031073.3    |
| NGF    | CGCATCGCTCTCCTTCATA      | GCCCAGACACTGAGGTG        | NM_001277055.1 |
| RPLP0  | TTCTCCTTCGGGCTGAT        | ATTCGGGACACCCTCTA        | NM_022402.2    |

All primer sets used in this dissertation were designed against rat genes. GNDF – glial derived neurotrophic factor; IGF-1 – insulin-like growth factor 1; BDNF – brain derived neurotrophic factor; NTF3 – neurotrophin 3; NGF – nerve growth factor; RPLP0 – ribosomal protein lateral stalk subunit P0.
Table S2. Antibodies.
| Target      | Conjugate | Supplier                  | Catalog No. | Application(s) | Concentration |
|-------------|-----------|---------------------------|-------------|----------------|---------------|
| Sox2        | -         | Santa Cruz                | sc-17320    | WB             | 0.4 µg/mL     |
| GFAP        | -         | Dako                      | Z0334       | WB             | 5.8 µg/mL     |
| PDGFRα      | -         | Santa Cruz                | sc-338      | IHC            | 0.4 µg/mL     |
|             |           |                           |             | WB             | 0.2 µg/mL     |
| Actin       | -         | ThermoFisher Scientific   | MA5-11869   | WB             | 0.04 µg/mL    |
| B-III-tubulin| -         | ThermoFisher Scientific   | 32-2600     | WB             | 0.1 µg/mL     |
| BrdU        | -         | Abcam                     | ab6326      | IHC            | 2 µg/mL       |
| DCX         | -         | Santa Cruz                | sc-8066     | IHC            | 0.2 µg/mL     |
|             |           |                           |             | WB             | 0.2 µg/mL     |
| Cyclin D1   | -         | Abcam                     | ab74646     | WB             | 5 µg/mL       |
| Iba1        | -         | Wako                      | 019-19741   | IHC            | 0.5 µg/mL     |
| CD68        | -         | Bio-Rad                   | MCA341R     | IHC            | 2 µg/mL       |
| CNPase      | -         | Gift from Dr. Ernesto     |             | IHC            | 1:10 dilution |
|             |           | Bongarzone                |             |                |               |
| MBP         | -         | Santa Cruz                | sc-376995   | WB             | 0.4 µg/mL     |
| CD11b       | Biotin    | BioLegend                 | 201803      | MSC Purification | 10 µg/mL |
| CD45        | Biotin    | BioLegend                 | 202203      | MSC Purification | 10 µg/mL |
| CD11b       | V450      | BD Biosciences            | 562108      | FC             | 0.5 µg/test   |
| RT1D        | FITC      | BD Biosciences            | 550982      | FC             | 0.5 µg/test   |
| CD29        | PE        | BD Biosciences            | 562154      | FC             | 0.5 µg/test   |
| CD45        | PE-Cy7    | BD Biosciences            | 561588      | FC             | 0.5 µg/test   |
| CD90        | APC       | eBioscience               | 17-0900     | FC             | 0.5 µg/test   |
| Ms IgA      | V450      | BD Biosciences            | 5262142     | FC             | 0.5 µg/test   |
| Ms IgG1     | FITC      | BD Biosciences            | 550616      | FC             | 0.5 µg/test   |
| Hm IgM      | PE        | BD Biosciences            | 562114      | FC             | 0.5 µg/test   |
| Ms IgG1     | PE-Cy7    | BD Biosciences            | 561588      | FC             | 0.5 µg/test   |
| Ms IgG2a    | APC       | eBioscience               | 17-4724     | FC             | 0.5 µg/test   |
| Rabbit IgG  | Biotin    | Jackson ImmunoResearch    | 711-065-152 | IHC            | 6 µg/mL       |
| Goat IgG    | Biotin    | Jackson ImmunoResearch    | 711-065-152 | WB             | 0.05 µg/mL    |
| Mouse IgG   | Biotin    | Jackson ImmunoResearch    | 711-065-152 | WB             | 0.05 µg/mL    |
| Mouse IgG   | Biotin    | Jackson ImmunoResearch    | 711-065-152 | WB             | 0.05 µg/mL    |
| Rabbit IgG  | Biotin    | Jackson ImmunoResearch    | 711-065-152 | IHC            | 6 µg/mL       |
| Rabbit IgG  | Biotin    | Jackson ImmunoResearch    | 711-065-152 | WB             | 0.05 µg/mL    |
| Goat IgG    | Biotin    | Jackson ImmunoResearch    | 711-065-152 | WB             | 0.05 µg/mL    |
| Mouse IgG   | Biotin    | Jackson ImmunoResearch    | 711-065-152 | WB             | 0.05 µg/mL    |
| Rabbit IgG  | Biotin    | Jackson ImmunoResearch    | 711-065-152 | IHC            | 6 µg/mL       |
| Primary Antibody | Secondary Antibody | Supplier | Catalog Number | Application | Dilution |
|------------------|--------------------|----------|----------------|-------------|----------|
| Streptavidin     | AF488              | Jackson ImmunoResearch | 016-540-084 | IHC         | 1.5 µg/mL|
| Goat IgG         | AF488              | Jackson ImmunoResearch | 705-545-147 | IHC         | 3 µg/mL  |
| Rat IgG          | Cy3                | Jackson ImmunoResearch | 712-165-153 | IHC         | 3 µg/mL  |
| Rabbit IgG       | Cy3                | Jackson ImmunoResearch | 711-585-152 | IHC         | 3 µg/mL  |
| Mouse IgG        | Cy3                | Jackson ImmunoResearch | 715-165-151 | IHC         | 3 µg/mL  |
| Goat IgG         | Cy5                | Jackson ImmunoResearch | 705-175-147 | IHC         | 6 µg/mL  |

Primary antibodies are listed above the black line and secondary antibodies are listed below the black line. Sox2 – SRY box 2; GFAP – glial fibrillary acidic protein; PDGFRα – platelet derived growth factor receptor α; BrdU – 5-bromo-2′-deoxyuridine; DCX – doublecortin; Iba1 – ionized calcium-binding adapter molecule 1; CD – cluster of differentiation; RT1D – MHC class II RT1D alpha chain; Ig – immunoglobulin; Ms – mouse; Hm – hamster; WB – Western blot; IHC – immunohistochemistry; MSC – mesenchymal stem cell; FC – flow cytometry.
Table S3. Proteins identified by mass spectrometry.

