Supplemental Information

Endogenous Matrix Metalloproteinase (MMP)-3 and MMP-9 Promote the Differentiation and Migration of Adult Neural Progenitor Cells in Response to Chemokines

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Supplementary Methods

Supplementary Figures and Figure Legends

Immunohistochemistry

Primary antibodies used were mouse anti-CXCR4 (1:1000, eBioscience, #14-6009-81), mouse anti-VEGFR2 (1:1000, eBioscience, #14-5821-81), mouse immunoglobulin G (IgG) (1:1000, Sigma, #15381), mouse anti-Nestin (1:1000, BD Pharmingen, #556309), rabbit anti-Sox2 (1:1000, Cell Signaling, #2748), goat anti-DCX (1:1000, Santa Cruz, #sc-8066), rabbit anti-type III β-tubulin (1:4000, Babco, Richmond, #PRB-435P), guinea pig anti-GFAP (1:5000, Advanced Immunochemical, Inc., Long Beach, CA), rabbit anti-MMP-3 (1:1000, Chemicon, #AB810), rabbit anti-MMP-9 (1:500, Chemicon, # AB19106), mouse anti-GFP (1:500, Molecular Probes, #A11120), mouse anti-NG2 (1:1000, Chemicon, #AB5320), and rabbit immunoglobulin G (IgG) (1:1000, Sigma, #15006). Secondary antibodies were all used at 1:250 dilutions: donkey anti-rabbit Cy3, donkey anti-mouse Alexa 488 (Invitrogen), and donkey anti-guinea pig Cy5 (Jackson ImmunoResearch, West Grove, PA). Cell quantification was performed as described above.

Cell Proliferation Assay

Briefly, at 8 h post-plating of NPCs, BrdU was added to the NPC culture at a 2.5-μM final concentration for an additional 8 h, followed by fixation using 4% PFA. Cells were then stained using rat anti-BrdU ascites (1:500; Accurate Chemicals, Westbury, NY) and counterstained with 1 μg/ml DAPI. The percentage of $BrdU^+$ cells among total $DAPI^+$ cells was quantified using an unbiased stereology method as described above.

Real-Time Quantitative PCR

Real time PCR was performed using established methods [1, 2]. Briefly, total RNA was isolated from cells using either TRIzol (Gibco BRL, Gaithersburg, MD) for cells grown on culture dishes

or the Ambion RNAqueous® Kit (Ambion, Austin, TX, #AM1912) for cells on transwells. The cDNA was synthesized using a Superscript II kit (Invitrogen, CA). Each RNA sample was generated from a single dish or well and at least 3 independent samples were analyzed for each experimental condition. PCR primers were designed using Primer Express software (Applied Biosystems) and ordered from Integrated DNA Technology (Coralville, IA). The primers and subsequent PCR products were first evaluated by gel electrophoresis to determine that a single PCR product of the predicted size was generated. The real-time PCR reactions were performed in an ABI 7700 Detection System (Applied Biosystems). Each sample was acquired in at least triplicate. Data analyses were performed according to the protocol provided by Applied Biosystems. Standard curves were generated using total RNA isolated from mouse CNS (a mixture of brain and spinal cord). The amount of each mRNA for tested genes was calculated according to the standard curve for that particular primer set. Finally, the relative amount of the tested message was normalized to the level of an internal control message, glyceraldehyde-3-phosphate dehydrogenase (Gapdh). The results were statistically analyzed using a two-tailed, unpaired Student's *t*-test. PCR primers used were as follows:

Gene (RefSeq Accession)	Forward	Reverse
mouse CXCR4 (NM_009911)	ACGCCATGGCTGACTGGTAC	CCAGGATAAGGATCACCGTA
mouse VEGF-R2 (NM_010612)	TCCGGAGCCATCCACTTCAA	TTGGACAGCATCACCAGCAGT
mouse MMP-3 (NM_010809)	AGTCTACAAGTCCTCCACAG	TTGGTGATGTCTCAGGTTCC
mouse MMP-2 (NM_011594)	GATGTCCAGCAAGTAGATGC	TGAAGTCACCAGGTGAAGGA
mouse MMP-7 (NM_010810)	GAGATCATGGAGACAGCTTC	TGTTGATGTCTCGCAACTTA

mouse MMP-9 (NM_013599)	TGAGTCCGGCAGACAATCCT	TCTTGGTCTGCGGATCCTCA
mouse MMP-10 (NM_019471)	CTTAGATGCTGCCTATGAGG	CATGATGATCAGCACAGCAG