| Protein Descriptions | UniProt Accession ID | Gene Symbol |
|-----------------------|----------------------|-------------|
| 72 kDa type IV collagenase OS=Rattus norvegicus GN=Mmp2 PE=2 SV=2 | P33436 | Mmp2 |
| 78 kDa glucose-regulated protein OS=Rattus norvegicus GN=Hspa5 PE=1 SV=1 | P06761 | Hspa5 |
| A disintegrin and metalloproteinase with thrombospondin motifs 1 OS=Rattus norvegicus GN=Adams1 PE=2 SV=1 | Q9WUQ1 | Adams1 |
| Actin, aortic smooth muscle OS=Rattus norvegicus GN=Acta2 PE=2 SV=1 | P62738 | Acta2 |
| Actin, beta-like 2 OS=Rattus norvegicus GN=Actb2 PE=1 SV=1 | D3ZRN3 | Actb2 |
| Actinin alpha 2 OS=Rattus norvegicus GN=Actn2 PE=1 SV=1 | D3ZCV0 | Actn2 |
| Actinin alpha 3 OS=Rattus norvegicus GN=Actn3 PE=1 SV=1 | Q8R4I6 | Actn3 |
| Actn1 protein OS=Rattus norvegicus GN=Actn1 PE=1 SV=1 | Q6GMN8 | Actn1 |
| ADAM metallopeptidase with thrombospondin type 1 motif, 1 OS=Rattus norvegicus GN=Adams12 PE=1 SV=1 | D3ZTJ3 | Adams12 |
| ADAMTS-like 3 OS=Rattus norvegicus GN=Adams13 PE=4 SV=3 | A2RU9V | Aebp1 |
| Aggrecan core protein OS=Rattus norvegicus GN=Acan PE=1 SV=2 | P07897; D4A7Y1 | Acan |
| AHNAK nucleoprotein OS=Rattus norvegicus GN=Ahnak PE=1 SV=1 | A0A0G2JU96 | Ahnak |
| Alpha 4 type V collagen OS=Rattus norvegicus GN=Col5a3 PE=2 SV=1 | Q9JI04 | Col5a3 |
| Alpha-1-macroglobulin OS=Rattus norvegicus GN=A1m PE=1 SV=1 | Q63041 | A1m |
| Alpha-2-antiplasmin OS=Rattus norvegicus GN=Serpinf1 PE=1 SV=1 | Q80ZA3 | Serpinf1 |
| Alpha-2-macroglobulin OS=Rattus norvegicus GN=A2m PE=2 SV=2 | P06238 | A2m |
| Alpha-actinin-4 OS=Rattus norvegicus GN=Actn4 PE=1 SV=2 | Q9QQX0 | Actn4 |
| Alpha-internexin OS=Rattus norvegicus GN=Ina PE=1 SV=2 | P23565; G3V8Q2 | Ina |
| Amyloid beta A4 protein OS=Rattus norvegicus GN=App PE=1 SV=2 | P08592 | App |
| Apolipoprotein B-100 OS=Rattus norvegicus GN=Apob PE=1 SV=1 | Q7TMA5 | Apob |
| Beta-2-glycoprotein 1 OS=Rattus norvegicus GN=Apoh PE=2 SV=2 | P26644 | Apoh |
| Beta-2-microglobulin OS=Rattus norvegicus GN=B2m PE=1 SV=1 | P07151 | B2m |
| Biglycan OS=Rattus norvegicus GN=Bgn PE=2 SV=1 | P47853 | Bgn |
| C1r protein OS=Rattus norvegicus GN=C1r PE=1 SV=1 | B5DEH7 | C1r |
| Cadherin 13 OS=Rattus norvegicus GN=Cdh13 PE=2 SV=1 | Q8R490 | Cdh13 |
| Calreticulin OS=Rattus norvegicus GN=Calr PE=1 SV=1 | P18418 | Calr |

Table S3. Proteins identified by mass spectrometry.

| Protein Descriptions | UniProt Accession ID | Gene Symbol |
|-----------------------|----------------------|-------------|
| Calsequestrin OS=Rattus norvegicus GN=Casq2 PE=1 SV=2 | F1M944 | Casq2 |
| Calsyntenin-1 OS=Rattus norvegicus GN=Clstn1 PE=2 SV=1 | Q6Q0N0 | Clstn1 |
| Carboxypeptidase E OS=Rattus norvegicus GN=Cpe PE=1 SV=1 | P15087; D4A8X4 | Cpe |
| Carboxypeptidase OS=Rattus norvegicus GN=Ctsa PE=1 SV=1 | Q6AYS3 | Ctsa |
| Carboxypeptidase Q OS=Rattus norvegicus GN=Cpq PE=1 SV=1 | Q6IK9 | Cpq |
| Protein Description                                                                 | UniProt Accession ID | Gene Symbol |
|-----------------------------------------------------------------------------------|----------------------|-------------|
| Collagen type VIII alpha 1 chain OS=Rattus norvegicus GN=Col8a1 PE=1 SV=1          | Q00657               | Cspg4       |
| Collagen type VIII alpha 1 chain OS=Rattus norvegicus GN=Col8a1 PE=1 SV=1          | D4AC70               | Col8a1      |
| Collagen type VIII alpha 2 chain OS=Rattus norvegicus GN=Col8a2 PE=4 SV=1          | D4ADG9               | Col8a2      |
| Collagen type XV alpha 1 chain OS=Rattus norvegicus GN=Col15a1 PE=1 SV=1           | A0A0G2JV12           | Col15a1     |
| Collagen type XVI alpha 1 chain OS=Rattus norvegicus GN=Col16a1 PE=1 SV=2          | F1LND0               | Col16a1     |
| Collagen type XVIII alpha 1 chain OS=Rattus norvegicus GN=Col18a1 PE=1 SV=2         | F1LR02               | Col18a1     |
| Complement C1q tumor necrosis factor-related protein 5 OS=Rattus norvegicus GN=C1qtnf5 PE=2 SV=1 | Q5FVH0               | C1qtnf5     |
| Complement C1s subcomponent OS=Rattus norvegicus GN=C1s PE=1 SV=1                 | G3V7L3;D4A1S0        | C1s         |