Western Blotting

Briefly, aNPCs were collected using homogenization buffer. After centrifugation to eliminate insoluble membranes, the protein concentration was determined using Bradford reagent (BioRad, CA). Protein size was resolved by a 4-12% Bis-Tris gel (Invitrogen, #NB0341BOX) and transferred to a nitrocellulose membrane (BioRad, #162-0115). After preblocking, the membrane was incubated overnight with appropriate primary antibodies: either goat anti-MMP-3 (1:1000, Abcam, #AB18898) or rabbit anti-MMP-9 (1:1000, Chemicon, #AB19106) in TBST containing 1% nonfat milk. Membrane was then rinsed and incubated for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated rabbit or goat anti-rabbit secondary antibody (Pierce, #1858415) diluted 1:5000 in 1% milk solution. Peroxidase activity was assessed using the enhanced chemiluminescence detection system (ECL and SuperSignal, Pierce) on X-ray films (FUJI Photo Film Co. LTD). The membrane was then stripped using Restore Western Blot Stripping Buffer (Pierce, #21059) for 60 min at 37°C, followed by incubation with a mouse antibody against β-actin (Sigma, 1:2000, #A5441) or Cyclophilin A (1:2000, Upstate, #07-313) as a loading control. The films were scanned using a BioRad Image station (BioRad, Hercules, CA) and quantified using Image J software (http://rsb.info.nih.gov/ij/).

Recombinant Lentiviruses and in vitro Gene Knock Down

The vectors expressing MMP-siRNA driven by a U1 promoter were purchased from SuperArray (SureSilencing shRNA plasmid: Mmp-3, #KM03673G; Mmp-9, #KM03661G). The efficiency

of the siRNAs was validated by cotransfecting MMP-expression plasmid (Open Biosystems) and siRNA plasmid into HEK293 cells. The U1-siRNA cassette were subsequently cloned into a third-generation lentiviral vector [3]. Lentivirus production was performed as described previously [2]. Briefly, lentiviral transfer vector DNA and packaging plasmid DNA were transfected into cultured 293T cells using calcium phosphate methods. The medium containing lentivirus was collected at 40, 64, and 88 hours post-transfection, pooled, filtered through a 0.2-μm filter, and concentrated using an ultracentrifuge at 19.4k rpm for 2 hours at 10°C using a SW27 Rotor (Beckman). The virus was washed once and then resuspended in 150 μl of phosphate-buffered saline. We routinely obtained 0.5-1 x 10^9 infectious viral particles/ml. 1 x 10^7 aNPCs were infected with 60 μl of virus for 24 hours, followed by subsequent migration and differentiation analyses as described above.

Mouse Middle Cerebral Artery Occlusion

Middle cerebral artery occlusion (MCAO) followed by reperfusion was conducted using the intraluminal method as described [4]. The protocol was approved by Institutional Animal Care and Use Committees (IACUC). Briefly, adult male C57BL/6 mice (3 month of age, weigh ~25 grams) were anesthetized with 1.5% isoflurane and maintained with 1.0% isoflurane in 1-L O_2 using a vaporizer (Summit Medical Equipment, Bend, OR, USA). The right common carotid artery was exposed by a midline incision in the neck, and the internal and external carotid arteries were isolated. The external carotid artery was ligated with a 6-0 silk suture. A 6-0 rounded tip nylon suture was introduced into the common carotid 10 to 11 mm distal to the bifurcation. The suture was advanced through the internal carotid artery to occlude the middle cerebral artery. After 60 minutes, the suture is withdrawn and the common carotid ligated with a

silk suture above the point of suture insertion. Mice were subcutaneously injected with 0.05 mg/kg bupronex for analgesia before surgery and 1 h after surgery. Mice were allowed to undergo reperfusion via the Circle of Willis for 14 days (n = 5/time point) before being sacrificed for analysis. Mice were killed by decapitation and brains were rapidly removed and quick-frozen in a beaker of isopentane equilibrated in dry ice/ethanol slurry and stored at −80°C until further processing. Coronal brain sections (20 μm) were prepared using a cryostat. Slides were air dried and stored frozen at −80°C until ready for use.

Primers used in Fluorescent In Situ Hybridization (FISH)