**Protein Descriptions**

| Protein Description                                                                 | UniProt Accession ID | Gene Symbol |
|-----------------------------------------------------------------------------------|----------------------|-------------|
| Cartilage oligomeric matrix protein OS=Rattus norvegicus GN=Comp PE=1 SV=1        | P35444               | Comp        |
| Catenin beta-1 OS=Rattus norvegicus GN=Ctnnb1 PE=1 SV=1                           | Q9WU82               | Ctnnb1      |
| Cathepsin B OS=Rattus norvegicus GN=Ctsb PE=1 SV=1                                | Q6IN22               | Ctsb        |
| Cathepsin L1 OS=Rattus norvegicus GN=Ctsl PE=1 SV=2                               | P07154               | Ctsl        |
| CD44 antigen OS=Rattus norvegicus GN=Cd44 PE=1 SV=2                               | P26051               | Cd44        |
| Chondroitin sulfate proteoglycan 4 OS=Rattus norvegicus GN=Cspg4 PE=1 SV=2         | Q00657               | Cspg4       |
| Class I histocompatibility antigen, Non-RT1.A alpha-1 chain OS=Rattus norvegicus GN=RT1-Aw2 PE=1 SV=1 | P15978               | RT1-Aw2     |
| Coiled-coil domain-containing protein 80 OS=Rattus norvegicus GN=Ccdc80 PE=1 SV=1  | Q6QD51               | Ccdc80      |
| Collagen alpha-1(I) chain OS=Rattus norvegicus GN=Col1a1 PE=1 SV=5                 | P02454               | Col1a1      |
| Collagen alpha-1(II) chain OS=Rattus norvegicus GN=Col2a1 PE=1 SV=2                | P05539               | Col2a1      |
| Collagen alpha-1(III) chain OS=Rattus norvegicus GN=Col3a1 PE=2 SV=3               | P13941               | Col3a1      |
| Collagen alpha-1(V) chain OS=Rattus norvegicus GN=Col5a1 PE=1 SV=1                 | Q9JI03;A0A0G2JX47    | Col5a1      |
| Collagen alpha-1(XI) chain OS=Rattus norvegicus GN=Col11a1 PE=1 SV=2               | P20909               | Col11a1     |
| Collagen alpha-1(XII) chain (Fragment) OS=Rattus norvegicus GN=Col112a1 PE=2 SV=1   | P70560;A0A0G2KAJ7    | Col12a1     |
| Collagen alpha-2(I) chain OS=Rattus norvegicus GN=Col1a2 PE=1 SV=3                 | P02466;A0A0G2K5E8    | Col1a2      |
| Collagen type IV alpha 1 chain OS=Rattus norvegicus GN=Col4a1 PE=1 SV=1            | F1MA59               | Col4a1      |
| Collagen type IV alpha 2 chain OS=Rattus norvegicus GN=Col4a2 PE=1 SV=3            | F1M6Q3               | Col4a2      |
| Collagen type IV alpha 5 chain OS=Rattus norvegicus GN=Col4a5 PE=3 SV=3            | F1LUN5               | Col4a5      |
| Collagen type V alpha 2 chain OS=Rattus norvegicus GN=Col5a2 PE=1 SV=3             | F1LQ00               | Col5a2      |
| Collagen type VI alpha 1 chain OS=Rattus norvegicus GN=Col6a1 PE=1 SV=2             | D3ZUL3               | Col6a1      |
| Collagen type VI alpha 2 chain OS=Rattus norvegicus GN=Col6a2 PE=1 SV=3             | F1LNH3               | Col6a2      |
| Collagen type VIII alpha 1 chain OS=Rattus norvegicus GN=Col8a1 PE=1 SV=3           | D4AC70               | Col8a1      |
| Protein Name                              | UniProt Accession ID | Gene Symbol |
|-------------------------------------------|----------------------|-------------|
| Complement C2 OS=Rattus norvegicus GN=C2 PE=1 SV=1 | Q6MG73;A0A0U1RRP9 | C2          |
| Complement C3 OS=Rattus norvegicus GN=C3 PE=1 SV=3 | P01026;M0RBF1       | C3          |
| Complement C4B (Chido blood group) OS=Rattus norvegicus GN=C4b PE=1 SV=1 | Q6MG90              | C4b         |
| Complement component 4A (Rodgers blood group) OS=Rattus norvegicus GN=C4a PE=1 SV=2 | M0RB00              | C4a         |
| Connective tissue growth factor OS=Rattus norvegicus GN=Ctgf PE=2 SV=1 | Q9R1E9              | Ctgf        |
| Cystatin-C OS=Rattus norvegicus GN=Cst3 PE=1 SV=2 | P14841              | Cst3        |
| Cysteine and glycine-rich protein 1 OS=Rattus norvegicus GN=Csrp1 PE=1 SV=2 | P47875              | Csrp1       |
| Desmin OS=Rattus norvegicus GN=Des PE=1 SV=2 | P48675              | Des         |
| Dystroglycan 1 OS=Rattus norvegicus GN=Dag1 PE=1 SV=1 | A0A0G2K2R5          | Dag1        |
| EGF-containing fibulin-like extracellular matrix protein 2 OS=Rattus norvegicus GN=Efemp2 PE=1 SV=1 | Q99372              | Efemp2      |
| Elastin microfibril interfacer 1 OS=Rattus norvegicus GN=Emilin1 PE=1 SV=1 | D3Z9E1              | Emilin1     |
| Elastin OS=Rattus norvegicus GN=Eln PE=1 SV=2 | Q99372              | Eln         |