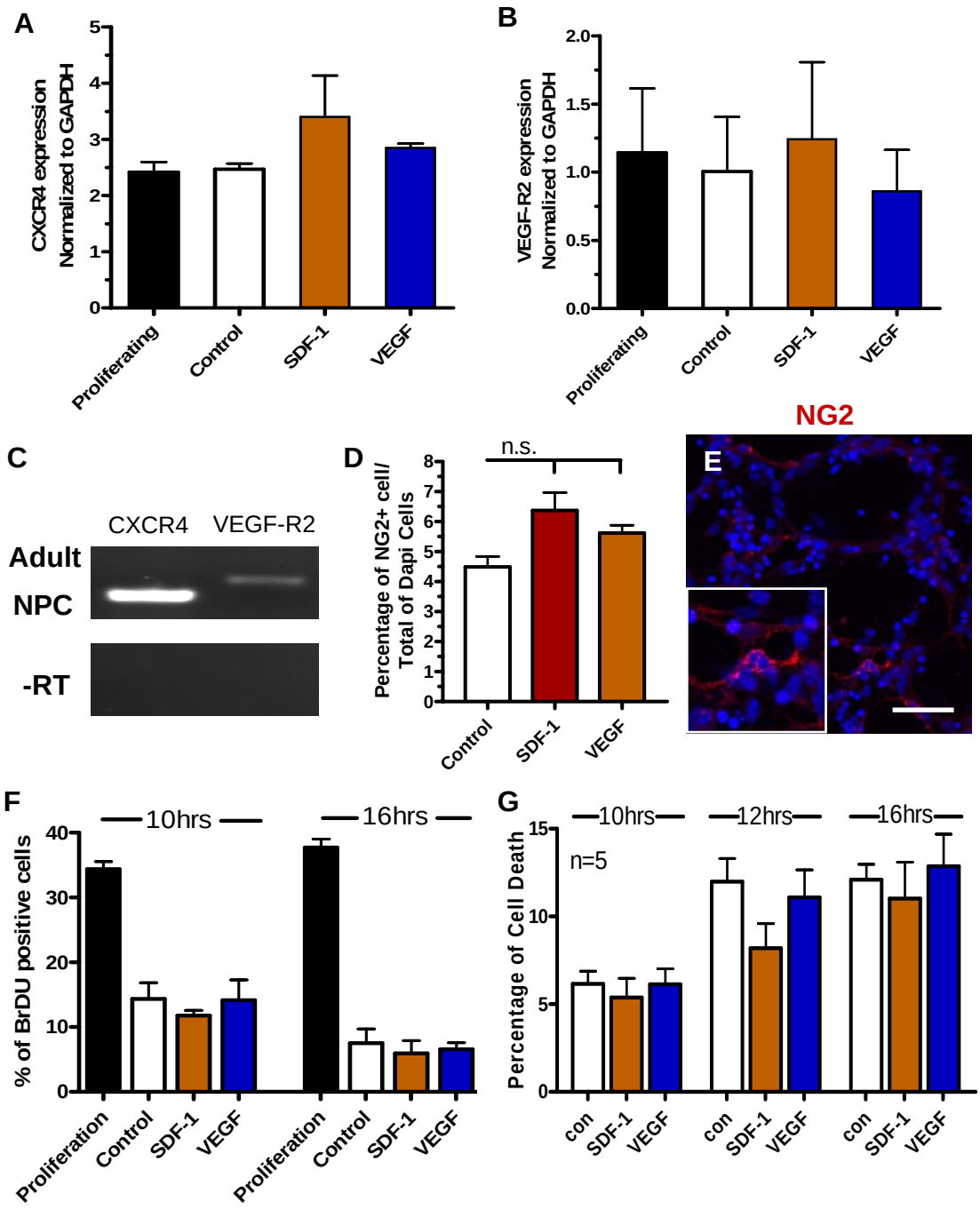
DNA templates for riboprobe synthesis were cloned using PCR based on GenBank sequences (RefSeq Accession): mouse MMP-3 (NM_010809): forward-TGGATCTTCGCAGTTGGA, reverse-GTTTGTGGCCCAGGAGTG; mouse MMP-9 (NM_013599): forward-CCGCTATGGTTACACCCG, reverse-GGGTAGGGCAGAAGCCAT; and mouse doublecortin (DCX, NM_010025): forward-TAACACCAACTGTTCACGATCC, reverse-AGAAACTCTTGGCCCCTAAATC.

REFERENCES

1. Zhao X, Ueba T, Christie BR et al. Mice lacking methyl-CpG binding protein 1 have deficits in adult neurogenesis and hippocampal function. Proc Natl Acad Sci U S A, 2003;100:6777-82.
2. Barkho BZ, Song H, Aimone JB et al. Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation. Stem Cells Dev, 2006. 15(3): p. 407-21.
3. Verma IM, Gage FH, Genome and gene therapy. Mol Ther, 2000;2:95.