| Elongation factor 1-alpha 1 OS=Rattus norvegicus GN=Eef1a1 PE=2 SV=1 | P62630              | Eef1a1      |
| Elongation factor 1-alpha 2 OS=Rattus norvegicus GN=Eef1a2 PE=1 SV=1 | P62632              | Eef1a2      |
| Elongation factor 2 OS=Rattus norvegicus GN=Eef2 PE=1 SV=4 | P05197              | Eef2        |
| Endothelial differentiation-related factor 1 OS=Rattus norvegicus GN=Edf1 PE=1 SV=1 | P69736              | Edf1        |
| Extracellular matrix protein 1 OS=Rattus norvegicus GN=Ecm1 PE=1 SV=1 | Q62894              | Ecm1        |
| Extracellular sulatase Sulf-1 OS=Rattus norvegicus GN=Sulf1 PE=1 SV=1 | Q8WI60              | Sulf1       |
| FAT atypical cadherin 1 OS=Rattus norvegicus GN=Fat1 PE=1 SV=1 | A0A0G2K5L1          | Fat1        |
| Fibrillin 1 OS=Rattus norvegicus GN=Fbn1 PE=1 SV=1 | G3V9M6              | Fbn1        |
| Fibrillin 2 OS=Rattus norvegicus GN=Fbn2 PE=4 SV=3 | F1M5Q4              | Fbn2        |
| **Protein Descriptions**                         | **UniProt Accession ID** | **Gene Symbol** |
| Fibronectin OS=Rattus norvegicus GN=Fn1 PE=1 SV=3 | F1LST1;A0A096P6L8   | Fn1         |
| Fibrillin 2 OS=Rattus norvegicus GN=Fbn2 PE=1 SV=1 | G3V6X1              | Fbn2        |
| Fibrillin-1 OS=Rattus norvegicus GN=Fbn1 PE=1 SV=1 | D3ZQ25              | Fbn1        |
| Fibrillin-5 OS=Rattus norvegicus GN=Fbn5 PE=2 SV=1 | Q9VVH8              | Fbn5        |
| Filamin A OS=Rattus norvegicus GN=Flna PE=1 SV=1 | C0JPT7              | Flna        |
| Follistatin-related protein 1 OS=Rattus norvegicus GN=Fstl1 PE=1 SV=1 | Q62632;F8WG88       | Fstl1       |
| Follistatin-related protein 3 OS=Rattus norvegicus GN=Fstl3 PE=2 SV=1 | Q99PW7              | Fstl3       |
| Fructose-bisphosphate aldolase OS=Rattus norvegicus GN=Aldoart2 PE=2 SV=1 | Q6AY07              | Aldoart2    |
| Galectin-3-binding protein OS=Rattus norvegicus GN=Lgals3bp PE=1 SV=2 | O70513              | Lgals3bp    |
| Gelsolin OS=Rattus norvegicus GN=Gsn PE=1 SV=1 | Q68FP1              | Gsn         |
| Gliomedin OS=Rattus norvegicus GN=Gldn PE=1 SV=1 | Q80WL1              | Gldn        |
| Protein Description                                                                 | UniProt Accession ID | Gene Symbol   |
|----------------------------------------------------------------------------------|----------------------|---------------|
| Glyceraldehyde-3-phosphate dehydrogenase, testis-specific OS=Rattus norvegicus GN=Gapdhs PE=1 SV=1 | Q9ESV6               | *Gapdhs       |
| Glypican 4 OS=Rattus norvegicus GN=Gpc4 PE=1 SV=1                                  | Q642B0               | Gpc4          |
| GM2 ganglioside activator OS=Rattus norvegicus GN=Gm2a PE=1 SV=1                   | Q6IN37               | Gm2a          |
| Granulin, isoform CRA_c OS=Rattus norvegicus GN=Grn PE=4 SV=1                      | G3V8V1               | Grn           |
| Haptoglobin OS=Rattus norvegicus GN=Hp PE=1 SV=3                                   | P06866               | Hp            |
| Heat shock protein HSP 90-alpha OS=Rattus norvegicus GN=Hsp90aa1 PE=1 SV=3         | P82995               | Hsp90aa1      |
| Heat shock protein HSP 90-beta OS=Rattus norvegicus GN=Hsp90ab1 PE=1 SV=4          | P34058               | Hsp90ab1      |
| Hemicentin 1 OS=Rattus norvegicus GN=Hmcn1 PE=1 SV=3                               | F1M4Q3               | Hmcn1         |
| Hepatoma-derived growth factor-related protein 3 OS=Rattus norvegicus GN=Hdgfrp3 PE=1 SV=1 | Q923W4               | Hdgfl3        |
| Heterogeneous nuclear ribonucleoprotein K OS=Rattus norvegicus GN=Hnrnpk PE=1 SV=1 | P61980               | Hnrnpk        |
| Histone cluster 1 H1 family member c OS=Rattus norvegicus GN=Hist1h1c PE=1 SV=1     | A0A0G2K654           | Hist1h1c      |
| Histone H1.0 OS=Rattus norvegicus GN=Hist1f0 PE=2 SV=2                              | P43278               | Hist1f0       |
| Histone H1.1 OS=Rattus norvegicus GN=Hist1h1a PE=1 SV=1                             | D4A3K5               | Hist1h1a      |
| Histone H1.4 OS=Rattus norvegicus GN=Hist1h1e PE=1 SV=3                             | P15865               | Hist1h1e      |
| Histone H1.5 OS=Rattus norvegicus GN=Hist1h1b PE=1 SV=1                             | D3ZBN0               | Hist1h1b      |
| Histone H1t OS=Rattus norvegicus GN=Hist1h1t PE=1 SV=2                              | P06349               | Hist1h1t      |