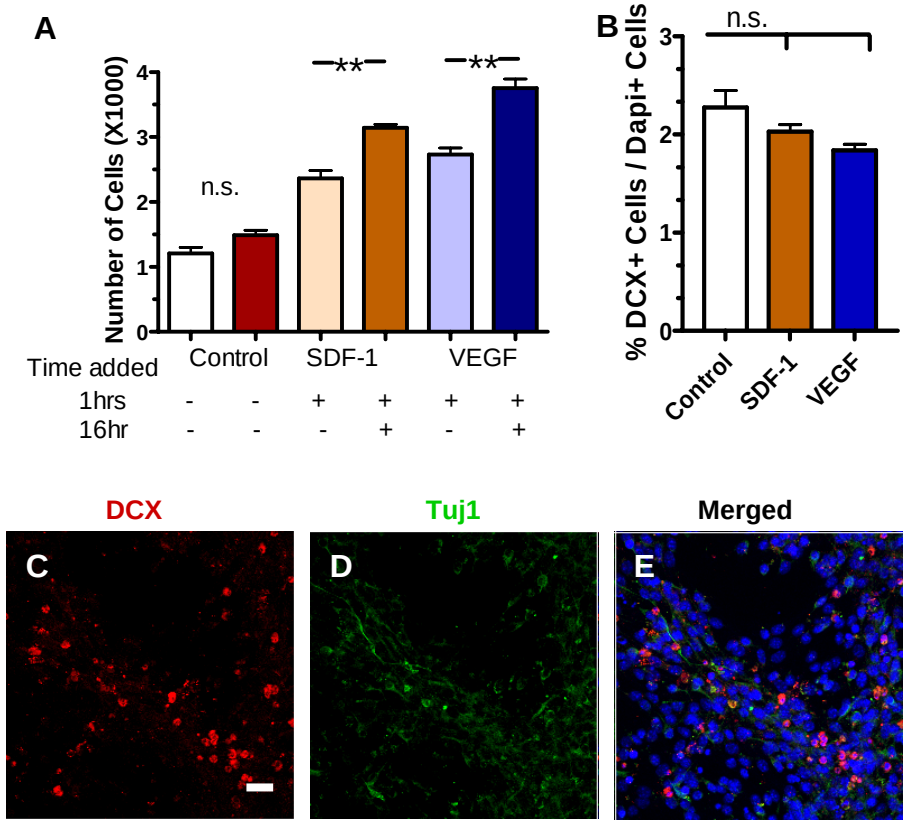
4. Kokovay E, Cunningham LA, Bone marrow-derived microglia contribute to the neuroinflammatory response and express iNOS in the MPTP mouse model of Parkinson's disease. Neurobiol Dis, 2005;19:471-8.

Figure S1


A
CXCR4 expression Normalized to GAPDH
Proliferating
Control
SDF-1
VEGF
B
VEGF-R2 expression Normalized to GAPDH
Proliferating
Control
SDF-1
VEGF
C
CXCR4
VEGF-R2
Adult NPC
-RT
D
n.s.
Percentage of NG2+ cell/ Total of Dapi Cells
Control
SDF-1
VEGF
NG2
E
F
10hrs
16hrs
% of BrDU positive cells
Proliferation
Control
SDF-1
VEGF
G
10hrs
12hrs
16hrs
n=5
Percentage of Cell Death
con
SDF-1
VEGF

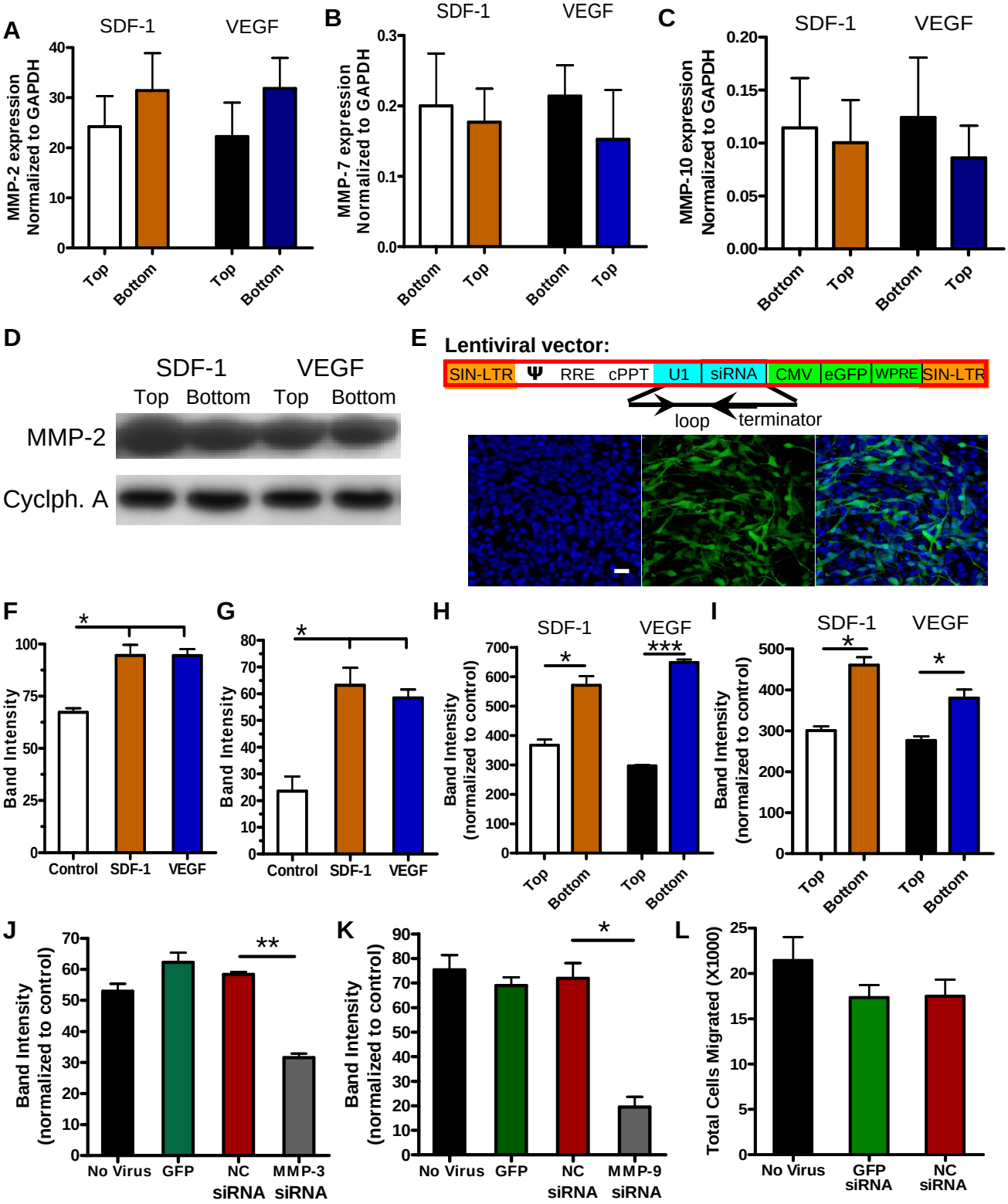
Supplementary Figure S1: SDF-1 and VEGF do not affect aNPC proliferation or survival and do not affect oligodendrocyte differentiation. (A, B) qPCR analysis of cultured NPCs demonstrated that **(A)** CXCR4 or **(B)** VEGF-R2 did not show differential gene expression across experimental conditions (n = 3). **(C)** Standard PCR representing expression of CXCR4 and VEGF-R2 in aNPCs cultured under proliferating conditions. Negative control, no reverse transcriptase (-RT) was added in cDNA synthesis. **(D)** Neither SDF-1 nor VEGF has a significant effect on oligodendrocyte differentiation of aNPCs after 16 hours of treatment (n = 3). **(E)** A representative image of $NG2^{+}$ oligodendrocytes quantified in (D). Scale bar = 50 μm. **(F)** Proliferation analysis using BrdU pulse labeling showed no change in cell division when aNPCs were treated with either SDF-1 or VEGF compared with the negative control (growth factor withdrawal alone) at 10 or 16 hours. aNPCs grown in the presence of the growth factors FGF-2 and EGF were used as a positive control for BrdU incorporation (n = 3). **(G)** Cell death analysis using propidium iodide permeability showed no change in the percentage of dead cells after either SDF-1 or VEGF treatment compared with the negative control (growth factor withdrawal alone) at 10, 12, and 16 hours (n = 3).

Figure S2


A
Number of Cells (X1000)
0
1
2
3
4
n.s.
**
**
Time added
Control
SDF-1
VEGF
1hrs - - + + + +
16hr - - - + - +
B
% DCX+ Cells / Dapi+ Cells
0
1
2
3
n.s.
Control
SDF-1
VEGF
DCX
Tuj1
Merged
C
D
E

Supplementary Figure S2: The plateau of cell migration at 16 hours is partially due to chemokine equilibrium. (A) In the cell migration assay, administering second doses of SDF-1 or VEGF at 16-hours post-plating resulted in significantly more cells migrated to the bottom chamber, compared with only one application of chemokines at 1 hour post-plating. ** $p < 0.01$ **(B)** When aNPCs were plated onto laminin-coated coverslips, rather than a migration chamber, neither SDF-1 nor VEGF had any effect on the differentiation of aNPCs into DCX^+ after 16 hours of treatment (n = 3). **(C-E)** In the same assay as **(B)**, no colocalization of **(C)** DCX^+ and **(D)** $Tuj1^+$ cells was observed after 16 hours of treatment with SDF-1. Scale bar = 20 μm. **(E)** Merged image showing the DCX- and Tuj1-expressing cells.