## Protein Descriptions

| Protein Description                                                                 | UniProt Accession ID | Gene Symbol   |
|----------------------------------------------------------------------------------|----------------------|---------------|
| Histone H2B OS=Rattus norvegicus GN=Hist1h2bo PE=3 SV=1                           | A0A0G2JXI9           | Hist1h2bo     |
| Histone H2B OS=Rattus norvegicus GN=Hist2h2be PE=3 SV=3                           | D4A817               | Hist2h2be     |
| Histone H2B OS=Rattus norvegicus GN=LOC102549061 PE=3 SV=1                        | A0A0G2JXE0           | *LOC10254 9061|
| Inactive serine protease 35 OS=Rattus norvegicus GN=Prss35 PE=2 SV=1              | Q5R212               | Prss35        |
| Insulin-like growth factor binding protein 7, isoform CRA_b OS=Rattus norvegicus GN=Igfbp7 PE=1 SV=2 | F1M9B2               | Igfbp7        |
| Inter-alpha-trypsin inhibitor heavy chain H3 OS=Rattus norvegicus GN=Ith3 PE=2 SV=1 | Q63416               | Ith3          |
| Interleukin-1 receptor-like 1 OS=Rattus norvegicus GN=Il1rl1 PE=2 SV=1            | Q62611;F1LR63        | Il1rl1        |
| Lactadherin OS=Rattus norvegicus GN=Mfge8 PE=2 SV=1                               | P70490;Q1PBJ1        | Mfge8         |
| Laminin subunit alpha 4 OS=Rattus norvegicus GN=Lama4 PE=1 SV=3                   | F1LTF8               | Lama4          |
| Laminin subunit alpha 5 OS=Rattus norvegicus GN=Lama5 PE=1 SV=2                   | F1MAN8               | Lama5          |
| Laminin subunit beta 1 OS=Rattus norvegicus GN=Lamb1 PE=1 SV=3                    | D3ZQN7               | Lamb1          |
| Laminin subunit beta-2 OS=Rattus norvegicus GN=Lamb2 PE=2 SV=1                    | P15800               | Lamb2          |
| Laminin subunit gamma 1 OS=Rattus norvegicus GN=Lamc1 PE=1 SV=1                   | F1MAA7               | Lamc1          |
| Latent-transforming growth factor beta-binding protein 1 OS=Rattus norvegicus GN=Ltpb1 PE=1 SV=1 | Q00918               | Ltpb1          |
| Latent-transforming growth factor beta-binding protein 2 OS=Rattus norvegicus GN=Ltpb2 PE=1 SV=1 | O35806;A0A0G2K1G5    | Ltpb2          |
| Latent-transforming growth factor beta-binding protein 3 OS=Rattus norvegicus GN=Ltpb3 PE=4 SV=1 | Q9ESV6               | Ltpb3          |
| Gene Name | Gene Symbol | UniProt Accession ID | Gene Name | Gene Symbol | UniProt Accession ID |
|-----------|-------------|----------------------|-----------|-------------|----------------------|
| Lipocalin 7, isoform CRA_a | Tinagl1 | Q4V8N0;Q9EQT5 | Lactate dehydrogenase A chain | Ldha | P04642 |
| | | | Lactate dehydrogenase B chain | Ldhb | P42123 |
| Lumican | Lum | P51886 | Lysyl oxidase homolog 2 | Loxl2 | B5DF27;A0A0G2K4P0 |
| | | | Lysyl oxidase-like 1 | Loxl1 | Q5FWS5 |
| | | | Lysyl oxidase-like 3 | Loxl3 | D3ZP82 |
| | | | Lysyl oxidase-like 4 | Loxl4 | D4A9V5 |
| Macrophage colony-stimulating factor 1 | Csf1 | Q8JZQ0 | Mannan-binding lectin serine protease 1 | Masp1 | Q8CHN8 |
| Matrix Gla protein | Mgp | P08494 | Matrix metallopeptidase 19 | Mmp19 | C0M4B0 |
| | | | Matrix metalloproteinase-14 | Mmp14 | Q10739 |
| | | | Matrix metalloproteinase-23 | Mmp23 | O88272 |
| | | | Metalloendopeptidase | Bmp1 | F1M798 |
| | | | Metalloendopeptidase OS=Rattus norvegicus GN=Tll1 PE=2 SV=3 | Tll1 | D3Z8U5 |
| | | | Metalloproteinase inhibitor 1 | Timp1 | P30120 |
| | | | Metalloproteinase inhibitor 2 | Timp2 | P30121 |
| | | | Multiple inositol polyphosphate phosphatase | Minpp1 | O35217 |
| | | | Myosin, heavy polypeptide 9, non-muscle | Myh9 | G3V6P7;Q62812 |
| | | | Neurofilament heavy polypeptide | Nefh | P16884;F1LRZ7 |
| | | | Neurofilament light polypeptide | Nefl | P19527 |
| | | | Neurofilament medium polypeptide | Nefm | G3V7S2 |
| | | | Nidogen-1 | Nid1 | F1LM84 |
| | | | Nidogen-2 | Nid2 | B5DFC9 |
| | | | Non-muscle caldesmon | Cald1 | Q62736;A0A0G2JTV2 |
| | | | NPC intracellular cholesterol transporter 2 | Npc2 | F7FJQ3 |
| | | | Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1 | Nucks1 | Q9EPJ0 |
| | | | Nucleobindin-1 | Nucb1 | Q63083 |
| UniProt Accession ID | Gene Symbol |
|----------------------|-------------|
| Q9JJ85               | Nucb2       |
| Q05982               | Nme1        |
| P19804               | Nme2        |
| D4A0J7               | Olfml2b     |
| B0BN15               | Olfml3      |
| D3ZVB7               | Ogn         |
| P08721               | Spp1        |
| Q6AYE5               | Oaf         |
| P396x566             | Protaglycin |
| P14925               | Pam         |
| P10111               | Ppia        |
| P24368               | Ppib        |
| Q5U2V1;A0A096MJW1    | Fkbp10      |
| D3ZAF5;A0A097BW25    | Postn       |
| A0A0G2JWB6           | Pxdn        |
| Q63716               | Prdx1       |
| Q6P734               | Serping1    |
| P20961               | Serpine1    |
| Q5RP7                | Pdgfr1      |
| B5DEZ8               | Plxdc2      |
| O08628               | Pcolce      |
| Q63321               | Plod1       |
| Q811A3               | Plod2       |
| Q5U367               | Plod3       |
| P62963               | Pfn1        |
| P07808               | Npy         |
| P10960;F7EPE0        | Psap        |
| Q9ES72               | Cyr61       |
| P11598               | Pdia3       |
| Q9QZQ5               | Nov         |
| Q6B345               | S100a11     |

**Protein Descriptions**

- **Nucleobindin-2** OS=Rattus norvegicus GN=Nucb2 PE=1 SV=1
- **Nucleoside diphosphate kinase A** OS=Rattus norvegicus GN=Nme1 PE=1 SV=1
- **Nucleoside diphosphate kinase B** OS=Rattus norvegicus GN=Nme2 PE=1 SV=1
- **Olfactomedin-like 2B** OS=Rattus norvegicus GN=Olfml2b PE=4 SV=1
- **Olfactomedin-like protein 3** OS=Rattus norvegicus GN=Olfml3 PE=2 SV=2
- **Osteoglycin** OS=Rattus norvegicus GN=Ogn PE=1 SV=1
- **Osteopontin** OS=Rattus norvegicus GN=Spp1 PE=1 SV=2
- **Out at first protein homolog** OS=Rattus norvegicus GN=Oaf PE=2 SV=1
- **Pappalysin 2** OS=Rattus norvegicus GN=Pappa2 PE=4 SV=2
- **Peptidyl-glycine alpha-amidating monooxygenase** OS=Rattus norvegicus GN=Pam PE=1 SV=1
- **Peptidyl-prolyl cis-trans isomerase A** OS=Rattus norvegicus GN=Ppia PE=1 SV=2
- **Peptidyl-prolyl cis-trans isomerase B** OS=Rattus norvegicus GN=Ppib PE=1 SV=3
- **Peptidylprolyl isomerase** OS=Rattus norvegicus GN=Fkbp10 PE=1 SV=1
- **Periostin** OS=Rattus norvegicus GN=Postn PE=1 SV=1
- **Peroxidasin** OS=Rattus norvegicus GN=Pxdn PE=4 SV=1
- **Peroxiredoxin-1** OS=Rattus norvegicus GN=Prdx1 PE=1 SV=1
- **Plasmib C1 inhibitor** OS=Rattus norvegicus GN=Serpine1 PE=2 SV=1
- **Plasminogen activator inhibitor 1** OS=Rattus norvegicus GN=Serpine1 PE=2 SV=1
- **Platelet-derived growth factor receptor-like protein** OS=Rattus norvegicus GN=Pdgfr1 PE=2 SV=1
- **Plexin domain containing 2** OS=Rattus norvegicus GN=Plxdc2 PE=1 SV=1
- **Procollagen C-endopeptidase enhancer 1** OS=Rattus norvegicus GN=Pcolce PE=1 SV=1
- **Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1** OS=Rattus norvegicus GN=Plod1 PE=2 SV=1
- **Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2** OS=Rattus norvegicus GN=Plod2 PE=2 SV=1
- **Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3** OS=Rattus norvegicus GN=Plod3 PE=2 SV=1
- **Profilin-1** OS=Rattus norvegicus GN=Pfn1 PE=1 SV=2
- **Pro-neuropeptide Y** OS=Rattus norvegicus GN=Npy PE=1 SV=1
- **Prosaposin** OS=Rattus norvegicus GN=Psap PE=1 SV=1
- **Protein CYR61** OS=Rattus norvegicus GN=Cyr61 PE=2 SV=1
- **Protein disulfide-isomerase A3** OS=Rattus norvegicus GN=Pdia3 PE=1 SV=2
- **Protein NOV homolog** OS=Rattus norvegicus GN=Nov PE=1 SV=1
- **Protein S100-A11** OS=Rattus norvegicus GN=S100a11 PE=3 SV=1
| Accession ID | Gene Symbol | Protein Description |
|-------------|-------------|---------------------|
| A0A0G2K447  | Shisa5      | Protein shisa-5     |
| A5I9F0      | Ptprk       | Protein tyrosine    |
| M0RB22      | Ptprd       | Protein tyrosine    |
| A0A0G2K561  | Ptprg       | Protein tyrosine    |
| P16636      | Lox         | Protein-lysine      |
| D3ZPK4      | Ssc5d       | Putative uncharacterized protein RGD1565772_predicted |
| P11980      | Pkm         | Pyruvate kinase PKM |
| Q6AYQ9      | Ppic        | Q6AYQ9|Q6AYQ9_RAT |
| Q6MFZ8      | RT1-N2      | RCG41803, isoform CRA_a |
| M0R7B4      | LOC684828   | RCG45259 OS=Rattus norvegicus GN=LOC684828 |
| G3V852      | Tln1        | RCG55135, isoform CRA_b |
| O55004      | Rnase4      | Ribonuclease 4 OS=Rattus norvegicus GN=Rnase4 |
| P16391      | RT1 class I histocompatibility antigen, AA alpha chain |
| Q6MG01      | RT1-N3      | RT1 class I, N3 OS=Rattus norvegicus GN=RT1-N3 |
| A0A0G2K1E1  | RT1-A1      | RT1 class Ia, locus A1 OS=Rattus norvegicus GN=RT1-A1 |
| D3ZLH8      | Rtf1        | Rtf1, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae) |
| D3ZQP6      | Sema7a      | Sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (Semaphorin) 7A (Predicted) OS=Rattus norvegicus GN=Sema7a |
| F7FHT4      | Sema3c      | Semaphorin 3C OS=Rattus norvegicus GN=Sema3c |
| F1IMAG8     | Sema3d      | Semaphorin 3D OS=Rattus norvegicus GN=Sema3d |
| Q5M7T5      | Serpin1     | Serine (Or cysteine) peptidase inhibitor, clade C (Antithrombin), member 1 OS=Rattus norvegicus GN=Serpin1 |
| A0A0G2JU46  | Prss23      | Serine protease 23 OS=Rattus norvegicus GN=Prss23 |
| Q9QQZK5     | Htra1       | Serine protease HTRA1 OS=Rattus norvegicus GN=Htra1 |
| P29457      | Serpin1     | Serpin H1 OS=Rattus norvegicus GN=Serpin1 |
| Q498M9      | LOC30344     | Similar to glyceraldehyde-3-phosphate dehydrogenase OS=Rattus norvegicus GN=LOC30344 |