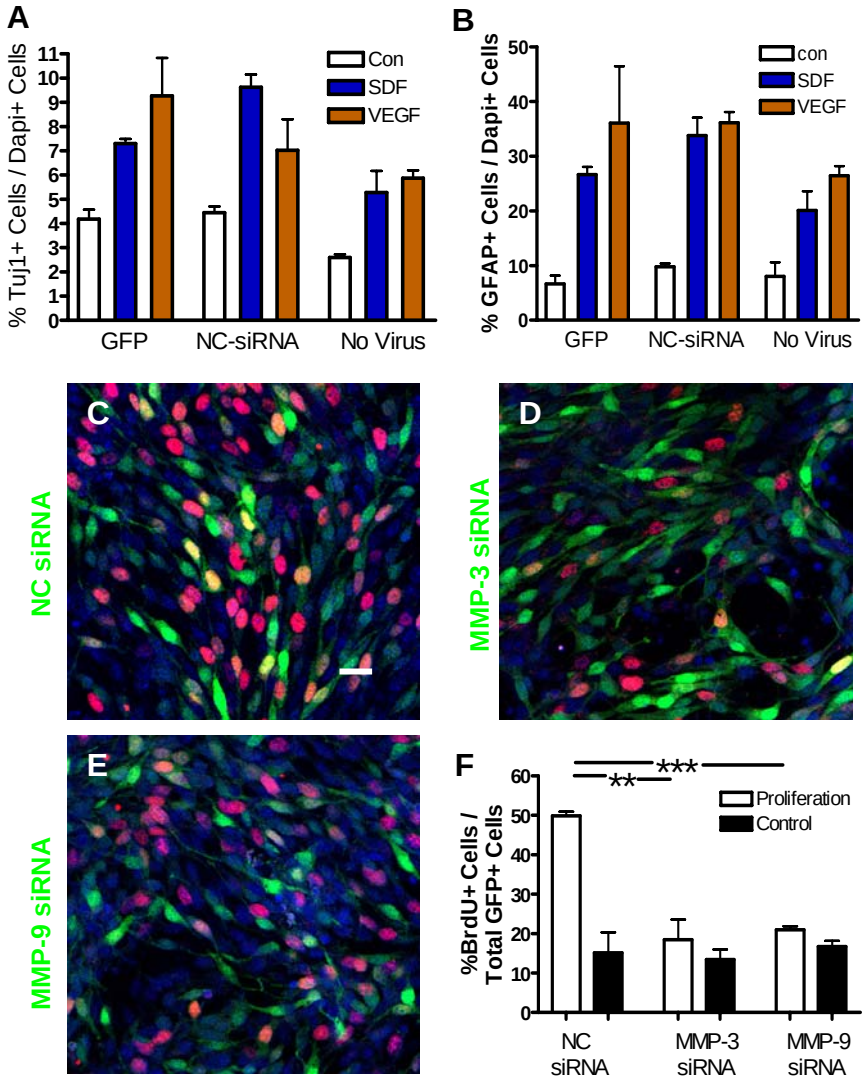
Figure S3


A
SDF-1
VEGF
MMP-2 expression Normalized to GAPDH
40
30
20
10
0
Top
Bottom
Top
Bottom
B
SDF-1
VEGF
MMP-7 expression Normalized to GAPDH
0.3
0.2
0.1
0.0
Bottom
Top
Bottom
Top
C
SDF-1
VEGF
MMP-10 expression Normalized to GAPDH
0.20
0.15
0.10
0.05
0.00
Bottom
Top
Bottom
Top
D
SDF-1
VEGF
Top
Bottom
Top
Bottom
MMP-2
Cyclph. A
E
Lentiviral vector:
SIN-LTR
Ψ
RRE
cPPT
U1
siRNA
CMV
eGFP
WPRE
SIN-LTR
loop
terminator
F
*
Band Intensity
100
75
50
25
0
Control
SDF-1
VEGF
G
*
Band Intensity
80
70
60
50
40
30
20
10
0
Control
SDF-1
VEGF
H
SDF-1
VEGF
*
***
Band Intensity (normalized to control)
700
600
500
400
300
200
100
0
Top
Bottom
Top
Bottom
I
SDF-1
VEGF
*
*
Band Intensity (normalized to control)
500
400
300
200
100
0
Top
Bottom
Top
Bottom
J
**
Band Intensity (normalized to control)
70
60
50
40
30
20
10
0
No Virus
GFP
NC siRNA
MMP-3 siRNA
K
*
Band Intensity (normalized to control)
90
80
70
60
50
40
30
20
10
0
No Virus
GFP
NC siRNA
MMP-9 siRNA
L
Total Cells Migrated (X1000)
25
20
15
10
5
0
No Virus
GFP siRNA
NC siRNA

Supplementary Figure S3: MMP-3 and MMP-9, but not MMP-2, -7, or -10, were differentially expressed in migratory versus stationary cells. (A-C) qPCR analysis demonstrated that mRNA levels of **(A)** MMP-2, **(B)** MMP-7, and **(C)** MMP-10 exhibited no significant difference between migratory and stationary cells (n = 4). **(D)** The protein levels of MMP-2 in the cell lysate of migrating cells (Bottom) were not significantly different compared with that in stationary cells (Top). Cyclophilin A antibody was used for loading control. **(E)** Lentivirus expressing both siRNA and GFP infected (see methods for details) aNPCs with good efficiency without apparent adverse effect. Structure of lentiviral vector used to express siRNAs is shown: SIN-LTR, self-inactivating long terminal repeat; Ψ, HIV packaging signal; cPPT, central polypurine track; CMV, cytomegalovirus promoter; WRE, woodchuck hepatitis virus response element. Loop, the loop of the siRNA hairpin; terminator, the terminator sequence of siRNA. Representative images of lentiviral NC-siRNA–infected aNPCs at 24 hours post-infection. Blue, Dapi; Green, GFP. **(F, G)** Quantification of Western blot band intensities (for Figure 3D) demonstrates higher protein levels of **(F)** MMP-3 and **(G)** MMP-9 in the culture media of migratory cells compared with stationary cells responding to SDF-1 or VEGF. Equal volume of cell culture medium was loaded onto each lane. **(H, I)** Quantification of Western blot band intensities (for Figure 3E) demonstrates higher protein levels of MMP-3 and MMP-9 in migratory cells compared with stationary cells responding to SDF-1 or VEGF (compared with loading control Cyclophilin A). **(J, K)**. Quantification of Western blot band intensities (for Figure 3I, J) demonstrates gene knockdown efficiency of siRNAs for MMP-3 (J) and MMP-9 (K) (β-actin was used as a loading control). **(L)** The total number of migrated cells was not significantly affected by

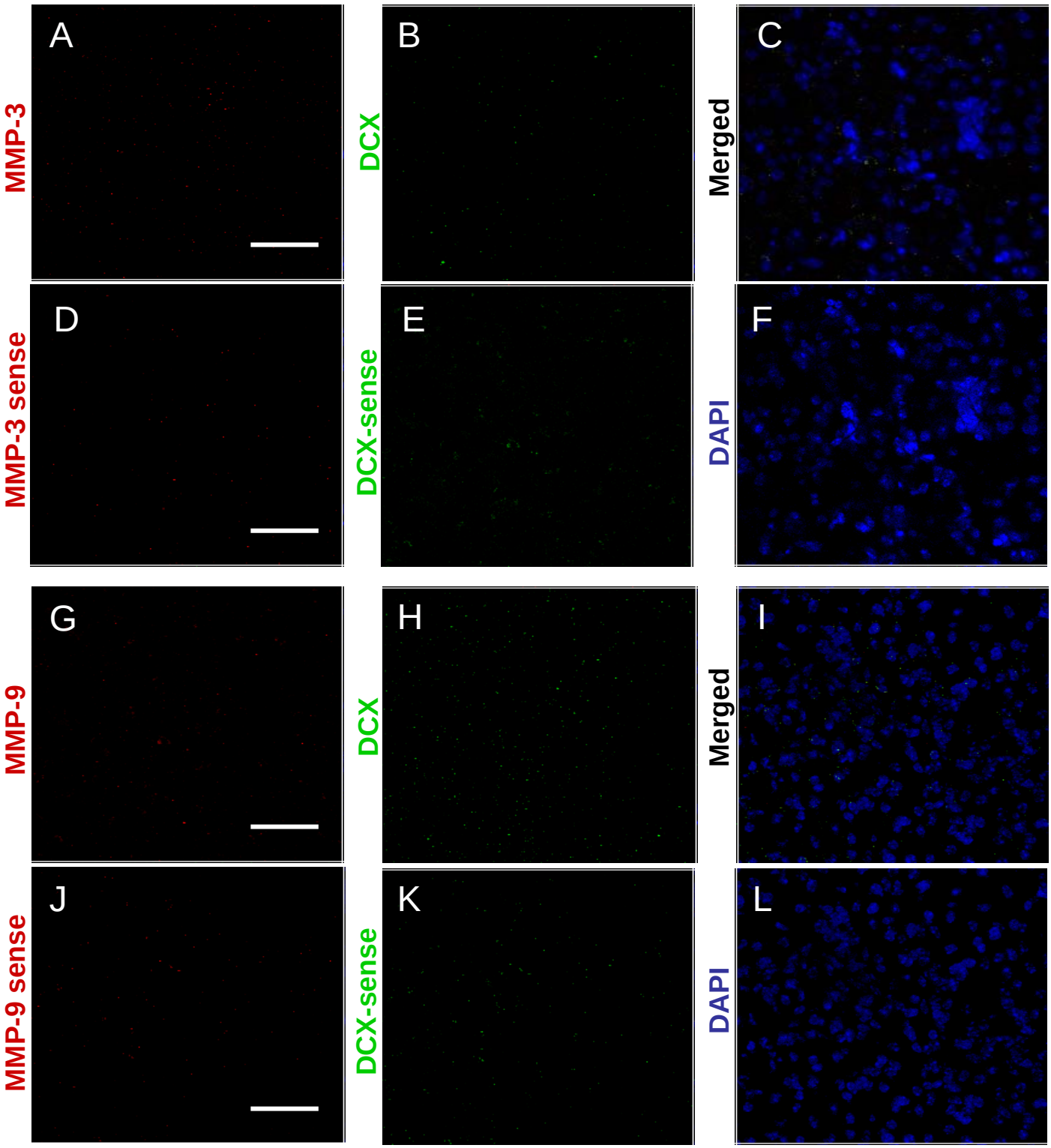
infection of two different control lentiviruses compared with uninfected aNPCs. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Figure S4