| E9PST1;A0A0G2K896 | RGD1310507   | Similar to RIKEN cDNA 1300017J02 OS=Rattus norvegicus GN=RGD1310507 |
| F1MAAQ9     | RT1-CE7     | Similar to RT1 class I, CE11 OS=Rattus norvegicus GN=LOC683761 |
| O88279      | Slt1        | Slit homolog 1 protein OS=Rattus norvegicus GN=Slt1 |
| F1MA79      | Slt2        | Slit homolog 2 protein OS=Rattus norvegicus GN=Slt2 |
| P16975      | Sparc       | SPARC OS=Rattus norvegicus GN=Sparc |
| Q6IE50      | Smoc1       | SPARC-related modular calcium binding protein 1 OS=Rattus norvegicus GN=Smoc1 |
| Protein Description                                                                 | UniProt Accession ID | Gene Symbol |
|------------------------------------------------------------------------------------|----------------------|-------------|
| Sulfhydryl oxidase 1 OS=Rattus norvegicus GN=Qsox1 PE=1 SV=1                        | Q6IUU3               | Qsox1       |
| Sushi domain containing 5 (Predicted) OS=Rattus norvegicus GN=Susd5 PE=4 SV=1       | D3ZSC1               | Susd5       |
| Sushi repeat-containing protein SRPX2 OS=Rattus norvegicus GN=Srpx2 PE=1 SV=1       | B5DF94               | Srpx2       |
| Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 OS=Rattus norvegicus GN=Svep1 PE=1 SV=1 | P0C6B8               | Svep1       |
| Tenascin C OS=Rattus norvegicus GN=Tnc PE=1 SV=1                                   | A0A0G2K1L0           | Tnc         |
| Thrombospondin 2 OS=Rattus norvegicus GN=Thbs2 PE=4 SV=2                           | D4A2G6               | Thbs2       |
| Thrombospondin 3 OS=Rattus norvegicus GN=Thbs3 PE=4 SV=1                           | A0A0G2JZH3           | Thbs3       |
| Transcobalamin-2 OS=Rattus norvegicus GN=Tcn2 PE=2 SV=1                            | Q9R0D6               | Tcn2        |
| Transforming growth factor beta-2 OS=Rattus norvegicus GN=Tgfb2 PE=2 SV=2          | Q07257               | Tgfb2       |
| Transforming growth factor beta-3 OS=Rattus norvegicus GN=Tgfb3 PE=2 SV=2          | Q07258               | Tgfb3       |
| Transgelin OS=Rattus norvegicus GN=Tagln PE=1 SV=1                                 | A0A0G2JWK7           | Tagln       |
| Transgelin-2 OS=Rattus norvegicus GN=Tagln2 PE=1 SV=1                              | Q55FX0               | Tagln2      |
| Triosephosphate isomerase OS=Rattus norvegicus GN=Tpi1 PE=1 SV=2                   | P48500               | Tpi1        |
| Tubulointerstitial nephritis antigen-like OS=Rattus norvegicus GN=Tinagl1 PE=2 SV=1 | Q9EQT5               | Tinagl1     |
| Uncharacterized protein OS=Rattus norvegicus GN=Gm5414 PE=3 SV=1                   | A0A0G2JUG1           | Gm5414      |
| Uncharacterized protein OS=Rattus norvegicus GN=Itih2 PE=1 SV=3                    | D3ZFH5               | Itih2       |
| Uncharacterized protein OS=Rattus norvegicus GN=RGD1559534 PE=4 SV=1               | F1M9V3               | RGD1559534  |
| Uncharacterized protein OS=Rattus norvegicus GN=RGD1564958 PE=1 SV=1               | A0A0G2K8S2           | RGD1564958  |
| Uncharacterized protein OS=Rattus norvegicus GN=RT1-CE2 PE=3 SV=3                  | D3ZGQG9;A0A0G2K8R6   | RT1-CE2     |
| Uncharacterized protein OS=Rattus norvegicus GN=Thbs1 PE=1 SV=2                    | M0R979;A0A0G2J3V4    | Thbs1       |
| Uncharacterized protein OS=Rattus norvegicus PE=1 SV=2                             | F1M2N4               | N/A         |
| Uncharacterized protein OS=Rattus norvegicus PE=1 SV=3                             | F1LITJ5              | N/A         |
| Uncharacterized protein OS=Rattus norvegicus PE=4 SV=3                             | F1M8C7               | N/A         |
| Uncharacterized protein OS=Rattus norvegicus PE=4 SV=3                             | F1LUI2               | N/A         |
| Versican core protein (Fragments) OS=Rattus norvegicus GN=Vcan PE=2 SV=2            | Q9ERB4;D3Z9N6        | Vcan        |
| Vimentin OS=Rattus norvegicus GN=Vim PE=1 SV=2                                     | P31000               | Vim         |
| Vinculin OS=Rattus norvegicus GN=Vcl PE=1 SV=1                                     | P85972               | Vcl         |

Gene symbols marked with n denote proteins unique to nMSC, gene symbols marked with γ denote proteins unique to aMSCγ.
Table S4. Metacore Pathway Enrichment.

| Pathway                                                                 | FDR   | Pathway Total | In Data | Object Name                                                                 |
|------------------------------------------------------------------------|-------|---------------|---------|-----------------------------------------------------------------------------|
| TGF-beta-induced fibroblast/myofibroblast migration and extracellular matrix production in asthmatic airways | 0.0001 | 35            | 3       | Tenascin-C, TGF-beta 3, COL1A2, Collagen IV, Thrombospondin 2               |
| Alternative complement cascade disruption in age-related macular degeneration | 0.0054 | 38            | 3       | Factor Ba, Factor Bb, Factor B                                              |
| Development: TGF-beta-dependent induction of EMT via SMADs             | 0.0054 | 42            | 3       | TGF-beta, Vimentin, TGF-beta 3                                             |
| WNT signaling in invasive-type melanoma cells                          | 0.0054 | 47            | 3       | Vimentin, Filamin A, Filamin-A (CTF)                                       |
| Stromal-epithelial interaction in Prostate Cancer                      | 0.0058 | 48            | 3       | Tenascin-C, Vimentin, TGF-beta 3                                           |
| Development: TGF-beta-dependent induction of EMT via MAPK             | 0.0058 | 50            | 3       | TGF-beta, Vimentin, TGF-beta 3                                             |
| Stimulation of TGF-beta signaling in lung cancer                       | 0.0058 | 53            | 3       | TGF-beta, Vimentin, TGF-beta 3                                             |
| Immune response: Lectin induced complement pathway                     | 0.0058 | 53            | 3       | C2b, C2a, C2                                                               |
| Immune response: Classical complement pathway                          | 0.0058 | 19            | 2       | C2b, C2a, C2                                                               |
| Immune response: Alternative complement pathway                         | 0.0058 | 25            | 2       | Factor Ba, Factor Bb, Factor B                                              |
| Stem cells Schema: Adult neurogenesis in the Subventricular Zone      | 0.0158 | 26            | 2       | Vimentin, CSPG4 (NG2)                                                      |
| Immune escape mechanisms in Prostate Cancer                           | 0.0251 | 28            | 2       | Beta-2-microglobulin, TGF-beta                                             |
| Stem cells: Hypothetical role of microRNAs in fibrosis development after myocardial infarction | 0.0251 | 35            | 2       | Fibrillin 1, COL1A2                                                        |
| Tumor-stroma interactions in pancreatic cancer                         | 0.0270 | 35            | 2       | COL1A2, Thrombospondin 2                                                    |
| Production and activation of TGF-beta in airway smooth muscle cells   | 0.0364 | 38            | 2       | TGF-beta, TGF-beta 3                                                       |
| Role of adhesion of SCLC cells in tumor progression                   | 0.0364 | 38            | 2       | Tenascin-C, Collagen IV                                                    |
| Cell adhesion: Cell-matrix glycoconjugates                             | 0.0364 | 38            | 2       | Tenascin-C, CSPG4 (NG2)                                                    |
| Stem cells: Role of TGF-beta 1 in fibrosis development after myocardial infarction | 0.0364 | 44            | 2       | Tenascin-C, COL1A2                                                        |
| Development: Neural stem cell lineage commitment (schema)             | 0.0364 | 45            | 2       | Vimentin, CSPG4 (NG2)                                                      |
| TGF-beta 1-mediated induction of EMT in normal and asthmatic airway epithelium | 0.0456 | 46            | 2       | Tenascin-C, Vimentin                                                      |
|   | Pathway                                                                 | p-value | Gene(s)                      |
|---|-------------------------------------------------------------------------|---------|-----------------------------|
| 21| LKB1 signaling pathway in lung cancer cells                           | 0.0456  | Vimentin, Collagen IV       |
| 22| Development: TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK | 0.0456  | Vimentin, TGF-beta 3        |
| 23| Cytoskeleton remodeling: Integrin outside-in signaling                 | 0.0491  | Filamin A, Collagen IV      |
| 24| Immune response IL-13 signaling via PI3K-ERK pathway                   | 0.0491  | Tenascin-C, COL1A2          |
Figure S1. Microglia viability studies after oxygen glucose deprivation (OGD) conditions.

(a) Microglia 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay after OGD. (b) Microglia release of lactate dehydrogenase (LDH) in media after OGD. (c) Microglia maximum LDH from cell lysate after OGD. Data were analyzed using two-way ANOVA with Tukey multiple comparison testing. *p≤0.05, ***p<0.001, ****p<0.0001.
Figure S2. Microglia Tunel Assay after oxygen glucose deprivation (OGD).

(a) Representative confocal images of Microglia positive and negative controls. (b) Representative confocal images of Microglia after OGD. DAPI (blue), SA-488 (green). Scale bar 10 μm.
Figure S3. Changes in subventricular zone (SVZ) composition at 1 week after MCAO.

(a) Bromodeoxyuridine (BrdU)$^+$ cell counts in the SVZ. (b) Doublecortin (DCX)$^+$ cell counts in the SVZ. (c) Platelet derived growth factor receptor alpha (PDGFRα)$^+$ cell counts in the SVZ. Data are mean ± S.E.M.; n = 3 animals for sham + vehicle group, n = 5-6 for the remaining groups. Data were analyzed using nonparametric Kruskal-Wallis one-way ANOVA.
Figure S4. Changes in subventricular zone (SVZ) composition at 3 weeks after middle cerebral artery occlusion (MCAO).

(a) Bromodeoxyuridine (BrdU)$^+$ cell counts in the SVZ. (b) Doublecortin (DCX)$^+$ cell counts in the SVZ. (c) 2’3’-Cyclic-nucleotide 3’ phosphodiesterase (CNPase$^+$) cell counts in the SVZ. Data are mean ± S.E.M.; n = 3 animals per group. Data were analyzed using nonparametric Kruskal-Wallis one-way ANOVA. *p≤0.05.
Figure S5. Neural stem cell (NSC) viability studies after oxygen glucose deprivation (OGD) conditions.

(a) NSC cell counts after OGD. (b) NSC cell counts normalized to 0 hours post OGD. (c) NSC 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay after OGD. (d) NSC release of lactate dehydrogenase (LDH) in media after OGD. (e) NSC maximum LDH from cell lysate after OGD. Data were analyzed using two-way ANOVA with Tukey multiple comparison testing. *p≤0.03, ** p≤0.002, ***p≤0.0002, ****p≤0.0001
Figure S6. Neural stem cell (NSC) Tunel Assay after oxygen glucose deprivation (OGD).

(a) Representative confocal images of NSC positive and negative controls. (b) Representative confocal images of NSC after OGD. DAPI (blue), SA-488 (green). Scale bar 10 μm
mRNA levels of brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), nerve growth factor (NGF), glial derived neurotrophic factor (GDNF), and insulin growth factor 1 (IGF-1) from nMSC and aMSC\(\gamma\). Data are mean \pm S.E.M.; \(n = 3\) independent experiments. Data were analyzed by two-tail unpaired t-test. ***(p<0.001.***
Figure S8. Mesenchymal stem cell (MSC) surface marker analysis.

(a-c) Flow cytometry gating strategy. (d-h) MSC surface marker (red lines on histograms) expression CD11b (d), RT1D (e), CD45 (f), CD29 (g), CD90 (h), and corresponding isotype controls (black lines on histograms).
Figure S9. Gating strategy for BrdU flow cytometry.
Supplemental Video Legend:

Video S1. T2-weighted magnetic resonance (MR) imaging from middle cerebral artery occlusion (MCAO) animals. Left panel is vehicle treated, middle panel is naïve mesenchymal stem cells (nMSC) treated, and right panel is aMSCγ treated animals. Best viewed with Windows Media Player.