A
% Tuj1+ Cells / Dapi+ Cells
Con
SDF
VEGF
GFP
NC-siRNA
No Virus
B
% GFAP+ Cells / Dapi+ Cells
con
SDF
VEGF
GFP
NC-siRNA
No Virus
C
NC siRNA
D
MMP-3 siRNA
E
MMP-9 siRNA
F
%BrdU+ Cells / Total GFP+ Cells
***
**
Proliferation
Control
NC siRNA
MMP-3 siRNA
MMP-9 siRNA

Supplementary Figure S4: MMP-3 and MMP-9 are important for mitogen-dependent aNPC proliferation. (A) Two control lentiviruses, Lenti-GFP and Lenti-NC-siRNA, had a mild effect on the neuronal differentiation of NPCs. However, the induction of neuronal differentiation by SDF-1 and VEGF was not affected by viral infection compared with uninfected cells, suggesting that this lentivirus system could be used for analyzing the effect of gene knockdown on SDF-1– and VEGF-induced neuronal differentiation. **(B)** Neither control virus had a significant effect on SDF-1– and VEGF-induced astrocyte differentiation. **(C-F)** Acute knockdown of MMP-3 or MMP-9 using siRNAs affect aNPC proliferation. **(C)** Lentivirus-infected aNPCs expressing NC-siRNA (green) incorporated BrdU (red) under proliferating conditions. Scale bar = 20 μm **(D, E)** Lentivirus-infected aNPCs expressing **(D)** MMP-3 or **(E)** MMP-9–siRNA (green) incorporated BrdU (red) under proliferating conditions. **(F)** Quantification of BrdU+ cells among total GFP+ cells indicated that MMP-3– or MMP9-siRNA–infected aNPCs had reduced proliferation compared with NC-siRNA–infected cells under proliferating conditions (white bars); however, neither siRNA had an effect on cell division in the absence of mitogens (black bars). ** $p < 0.01$ and *** $p < 0.001$.

Figure S5


A
B
C
MMP-3
DCX
Merged
D
E
F
MMP-3 sense
DCX-sense
DAPI
G
H
I
MMP-9
DCX
Merged
J
K
L
MMP-9 sense
DCX-sense
DAPI

Supplementary Figure S5: Sense riboprobes did not show detectable signals in either the ipsilateral or contralateral side of the brain. (A-C) Ribroprobes detected no **(A)** MMP-3 mRNA (red) and no **(B)** DCX mRNA (green) expression in the contralateral side after MCAO. **(C)** Merged image (A) and (B). Scale bar = 50 μm. **(D-F)** Sense ribroprobes detected no **(D)** MMP-3 mRNA (red) and no **(E)** DCX mRNA expression in the contralateral side after MCAO. **(F)** Dapi image. Scale bar = 50 μm. **(G-I)** Ribroprobes detected no **(G)** MMP-9 mRNA (red) and no **(H)** DCX mRNA (green) expression in the contralateral side after MCAO. **(I)** Merged image (G) and (H). **(J-L)** Sense ribroprobes detected no **(J)** MMP-9 mRNA (red) and no **(K)** DCX mRNA expression in the contralateral side after MCAO. **(L)** Dapi image. Scale bar = 50 μm.